A Study of Transcription Factors STAT3, SP1 and NFκβ in Breast Cancer.

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Why do some patients, who should survive their breast cancer, die from their disease?

This question was posed by my consultant during a breast review clinic. Our current knowledge of predictive markers and prognostic indexes go some way to stratify patients into groups of likely outcome but there are always patients who "buck the trend," the patient who doesn't follow the prediction. This patient is an indication that, despite our predictive models some tumours do not behave in the way we expect; small tumours sometimes metastasise, low grade tumours sometime recur and some estrogen receptor (ER) positive tumours don't respond to endocrine therapy. This clinical problem raised the question behind this study. Could we identify certain biological markers in breast cancer samples that would improve our stratification of those tumours?

The fact that some ER positive cancers do not respond to endocrine therapy suggests that other proliferative pathways are driving that tumour's growth. The end points of these pathways are the DNA transcription factors. Some of these transcription factors have been shown to have a role in increased proliferation of cancer cells. It was for this reason that the transcription factors STAT3, SP1 and NFKB were selected for further study in my research work.

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Abstract of submitted thesis.

Title: A Study of Transcription Factors STAT3, SP1 and NFkB in Breast Cancer.

Supervisors: Dr B. Shenton, Mr D Browell, Professor T Lennard.

Background and Aims: Breast cancer is the second most common cause of cancer deaths in women. It is a tumour which has been extensively studied at a molecular level and, compared to other solid tissue tumours, our understanding of its biology is extensive. There are however some patients who are considered to have good prognostic feature of their tumours who go on to die from their disease.

Transcription factors are the end point of many cell signalling pathways. They form the link between exogenous hormones and growth factors and DNA transcription. For the purpose of this study 3 different transcription factors have been selected for investigation.

STAT3 is activated by various growth factors and cytokines including EGF. It is classified as an oncoprotein as its activation can mediate tumorgenisis in nude mice. STAT3 has been shown to confer resistance to apoptosis in breast cancer cells and it is associated with poor outcome in high risk breast cancers. SP1 is a transcription factor which is essential in the expression and the action of estrogen receptors (ER). It is known to be over expressed in other solid tissue tumours but there has been little work into its role in breast cancer. NFkB is activated in many cell survival settings. It is involved in the transcription of anti-apoptotic genes and also plays a role in cell proliferation, angiogenisis and cell adhesion. It is associated in breast cancers with an

over expression of the oncogene Bcl-2. It has not been show to be a marker of prognosis but does appear to identify breast cancers with a poor response to chemotherapy.

The aim of this study is to investigate the role of these transcription factors in the behaviour of breast cancers and the outcome of the disease. It will also investigate the affect of EGF and estogen stimulation on STAT3 activation in breast cancer cell lines.

Methods: This study consists of 2 elements. Firstly an assessment of transcription factor expression in breast cancer samples and secondly a cell model experiment to investigate the stimulation of STAT3 activation.

A cohort of 213 patients who presented to the Queen Elizabeth Hospital with invasive breast cancer in 1999 was selected. Tumour samples from these patients were retrieved and using immunohistochemistry were tested for the expression of STAT3, SP1 and NFkB. These results were then correlated with pathological features of the tumours, tumour receptor status (ER, PR HER2 and EGFR) and outcome of the disease.

Two cell lines, MCF7 and SKBr3, were cultured in depleted medium. These cells were then stimulated with estrogen and EGF alone and in combination. Flow-cytometry was then used to quantify the levels of phosphorylated STAT3 in the 2 cell lines over a 3 day time course. The level of phosphorylation was then compared to the control lines to asses the effect of stimulation.

Results: 209 breast cancers were successfully analysed for the expression of STAT3, 27% of these cancers expressed nuclear STAT3. The results demonstrated a significant correlation of STAT3 expression with cancers of a high grade (p=<0.001), increasing tumour size (p=0.004), vessel space invasion (p=0.034) and lymph node metastases (p=0.015). STAT3 expression was shown to be significantly correlated to high Nottingham prognostic index (NPI) scores. With regards to receptor status it was show that STAT3 expression was significantly associated with ER negative and PR negative cancers (p=0.003), whereas there was no relationship with HER2 status. The results did show that there was a significant relationship between STAT3 expression and EGFR positive cancers (p=0.007). When disease outcome was investigated it was shown that there was a trend towards improved survival in the STAT3 negative group and a significant relationship between STAT3 expression and disease recurrence at 5 years (p=0.04).

SP1 expression was determined in 208 of the cancer samples with 33% of the tumours having strong nuclear staining. There was no significant relationship between SP1 expression and any of the pathological features mentioned. SP1 expression was related to ER positive tumours (p=0.015). Though there was no relationship with 5 year survival it appears that SP1 expression does reduce the risk of late (>2yr) disease recurrence (p=0.005).

NFkB was over expressed in 15% of the 208 cancers samples. Again a significant correlation was shown with high grade tumours (p=0.001) and large tumours (p=0.014). NFkB expression was also shown to be more prevalent in ER negative

cancers (p=0.006) and EGFR positive tumours (p=0.007). There was no significant relationship between NFkB expression and disease outcome.

The cell model results showed that in the EGFR positive ER negative cell line (SKBr3), EGF stimulation resulted in a biphasic response of STAT3 phosphorylation, whereas estrogen had no effect on phosphorylation. In the ER positive MCF7 cells, which express low levels of EGFR, again EGF stimulation resulted in a biphasic response curve. Estrogen stimulation does cause an increase in activation but when estrogen is added to EGF stimulation there is an inhibition of STAT3 phosphorylation.

Conclusions: This study has demonstrated that STAT3 and SP1 expression is important in disease outcome in breast cancer patients. Though there are differences in levels of expression, NFkB does not appear to have a role in breast cancer outcome. The cell model has show that EGF stimulation of EGFR positive cell lines results in increased STAT3 activation and also that this effect is inhibited by the addition of estrogen stimulation.

These results raise important questions which are discussed in the study and suggest areas for further investigation.

1 Introduction.

In the introduction of this thesis both the subject of breast cancer as a disease and on the endocrine aspects of breast cancer and its relevance to current therapies will be discussed. The subject the of the transcription factors STAT3, SP1 and NFKB will be introduced with a review of the current understanding of their role in breast cancer.

1.1. An Introduction to Breast Cancer.

This section is written as a background of breast cancer as a disease. It will review the anatomy and functional physiology of the breast and give a historical perspective of our changing understanding of breast cancer. It will then go on to outline the histological basis of breast cancer and its staging. The section will finish with a review of the current epidemiology of breast cancer and its aetiology.

1.1.1 The gross and functional anatomy of the breast.

The class of Mammalia is distinguished from other animals by the possession of mammae, breasts. These glands evolved for the feeding of offspring by the production of milk, termed lactation. Though only functional in the female sex they are present in a rudimentary form in the male. In the adult female the breast lies within the superficial fascia on the anterior chest wall. The base of the breast is attached to the chest wall longitudinally from the 2^{nd} or 3^{rd} rib to the 6^{th} rib. In the transverse plane it extends from the sternal edge to almost the mid axillary line. The superolateral quadrant of the breast extends towards the axilla (the axillary tail of Spence.) The arterial blood supply to the breast arises from the axillary artery suppling blood to the breast via the superior thoracic, the pectoral branch of the thoraco-acromial artery, the lateral thoracic and the subscapular arteries. The internal thoracic artery gives perforating branches to the anterior medial portion of the breast. There are also

perforating branches more laterally from the 2nd to 4th intercostal arteries. The venous drainage predominantly follows the arterial supply. The lymphatic drainage of the breast is much more variable. From a subareolar plexus there are lymphatic vessels draining to the following regions; the contra lateral breast, the internal mammary chain and the ipsilateral axilla. The axilla is the predominant site of lymphatic drainage. There are usually between 20 and 40 nodes in the axilla and these may be grouped into level 1 nodes, below pectoralis minor muscle, level 2 nodes, behind pectoralis minor and level 3 or apical nodes which are between pectoralis minor and the clavicle.(Grey, 1999)

The substance of the breast is composed of epithelial glandular tissue, fibrous connective tissue (stroma) surrounding the glandular tissue and interlobular adipose tissue.(Cowie, 1974) The glandular tissue of the breast consists of branching ducts connecting the terminal secretory lobules to larger lactiferous ducts which in turn end with an opening on the apex of the nipple. Just prior to this opening the duct dilates and forms the lactiferous sinus. Each breast contains 15-25 of these functional units (lobes.) The branching ducts eventually form terminal ducts each connected to a lobule. The lobule consists of multiple excretory acini. The lobules are separated by moderately dense collagenous interlobular tissue, whereas the supporting tissue within the lobule, the intralobular tissue, is less collagenous and more vascular. Ducts and acini are lined with 2 layers of cells, a luminal layer of epithelia cells and a basal layer of myoepithelial cells which rest on a basement membrane. The epithelial cells are columnar in the larger ducts and cuboidal in the smaller ducts and acini.(Wheater, 2000)

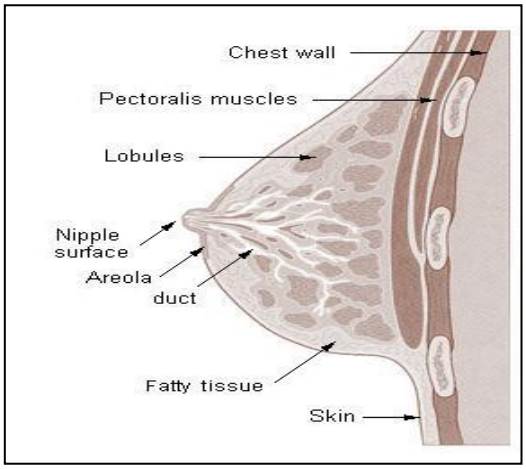

Fig. 1.1: The gross anatomy of the breast. (courtesy of the breastsite.com)

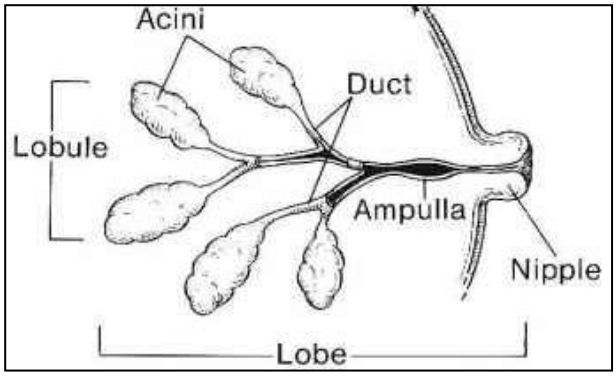

Fig. 1.2: The breast lobe. (http://www.kbsplit.hr/rakdojke/images/fig9.)

The embryological origins of the breasts develop in the 4th week of gestation as a pair of epidermal thickenings, mammary ridges. These run from the axilla to the inguinal region as what are known as the milk lines. These lines usually disappear apart from a remnant on the anterior chest wall which becomes the primary bud of the mammary gland by the 5th week. This primary bud grows down into the dermis beginning to branch by the 12th week forming several secondary buds. During the rest of gestation these buds lengthen, branch and canalize forming the 15-25 lactiferous ducts opening at the nipple. It is not unusual for more than one nipple to be formed along the milk line (polythelia,) due to accessory remnants of the mammary ridge. (Larsen, 2001)

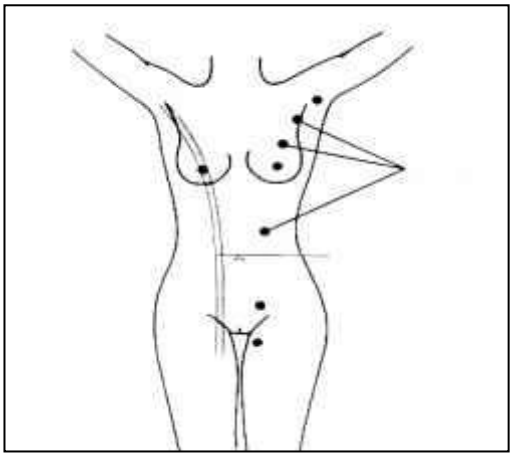

Fig. 1.3: The mammary ridge line. http://www.kbsplit.hr/rakdojke/images/fig1.

The next stage in breast development in the female commences with puberty. There are 4 described stages of this pubertal breast development; Stage 1. Breast bud elevation, Stage 2. Growth and protrusion of the nipple, Stage 3. Elevation of the secondary areolar mound, Stage 4. Regression of the areolar mound to form the general breast contour.(Zacharias and Schatzoff, 1970, Rees, 1995) This development is driven by increasing estradiol levels which during the first anovulatory cycles induce duct sprouting, and branching. Estrogen also stimulates the connective tissue and vascular growth required to support the developing breast. On the commencing of ovulatory cycles there is an increased production of progesterone which causes the differentiation of the terminal ductular bud to form the adult lobules (Graham and Clarke, 1997).

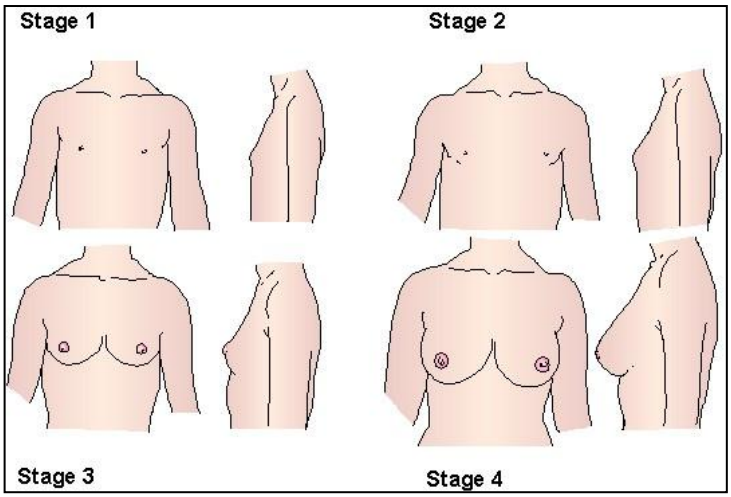

Fig. 1.4: The stages of breast development at puberty.

www.teenpuberty.com/images/puberty/breastdev.

Following puberty the breast is subject to a fluctuating hormone environment during the menstrual cycle leading to changes in the nature and structure of the breast tissue. In the follicular phase of menstruation (days 3-14) the stroma of the breast becomes less dense and there is an expansion of the lumen of the ducts. During the luteal phase the ducts fill with secretions as the stromal tissue density increases. There is an increase cellular proliferation until day 26 when apoptosis of the epithelial cells returns the breast to its resting state (Fanger and Ree, 1974).

Under the influences of the increased circulating sex steroid hormones the breast alters during pregnancy in preparation for lactation. The stromal elements in the breast tissue diminish to accommodate the hyperplasia of the lobular units. At the time of the third trimester there is an increase in the number of acini and the overall size of the lobules. The ductal epithelial cells differentiate and begin to synthesise and secrete milk. At birth there is a dramatic fall in the levels of oestrogen and progesterone allowing prolactin which has risen during pregnancy to act uninhibited

on the breast inducing lactation. The stimulus of suckling on the nipple acts along with inputs from the higher centres on the hypothalamus, this produces oxytocin which causes milk expulsion and maintains prolactin production. On cessation of breast feeding there is a fall in oxytocin and prolactin levels and the breast returns to its normal pre-pregnancy state.

During the approach of the menopause breast involution begins. The stromal tissue around the lobules becomes denser, there is loss of the cells of lining the acini and the basement membranes become thicker. This is not a uniform process throughout the breast and can lead to clinically palpable lumps within the breast. The major constituent of the remaining post menopausal breast is adipose tissue.

1.1.2 The history of breast cancer.

Breast cancer may be defined as a malignant proliferation of a cellular constituent of the breast (Schnitt and Guidi, 2004). This pathology has been studied and written about for the past 4000 years.

The Edwin Smith papyrus is thought to have been written in 1700BC based upon a text written in 3000BC. Within the 48 cases described there are two concerning tumours of the breast; the first of which feels hot under the hand is described as;

"One having tumors with prominent head in his breast, (and) they produce cysts of pus. An ailment which I will treat with the fire-drill."(Breasted, 1980)

An accurate description of a breast abscess and its treatment. The second case is that of a tumour thus described;

"If thou examinest a man having tumors on his breast, (and) thou findest that swelling have spread over his breast; if thou puttest thy hand upon his breast upon these tumors, (and) thou findest them very cool, there being no fever at all therein when thy hand touches him; they have no granulation, they form no fluid, they do not generate secretions of fluid, and they are bulging to thy hand. Thou shouldst say concerning him: One having tumors. An ailment with which I will contend."(Breasted, 1980) There is no treatment offered for this type of tumour likely to be a breast cancer.

The origin of the word cancer can be attributed to the Greek physician Hippocrates (460-370 B.C.) Hippocrates used the term “Karkinos” or “Karkinoma” for tumorous growths probably relating to the crab like invasion of a tumour. Hippocrates considered that these hidden cancers should not be treated as treatment hastened death. Celsus, first century AD, although better known for his description of inflammation (calor, dolor, rubor, tumor) described an operation for early breast cancer but advised against surgery for more advanced disease. Galen (A.D. 130-203) considered that the most common cancers arose in the female breast and were due to an excess of black bile again he recognised the need for surgery to control local disease. Following this period medicine along with the rest of intellectual thinking entered the dark ages, it was not until the renaissance that there were any further advances in medical science.

The 16th and 17th century saw a rebirth of the study of anatomy and surgery. Andreas Vesalius (1514 – 1564) published the first comprehensive anatomy text, De Humani Corporis Fabrica, in 1543. This replaced Galen’s writings which were based on the dissections of apes. Ambroise Paré (1510 – 1590) contributed to the advancement of surgery in numerous ways including the introduction of ligation of vessels during operations as opposed to cautery. These advances lead to the description of a number of breast operations for cancers. Based upon the work by Thomas Bartholin (1616 – 1680) on the lymphatic system and the introduction of the microscope to medicine, Henry François LeDran (1685 -1780) proposed in 1757 that breast cancer commenced as a local disease that then spread via the lymphatics to the rest of the body, hence early surgery may be curative. This centrifugal theory of metastatic spread was the basis of the treatment of breast cancer into the 20th century (Zenon Rater, 2003).

In 1867 Charles Moore published a paper describing local recurrences of breast cancer following inadequate operations (Moore, 1867). He stressed the importance of adequate resection and that the tumour should not be cut into or even seen during the operation. Following work in Germany during the 1880's by Kuster, Schmid and Heidenman on the value of mastectomy and en-bloc removal of the axilliary lymph nodes William Stewart Halsted (1852 – 1922) published his first description in 1898 of what was later to become known as the radical mastectomy bearing his name (Halsted, 1898).

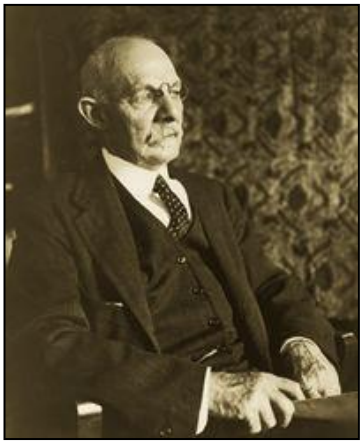

Fig. 1.5: William Stewart Halsted. http://www.hopkinsmedicine.org/hmn/W02/photos/Halsted.jpg.

Advances in the understanding and treatment of breast cancer during the last 100 years now underpin its current management. The surgical therapy has become less aggressive with an improved understanding of cancer biology. There is now an important role for radiotherapy in combination with surgery. Chemotherapy is an important adjuvant in advanced or metastatic disease and with advances in molecular biology endocrine manipulation is now one of the core elements of the treatment of breast cancers.

1.1.3 The histological basis of breast cancer.

Carcinoma of the breast may be considered as a heterogeneous group of diseases. The formal histological typing of breast cancer has been established by the World Health Organization (1982). This classification is based upon the histological properties of the cancer. It is not an indication of the origin of the tumour within the breast. The majority of invasive carcinomas originate from the terminal duct lobular unit irrespective of its histological type (Schnitt S.J., 2004)

Cancers of the breast may be divided in to invasive and non invasive or in situ lesions. The in situ lesions are defined as abnormal proliferation of cells that are not invading the basement membrane that the cells are attached to, whereas invasive lesions are those in which the cells transgress this membrane. For the purpose of this study we will consider only the invasive breast cancer

The prevalence of the different histological types of breast cancer is well documented. The most common type of invasive breast cancer is the default diagnosis of ductal carcinoma of no special type (NST) This is followed by lobular carcinoma, then the rarer special types of breast cancer (see table 1.1).

Histological Type	% of presenting lesions
Ductal	70%
Lobular	10%
Medullary	5%
Mucinous	2%
Tubular	3%
Mixed	2%
Other	8%

Table 1.1: Histological types of breast cancer(Schnitt S.J., 2004).

1.1.3.1 Ductal Carcinoma of no special type.

Ductal carcinoma of no special type (NST) is diagnosed when the lesion does not exhibit any histological features of the other breast cancer types. They commonly present with a mass or on screening mammography, rarely they can present as Paget's disease of the nipple (more commonly due to DCIS). Macroscopically they are commonly a hard mass with a grey or white surface on sectioning and a gritty texture. The lesion itself may be spiculated or stellate in nature infiltrating the surrounding tissue though some lesion may be more circumscribed. Fig 1.6 illustrates a classical ductal carcinoma with a white surface and an infiltrating stellate appearance. Microscopic examination of invasive ductal cancers can show marked variation between both individual cases and within a single tumour. Histologically the cells can be seen as glandular structures, sheets, cords or trabeculae. This may be associated with or without necrotic areas. The tumour cells themselves can exhibit a range of cytological features in the nucleus, cytoplasm and the mitotic rate.

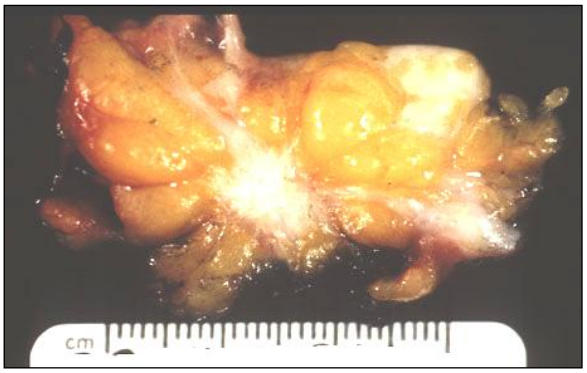

Fig. 1.6: Ductal cancer of the Breast. (Courtesy of Loyola University Chicago Stritch School of Medicine. Chicago.)

1.1.3.2 Special types of Breast Cancers.

As shown in table 1.1 lobular carcinoma of the breast is the most common type of special cancers. Clinically they may not present with a classical hard mass but more of a non discrete thickening, they are more commonly found to be multifocal and bilateral compared to the other types of breast cancers. On imaging, both mammographical and on ultrasound, the appearances may be quite subtle. These features of lobular cancer can present a challenge when diagnosed pre-operatively. It is well recognised that both clinical examination and classical imaging may undersize the pathological lesion and this can cause a higher rate of involved margins when the lesions are excised with breast conserving surgery. In the Queen Elizabeth Hospital Gateshead, women with a preoperative diagnosis of lobular carcinoma will, as standard practice, have a breast MRI scan which is considered to be more accurate guide to the overall size of the lesion than mammography or USS and diagnose

mammographically invisible multi focal disease. Histologically lobular carcinomas classically appear as an infiltration of the stroma with relatively uniform small cells with small nuclei. The cells usually appear loosely cohesive and some may have a signet ring appearance. There are a number of described variants of lobular carcinomas including the signet cell subtype but also the solid, alveolar, tubulolobular, pleomorphic and histocytoid variations. The lobular carcinoma may be less likely to metastasise to the classical site of lung, liver and brain though compared to ductal carcinoma but can be found as metastasis in unusual sites, such as the ovaries, many years after the primary disease. Its outcome is not as advantageous as some of the other special types of breast cancer.

Medullary carcinoma, now called medullary like carcinoma, is a cancer of the breast which presents commonly with a palpable mass or with mammographic appearances similar to a ductal cancer. Histologically it has a well circumscribed margin with an infiltration of lymphocytes and plasma cells. It is considered to have a lower rate of axillary metastases and an improved survival compare to ductal cancers.

Mucinous (colloid) carcinomas tend to present with poorly defined lobular masses. They consist of neoplastic cells in pools of mucin. They have lower rates of metastasis and an improved survival compared to ductal cancers.

Tubular carcinomas more commonly present as screening abnormalities rather than palpable lesions. They are characterised by the presence of well differentiated glands or tubules and have an excellent prognosis.

More recently has been the identification of the basal phenotype of breast cancer. These cancers are identified by the expression of CK5/14 on IHC. They are commonly associated with BRCA1 positive cancers and tend to be ER and HER2 negative (Jumppanen et al., 2007). These cancers tend to be more aggressive and have a poorer outcome than non-basal type cancers of the same grade (Rakha et al., 2006, Fulford et al., 2007). The ER and HER2 negative status of these cancers obviously have an impact on the use of adjuvant therapies available for their treatment.

There are also a number of much rarer cancers of the breast including cribiform carcinomas, papillary carcinomas, metaplastic carcinomas, and adenoid cystic carcinomas to mention but a few.

1.1.3.3 Tumour Grading.

This heterogeneous group of tumours are classified in a grading system. The grading of a cancer is a microscopic description of how similar the tumour is to normal breast tissue. The most commonly recommended histological grading system in use is the Elston and Ellis modification of the Bloom and Richardson system (Elston and Ellis, 1991). This grading system scores a tumour on three components; tubule formation, nuclear pleomorphism and mitotic rate. These three components are allocated a score (1-3) and the total score determines the overall histological grade: The scoring is determined as followed;

Tubule formation, % of the tumour forming normal duct structures >75% = 1 point, 10-75% = 2 points and <10% = 3 points.

Nuclear pleomorphism, this parameter is based upon whether the nucleus of the cell is of a normal shape, size and staining or an abnormal shape size and staining (pleomorphic.) minimal pleomorphic = 1 point, moderate pleomorphic = 2 points and marked pleomorphic 3 points.

Mitotic rate, this is a measure of the rate of division within the tumour and is assessed by counting the number of dividing cells in a field of view under the microscope. These mitotic figures are only counted at the periphery of the tumour in the most mitotically active areas seen. The score per count is dependent on the microscopic field used but again is allocated a 1,2 or 3 score as the number of mitotic cells increases.

Grade	Description	Score
I	Well differentiated	3-5
II	Moderately differentiated	6-7
III	Poorly differentiated	8-9

Table 1.2: Table showing the Elston and Ellis modification of the Bloom and Richardson grading of breast cancer(Elston and Ellis, 1991).

The grade of the ductal cancer has implications for the survival of the patient and treatment decisions may be based partly upon this grade. There are some issues with regards to this method of allocating a grade to the tumour as it is obviously dependent on the observer and it is known that there will be some intra observer variation or discordance. In a recent study it was shown that there was a discordance rate of 28% (195 of 689 samples) in the grade assigned the same tumour but 2 different pathology

centres (Bueno-de-Mesquita et al., 2009). These discordance rates can be reduced by using standardised protocols between units. It has to be remembered that despite this discordance rate grade is still a significant prognostic factor in the outcome of the disease. .

1.1.3.4 Molecular Classification of breast cancers.

With the advent of gene expression profiling in breast cancer tumours can now be classified based upon their genetic signature rather than histological phenotype. Using cDNA microarrays thousands of candidate genes in many archived breast cancers sample have been assessed for the significance of expression up and down regulation (Hyman et al., 2002). By the hierarchal analysis of gene expression in multiple tumours distinct sub-types of cancers have become apparent (SÃ¸rlie et al., 2001). In some of the latest work a sub-set of 306 gene micro array has been used to distinguish 5 and a possible 6^{th} subtype of breast cancers (Hu et al., 2006) see fig 1.5.5.

The following subtypes have been identified and now validated in many other studies;

The Luminal (ER positive) type.

Luminal A, an ER positive predominantly lower grade cancer expressing the highest level of ER α, GATA binding protein A,, X-box binding protein 1, Trefoil factor 3, Hepatocyte nuclear factor 3 α and estrogen-regulated LIV-1.

Luminal B, again an ER positive tumour but lower expression of the luminal type genes with a higher overall grade and poorer outcome than Luminal B cancers.

Lumina C, a subdivision of luminal B but showing expression of a gene subset similar to the Basal and ERBB2+ subtypes.

Non-luminal types (low to absent of ER α and other luminal cluster factors.)

Basal like subtype, characterised by high expression of keratin 5 and 17 and fatty acid binding protein.

ERBB2+ subtype, characterised by high expression of the genes in the ERBB2+ amplicon at 17q22.24 including ERBB2 and GRB7

Normal Breast like subtype, showing high expression of gene associated with adipose and other nonepithelial cell types. They also showed high expression of basal type genes with low expression of luminal epithelial genes. (Perou et al., 2000, SÃ¸rlie et al., 2001)

This molecular classification of breast cancer looking at the alterations in gene expression driving the cancer is important in two particular areas. Firstly the classification into these subtypes has been shown to have important prognostic significances beyond the traditional prognostic indexes discussed later and secondly it is now being realised that these subtype groups are important in predicting the response to adjuvant therapies including chemotherapy (Rouzier et al., 2005). These studies have shown that there is a greater response to chemotherapy in the non-luminal types of breast cancers rather that the luminal types. Factors such as the subtype group are now being taken into account when planning patients adjuvant treatment.

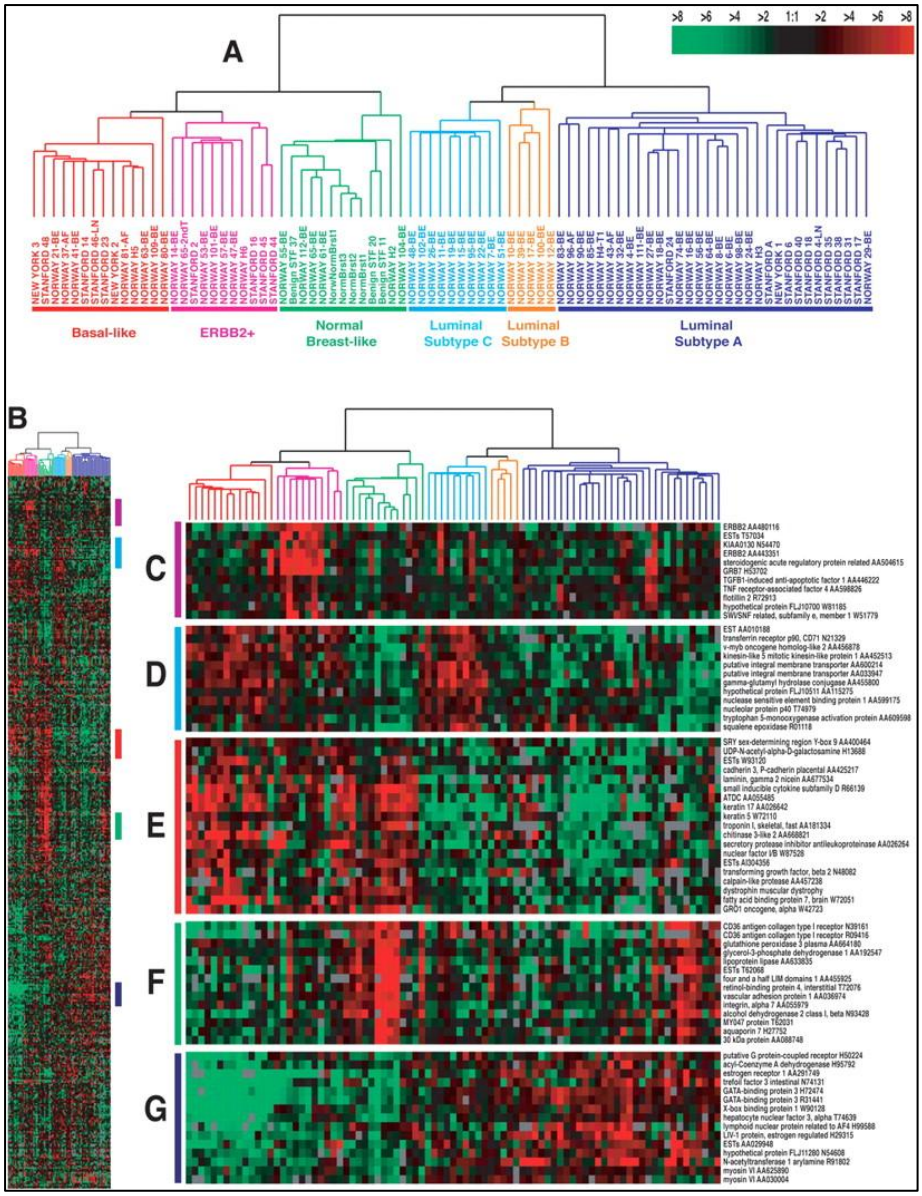

Fig 1.5.5 Gene expression patterns of 85 experimental samples representing 78 carcinomas, three benign tumors, and four normal tissues, analyzed by hierarchical clustering using the 476 cDNA intrinsic clone set. (A) The tumor specimens were divided into five (or six) subtypes based on differences in gene expression. The cluster dendrogram showing the five (six) subtypes of tumors are colored as: luminal subtype A, dark blue; luminal subtype B, yellow; luminal subtype C, light blue; normal breast-like, green; basal-like, red; and ERBB2+, pink. (B) The full cluster diagram scaled down (the complete 456-clone cluster diagram is available as Fig. 4). The colored bars on the right represent the inserts presented in C–G. (C) ERBB2 amplicon cluster. (D) Novel unknown cluster. (E) Basal epithelial cell-enriched cluster. (F) Normal breast-like cluster. (G) Luminal epithelial gene cluster containing ER(SÃ¸rlie et al., 2001).

1.1.4 The staging of breast cancer.

The staging of all types of cancers has a similar aim, that is to group patients according to their disease burden. This grouping of patients allows for prediction of clinical outcome, comparison of like for like patients for the purpose of research and provides a guide for treatment of their disease. To this extent breast cancer is no different. The staging of breast cancer is based on the TNM (tumour, nodes and metastases) system. Once the TNM status has been assessed the patients are placed in a Stage Group. The TNM staging is based on both clinical (examination and imaging) findings and on the pathological findings.

The T stage is based upon the size of the cancer or its invasion of local structures. The size primarily is taken from the pathological excision but, if not available, from the imaging results. The involvement of local structures may be seen on clinical examination, imaging or pathology specimen. There are 4 major groups T1-T4 with subset of both the T1 and T4 groups. See table 1.3.The T stage is also used in the Nottingham Prognostic Index (NPI) discussed later.

Major Heading	Subset	Descritpion.
T1		Tumour 2cm or less.
	T1is	In situ disease only.
	T1mic	Microinvasion < 0.1cm
	T1a	Tumour >0.1cm <0.5cm
	T1b	Tumour >0.5cm <1cm
	T1c	Tumour >1cm <2cm
T2		Tumour >2cm <5cm
T3		Tumour >5cm
T4		Tumour with extension.
	T4a	Chest wall extension not including pectoralis.
	T4b	Oedema or ulceration of the skin.
	T4c	Both T4a and T4b
	T4d	Inflammatory carcinoma.

Table 1.3: The Tumour staging groups for breast cancer

The lymph node stage again may be assessed clinically with examination and imaging but more commonly determined after surgical excision of a sample of lymph nodes. In the UK there has been a shift in the staging of the axillary lymph nodes. Traditionally a sample of nodes was excised during the operation on the breast cancer. This sample may have been a random 4 node sample or a formal level 1 or level 2 dissection. Due to the results of the ALMANAC trial (Mansel et al., 2006), the staging of the axillary lymph nodes is now a more targeted exercise. Pre-operatively the axilla is imaged with USS and any large or structurally abnormal lymph nodes sampled with FNA or core biopsy. If the preoperative staging of the axilla is negative the patient will then go on to a targeted sentinel node sample. This approach reduces the morbidity

associated with lymphoedema in the arm which was seen with axillary clearance (Mansel et al., 2006).

The sentinel node is identified intra-operatively with a combination of blue dye and radioactive isotope injection. The sentinel node or nodes are excised and examined pathologically. If positive sentinel nodes are identified, the axilla is managed with further surgery, an axillary clearance, and radiotherapy. Table 1.4 shows the 3 major pathological lymph node (N) groups and the relevant sub groups.

Distant metastasis (M) may be clinically apparent either on physical examination at the point of diagnosis or on staging imaging such as CT scans of the chest and abdomen or isotope bone scans. Routine staging imaging tends to be restricted to patients who are lymph node positive or are going on to chemotherapy. These results of the staging imaging are valid if carried out within 4 months of the diagnosis of the primary disease if no neo-adjuvant treatment has been used. There are only 3 major M groups; MX metastasis not assessed, M0 no distant metastasis and M1 distant metastasis present.

The TNM groups are then combined to place the patient into one of the overall staging groups see table 1.5. These stages of disease range from Stage 0 to Stage 4. It is more common for the disease stage to be used for the comparison of patient group response to treatment rather than the more involved TNM classification.

Major Heading	Subset	Description
pNX		Regional nodes not available for assessment
pN0		No regional lymph node metastasis.
pN1		Metastasis in 1-3 axillary nodes or internal mammary nodes (not clinically apparent.
	pN1mi	Micro metastasis >0.2mm <2mm.
	pN1a	Metastasis in 1-3 axillary nodes.
	pN1b	Metastasis in internal mammary nodes.
	pN1c	pN1a and pN1b
pN2		Metastasis in 4-9 axillary nodes or clinically apparent internal mammary nodes.
	pN2a	Metastasis in 4-9 axillary nodes (at least 1 deposit >2.0mm.
	pN2b	Clinically apparent internal mammary metastasis in absence of axillary metastasis.
pN3		10 or more involved axillary nodes or internal mammary nodes; or 1 or more ipsilateral supraclaviclar nodes.

Table 1.4: The Lymph node staging groups for breast cancer

Stage Group	**Tumour**	**Nodes**	**Metastasis**
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II A	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage II B	T2	N1	M0
	T3	N0	M0
Stage III A	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage III B	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage III C	Any T group	N3	M0
Stage IV	Any T group	Any N group	M1

Table 1.5: The Stage group of breast cancer.

1.1.4.1 Prognostic Indices.

There is an obvious need to be able to stratify patients into prognostic groups. The TNM staging is, as can be seen in table 1.5, cumbersome and difficult to interpret. Using factors which, independently are of important prognostic value and when combined into a prognostic index and subjected to multi-variant analysis still remain significant, a prognostic tool can be developed. The Nottingham prognostic index (NPI) uses this method to score patients and place them into prognostic group. The three factors which are used in the NPI are tumour size, tumour grade and lymph node status. They are combined in the following formula;

NPI = Tumour size (cm) x0.2 + histological grade (1-3) + Lymph node status*

(* 1= node negative, 2= 1-3 nodes positive, 3= >3 nodes positive)

This formula produced a range of values from 2.08 upwards. Arbitrary cut off points were made at 3.4 and 5.4 to create 3 prognostic groups. Now 6 prognostic groups are recognised. These are; an Excellent Prognostic group (EPG) with an observed NPI score of 2.08–2.4, Good (GPG) 2.42 to $\leqslant$3.4; Moderate I (MPG I) 3.42 to $\leqslant$4.4, Moderate II (MPG II) 4.42 to $\leqslant$5.4, Poor (PPG) 5.42 to $\leqslant$6.4 and very poor (VPG) 6.5–6.8 (Blamey et al., 2007).

Table 1.6 shows the 10 year breast cancer specific survival of patients from 1990. This index has been well validated in many studies and is robust and widely used (Balslev et al., 1994).

NPI Group	10 year survival (%)
EPG	96
GPG	93
MPG I	81
MPG II	74
PPG	50
VPG	38
All	80

Table 1.6: Table showing the 10 yr survival depending on NPI group(Blamey et al., 2007) .

There are a number of other tools used in the breast clinic to aid decision making on an individual patient basis. One of these used extensively by oncologists is "Adjuvant Online." (https://www.adjuvantonline.com.) This online program uses patient details (age and co-morbidities) tumour details (size, grade, lymph node status and hormone receptor status) and proposed adjuvant treatment to provide specific predicted survival and the survival benefit of adjuvant treatment.

1.1.5 An overview on the treatment of breast cancer.

This section gives a brief overview of the current treatment of breast cancer. Some areas will be further discussed in the relevant sections later in the thesis. The treatment of breast cancer is based upon five modalities; surgery, radiotherapy, chemotherapy, endocrine manipulation and biological inhibitors. These modalities can all be used in both the curative and the palliative setting. For simplicity this section will consider the treatment of curable (early) breast cancers.

1.1.5.1. Surgery.

The role of surgery in breast cancer if twofold, firstly it is for the excision of the primary tumour and secondly for the staging and control of the axilla. Primary surgery on the breast can be considered a 2 therapeutic options either a simple mastectomy or breast conserving surgery (BCS) the choice between mastectomy and BCS is dependent on a number of tumour and patient specific factors. Traditionally a mastectomy is advised if the tumour is greater than 40mm in size or is multi-focal (in more than one area of the breast). Some units will have a lower size threshold for mastectomy but what is probably more valid is the consideration of tumour volume to whole breast volume. When BCS is performed then the patient will require whole breast radiation. The combination of BCS and whole breast radiation has been shown in many meta-analysis studies to have an equivalent 10 year overall survival as a mastectomy (EBCTCG, 1995). The introduction of oncoplastic techniques using breast reduction surgery now is pushing the boundaries of what is possible to safely remove whilst preserving the breast. It is now recognised that neo-adjuvant chemotherapy may render some tumours suitable for BCS that were only operable with a simple mastectomy. There has been much discussion over the distance of clear

excision margin required in BCS but it is now generally accepted that >2mm is adequate (Singletary, 2002).

As mentioned earlier the staging of the axilla now involves targeted sentinel lymph node biopsy using blue dye and radio-isotopes. This is usually carried out at the time of the primary breast surgery but may be used alone prior to this if considering neo-adjuvant chemotherapy. The level III axillary clearance is now reserved for the patient who have been identified pre-operatively to have positive lymph nodes or as a second procedure following a positive sentinel node biopsy.

1.1.5.2 Radiotherapy.

Radiotherapy has a key role in the reduction of locoregional recurrence. It is used in the post operative or adjuvant setting commonly in BCS but also to the skin flaps and chest wall in mastectomies with a high risk of recurrence ie large tumours close to the chest wall. Radiotherapy is also used in the axilla and supra-clavicular regions in patients with lymph node involvement. The whole breast radiotherapy is given following CT planning and patent marking and the current regime locally is a dose of 40 Gy given over 3 weeks (15 sessions). Following radiotherapy the reported local recurrence rates in BCS vary from 6-20% at 10 years but it is however accepted that the use of radiotherapy in BCS leads to an 18.5% reduction in local recurrence at 10 years (Fisher et al., 1995)

1.1.5.3. Chemotherapy.

It is now recognised that the use of chemotherapy in the adjuvant setting reduced the risk of recurrence and death in breast cancers (EBCTCG, 2005). The important consideration in the use of this therapy which has a significant morbidity and mortality if the selection of patients who are likely to gain the most with a reduction in the over treatment of low risk patients. The St Gallen meeting 2007 categorised operable breast cancer into risk groups.

Based upon this risk group categorisation recommendations were then made upon the adjuvant treatment. These recommendations were not only for chemotherapy but also for the use of endocrine therapies (tamoxifen and AIs) and Trastuzumab (Herceptin). The consensus suggested that all HER2 positive case chemotherapy was considered. In HER2 negative cases patients of in the high risk group were offered chemotherapy, patients in the low risk group should not be offered chemotherapy. In the intermediate group the hormone receptor negative and the low receptor expressing tumours should be considered for chemotherapy (Goldhirsch et al., 2007).

Risk category	
Low risk[a]	**Node negative** AND **all** of the following features:
	pT* ≤2 cm, AND
	Grade 1**, AND
	Absence of extensive peritumoral vascular invasion[b], AND
	ER and/or PgR*** expressed[c], AND
	HER2/*neu* gene neither overexpressed nor amplified[d], AND
	Age ≥5 years
Intermediate risk[e]	**Node negative** AND **at least one** of the following features:
	pT* >2 cm, OR
	Grade 2-3**, OR
	Presence of extensive peritumoral vascular invasion[b], OR
	ER and PgR absent[c], OR
	HER2/*neu* gene overexpressed or amplified[d], OR
	Age <35 years
	Node positive (1-3 involved nodes) AND
	ER and/or PgR expressed, AND
	HER2/*neu* gene neither overexpressed nor amplified[d]
High risk	**Node positive (1-3 involved nodes)** AND
	ER and PgR absent, OR
	HER2/*neu* gene overexpressed or amplified[d]
	Node positive (4 or more involved nodes)

Table 1.7 Definition of risk categories (adapted from 2007 St Gallen Meeting (Goldhirsch et al., 2007)) Some Panel members view pT1a and pT1b (i.e. pT <1 cm) tumors with node-negative disease as representing low risk even if higher grade and/or younger age [b] Extensive peritumoral vascular invasion (i.e. neoplastic emboli seen in two or more blocks of the tumor) was recognized as a discriminatory feature of increased risk; its presence defined intermediate risk for node-negative disease, but did not influence risk category for node-positive disease [c] Some cases such as medullary carcinoma and apocrine carcinoma may be regarded as low risk despite the absence of steroid hormone receptor expression.[d] HER2/neu gene overexpression or amplification must be determined by quality-controlled assays using immunohistochemistry or FISH analysis.[e] Note that the intermediate risk category includes both node-negative and node-positive 1-3 disease. pT, pathological tumor size (i.e. size of the invasive component); **histologic and/or nuclear grade; ***ER, estrogen receptor; PgR, progesterone receptor.*

The types of chemotherapy prescribed predominantly now fall into two groups, Anthracycline based chemotherapy and Taxane based chemotherapy. The anthracycline agents have largely superseded the older CMF regimes as they have been show to be superior in randomised control trials such as the UK National Epirubicin Trial (NEAT). This trial compares 6 cycles of cyclophosphamide, methotrexate and flurouracil (CMF) with 4 cycles of epirubicin combined with 4 cycles of CMF. This showed that the epirubicin arm had a relapse free survival at 5 years of 91% compared with 85% in the CMF alone arm (Poole et al., 2006).

The taxanes paclitaxel (Taxol) and docetaxel (Taxotere) are active cytotoxic agents against breast cancer. In the metastatic setting they have been shown to be active against anthracycline resistant tumours (Ghersi et al., 2005). In the adjuvant setting the issue of superiority over anthracycline based chemotherapy is unclear and it is probably safe to say that in selected groups (HER2 positive ER negative) the taxanes may offer a survival advantage over anthracycline (Hayes et al., 2007).

1.1.5.4. Endocrine therapies.

The use of anti-estrogen therapies in breast cancer is discussed in detail in the later sections of the thesis. The use of Tamoxifen and Aromatase inhibitors as adjuvant therapy is the mainstay of the treatment in ER positive breast cancers.

1.1.5.5 Biological Modulators.

The use of the anti ERB-2neu receptor antagonist Trastuzumab and other pathway inhibitors are again discussed later in this thesis.

1.1.5.6 Neo-adjuvant treatment.

Radiotherapy, endocrine treatment and chemotherapy can be employed in the neo-adjuvant setting. The aim of this treatment is usually to render a locally advanced inoperable breast cancer operable. There is also the argument that neo-adjuvant chemotherapy and endocrine therapy may be used to move a patient from a mastectomy towards BCS. It has been shown that there is equivalence between neo-adjuvant and adjuvant chemotherapy in terms of survival (Deo et al., 2003). Neo-adjuvant treatment is now more widely accepted as a therapy for patients with borderline BCS of a relatively large tumour to breast volume ratio whom it is clear would be receiving adjuvant chemotherapy (i.e. young node positive high grade tumours.)

There are some issue which need to be considered regarding neo-adjuvant chemotherapy. Firstly it is sensible to mark the location of the tumour within the

breast using a marker clip at time of biopsy; this is because successful neo-adjuvant chemotherapy could render the tumour invisible with a complete radiological response rendering further surgery difficult. It is recognised that patients should undergo pre-treatment axillary staging as the axillary sample post neo-adjuvant therapy is not prognositcally valid. The ongoing assessment of response to the chemotherapy is important and it is suggested that MRI assessment is both a measure of size and metabolic response (Drew et al., 2001). It has to be agree how long to give the neo-adjuvant therapy for and when to decide that the response is not enough to justify further treatment a surgery should be performed. There is also some debate about post operative chemotherapy regimes for both the responders and non-responders to neo-adjuvant treatment.

1.1.6 The epidemiology of breast cancer.

Worldwide breast cancer represents 1/10 of all new cancers diagnosed. It accounts for a quarter of cancers diagnosed in women which equals approximately 1.1 million cases per year (Ferlay, 2004). It is the leading cause of cancer death in women and its incidence is increasing.

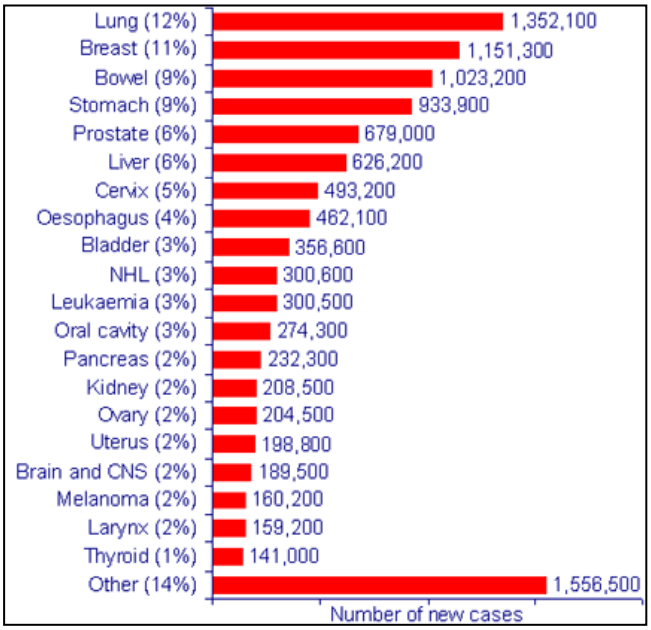

Fig. 1.7: Worldwide incidence of cancer by type(both sexes). (Ferlay, 2004)

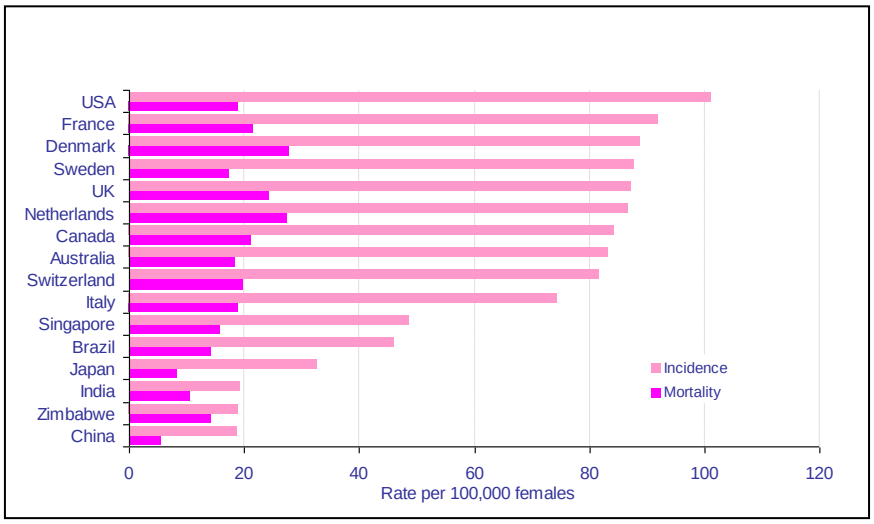

Fig. 1.8: Worldwide incidence and mortality of breast cancer 2002 (Ferlay, 2004)

Breast cancer is considered to be a disease of the western or developed world. The families of migrants from countries with a low incidence of breast cancer will acquire the same risk of developing the disease as the home population within 2 generations of moving to a country with a higher incidence (Ziegler, 1993). There is now also a dramatic rise in the rates of breast cancer in countries with a historically low incidence (Pompe-Kirn et al., 2000, Nagata et al., 1997, Leung, 2002).

In Europe breast cancer is the second most common cancer, to lung cancer, diagnosed with 245,000 women developing the disease in 2000. This represents ¼ of all female malignancies diagnosed (Becker N., 1998).

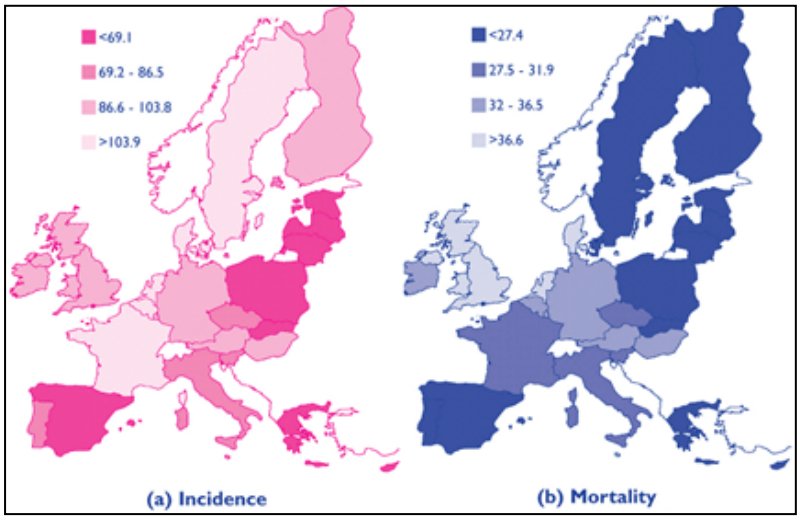

Fig. 1.9: Estimated incidences and mortality of Breast cancer in the year 2000 per 100,000 population.(Becker N., 1998)

In the UK Breast cancer is the most common cancer diagnosed in women. In 2005 there were 45947 new cases recorded, 99% of these cases are in women. This total represents 15% of all cancers diagnosed(2005a, 2005b, 2005c). In the UK due to its high incidence and 5 year survival rates approaching 75% there is a high prevalence

of women living with the diagnosis of breast cancer. It is estimated that there are 172,000 women in the UK alive with this diagnosis(Micheli, 2002).

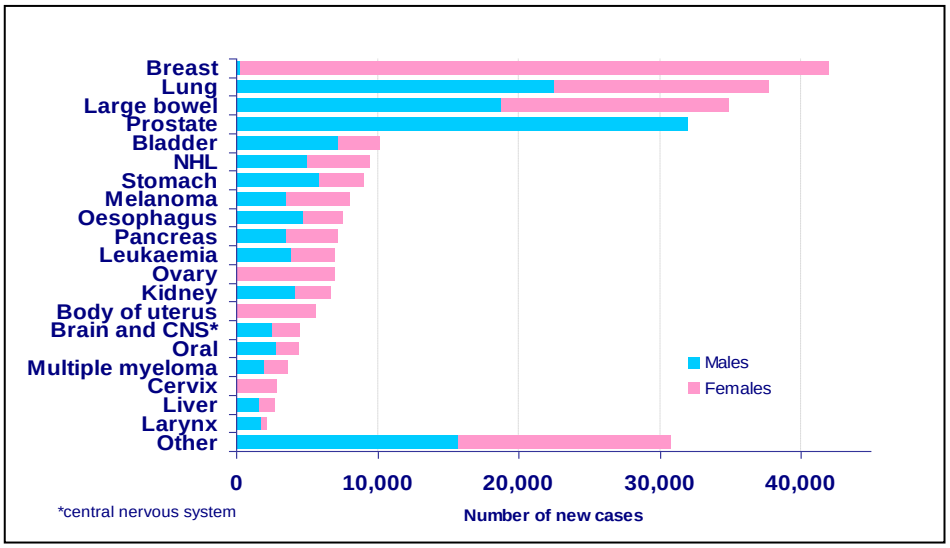

Fig 1.10: UK cancers by type 2002. (Statistics, 2005)

For the past 10 years there has been a fall in the mortality rates in breast cancer. Accounting for 12,696 deaths in 2003 breast cancer was until 1999 the leading cause of cancer deaths in women. It has since then been surpassed by lung cancer (1997b).

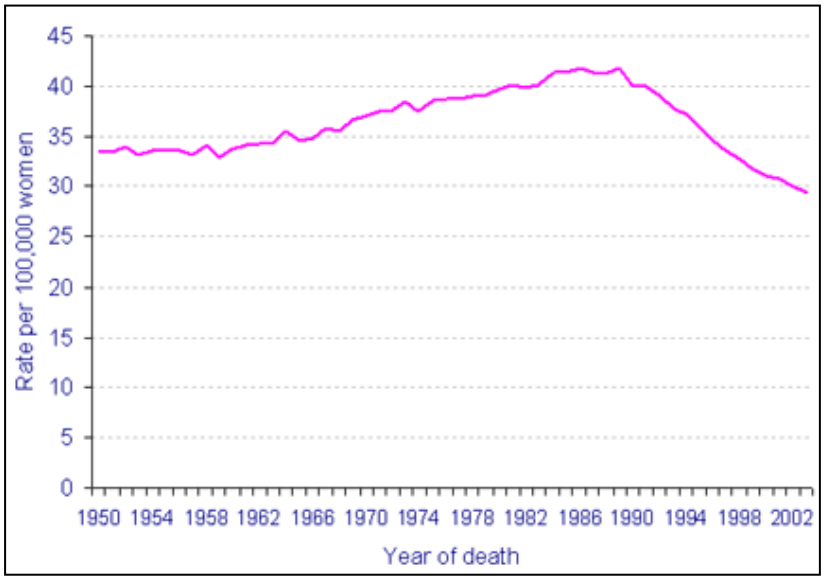

Fig. 1.11: Breast cancer mortality since 1950 per 100,000 population (UK) (1997b).

The survival rates for breast cancer have also been improving. The five year survival for patients diagnosed from 1971 -1975 was 52% compared with the estimated five year survival today of 80%(Coleman, 2004).

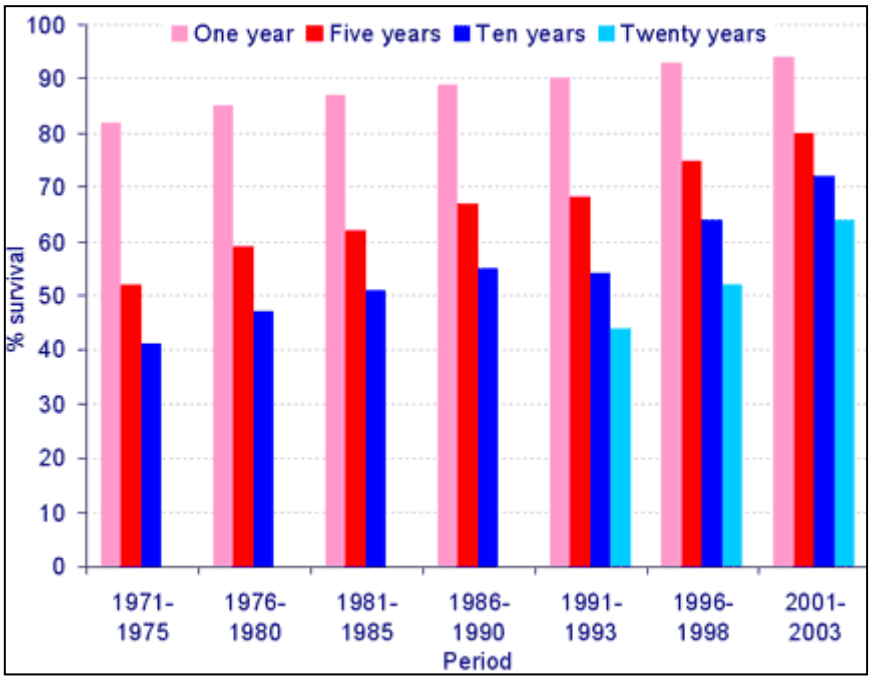

Fig. 1.12: Improvements in breast cancer survival (Coleman, 2004).

The prevalence of breast cancer on a national and worldwide scale and the increasing incidence of breast cancer in the developing world is an indication of the importance of this disease to the global population.

1.1.7 The aetiology of breast cancers.

The study of the epidemiological distribution of breast cancer is the basis of many of the declared risk factors for developing the disease. These risk factors are predominantly related to the breast's life time exposure to estrogen.

Breast cancer is 100 times more common in women than men, following sex age is the next most significant risk factor. As a woman's age increases then so does her likelihood of developing cancer. Before the age of 40 the risk of developing breast cancer is 1/200 this increases to 1/10 by the age of 85.(Statistics, 2005)

Lifetime estrogen exposure is directly related to a woman's reproductive history. Estrogen production by the ovaries increases dramatically after the menarche. If menarche takes place at an earlier age then this estrogen exposure will be more significant. The average age of menarche has been falling in the 20th century from 16-17 years to 12-13 today (Rees, 1995). For every year that menarche is delayed after the age of 12 it is estimated that the risk of developing pre and post menopausal breast cancer is reduced by 7% and 3% respectively.(Clavel-Chapelon, 2002) Estrogen production is suppressed during pregnancy and breast feeding. It has been shown that the younger the age a woman starts her family the lower the risk of developing breast cancer. There is also a risk reduction for every month that a women breast feeds (2002). Nulliparous women have been shown to have a 30% greater risk of developing breast cancer compared to parous women.(Ewertz M, 1990) The later the menopause the greater the risk of breast cancer (Sweeney et al., 2004) and pre-

menopausal women have a higher risk of breast cancer compared with post menopausal women of the same age (1997a).

The prescription of exogenous hormones, in the form of the oral contraceptive pill and hormone replacement therapy (HRT), affect an individual's breast cancer risk. It has been shown that during and immediately after taking an estrogen containing contraceptive pill the risk of developing breast cancer increases slightly, relative risk 1.24 (95% CI 1.15-1.33), but this risk returns to base line after 10 year of cessation(1996). HRT has long been associated with an increased risk of breast cancer. The degree of this was more accurately quantified with the publishing of the Million Women Study in 2003(2003). This concluded that there was an increase in the development of breast cancer if combined HRT was used for more than 10 years following the age of natural menopause with a relative risk increase of 2.31 (C.I. 2.08-2.56) (Collaborators, 2003). It has been estimated that there has been 15,000 extra cases of breast cancer due to estrogen/progesterone containing HRT over the last 10 years.(Collaborators, 2003)

In post menopausal women estrogen production predominantly occurs in adipose tissue but at a much reduced level than in the premenopausal women. A higher body mass index in post menopausal woman is associated with an increase in the risk of developing breast cancer (Bergstrom et al., 2001). This increase in risk is not seen in the pre-menopausal patient (Tehard and Clavel-Chapelon, 2006). It has also been demonstrated that increased physical activity is associated with a decrease in the risk of breast cancer (Tehard et al., 2006).

Alcohol has been associated with an increase in the risk of developing breast cancer. It has been shown that a moderate intake (2 units per day) over a life time increase the risk with an odds ratio of 1.33 (C.I. 1.01–1.74), this is more significant in women with a lower BMI.(Terry et al., 2006)

Diet, especially fat intake, has long thought to be important in the risk of developing breast cancer. The extent of its relationship is debated but it is probable safe to say that there is a causal link between high fat intake and breast cancer (Mattisson et al., 2004).

Unusually for most neoplastic diseases breast cancer is more common in women of a higher social economic group with an odds ratio of 1.2 (C.I. 1.05-1.37) (Robert et al., 2004). This difference is probably related to some confounding risk factors such as reproductive history and nutritional differences. It is suggested that there is a relationship between breast cancer and high protein diets. It is also more likely that women in higher socio-economic groups are older at the time of their first child and have fewer children, again increasing the risk of breast cancer.

Exposure to ionising radiation increases the risk of developing breast cancer. This has been seen in survivors of atomic bombs and those who have had therapeutic radiotherapy for Hodgkin's lymphoma (Carmichael et al., 2003), this later group are now offered additional screening for breast cancer. It has also been noted that air hostesses with a higher occupational exposure to cosmic radiation have correspondingly higher rates of breast cancer, but there may be some confounding factors such as reproductive history (Kojo et al., 2005).

A positive family history is associated with an increase risk of developing breast cancer. It is however important to recognise the over 85% of breast cancer patients have no family history of the disease. In women developing breast cancers aged below 40 who have a family history of breast cancers or other malignancies, the

possibility of an inherited susceptibility to malignancy should be considered. Most cases of hereditary breast cancers are due to mutations in BRCA1 and BRCA2 genes. There are other rarer syndromes associated with the development of breast cancer including Li-Fraumeni syndrome, Cowdens disease, Peutz-Jeghers syndrome and ataxia-telangiectasia heterozygosity (Isaacs et al., 2004). There has been much debate over the exact risk conferred by a BRCA1 or BRCA2 mutation. Early studies in families selected because of their hereditary history suggested a life time risk of breast cancer in BRCA1 carriers was 87% (95% CI = 72-95%)(Ford et al., 1994) and 84% (95% CI = 43-95%)(Ford et al., 1998) in BRCA2 carriers. These studies also demonstrated a high risk of pre-menopausal breast cancer, contra lateral breast cancer and ovarian cancer in BRCA1/2 carriers compares with general population risk. It is however possible that the risk of breast cancer in BRCA1/2 carriers, in a less selected population, may be lower due to reduced penetrance (Struewing et al., 1997).

It has to be remembered that prior to testing a patient for the carriage of one of the BRCA genes careful counselling is required. The patient needs to understand the impact of a positive result on both themselves and their offspring in terms of the risk of developing cancer and potential risk reducing strategies. These strategies may include increased surveillance using mammography or MRI, chemoprevention using tamoxifen or risk reducing surgery including prophylactic salpingo-oophorectomy and prophylactic mastectomy.

It has been shown that screening in BRCA patients using mammography has a lower sensitivity than the general population and it has been suggested that MRI screening should be considered in these patients (Stoutjesdijk et al., 2001). Tamoxifen use has

been associated with a decrease in breast cancers in BRCA2 mutation carriers when started at the age of 35 but this effect is not seen in BRCA1 carriers, it is suggested that this difference is due to the finding of high rates of ER negative cancers in BRCA1 carriers compared to BRCA2 carriers (King et al., 2001). Salpingo-oophorectomy is associated with a significantly statistic reduction in the development of breast cancers in both BRCA1/2 carriers (HR 0.47 95%CI = 0.35-0.64) as well of course reducing the risk of developing ovarian or fallopian tube cancers (Rebbeck et al., 2009).

It is now not uncommon in breast clinics to be referred patients via the genetics services for discussion of risk reducing surgery. There is also a population of patients who though they do not have a documented gene abnormality may well request this surgery on the basis of family history alone. This risk reducing surgery is usually in the form of a mastectomy. This may be a standard mastectomy or a skin sparing mastectomy in conjunction with reconstructive surgery. The reconstructive surgery may be immediate or delayed. It has to be remembered that, as its name suggests, this is risk reducing surgery not risk eliminating surgery. Despite best intentions there will be residual breast tissue remaining after the surgery and breast cancer can arise in this residual tissue. This along with the possibility of occult tumours being discovered within the specimen at pathological sampling has to be discussed with the patient prior to surgery.

Bilateral risk reducing mastectomy has been shown to reduce the risk of breast cancer by 90% alone or by 95% when in conjunction with an oopherectomy (Rebbeck et al., 2004). As the awareness of genetic screening and its use becomes more widespread it

is not unreasonable to expect these cases to become a more common feature in the breast clinic and it is likely that these patients will be best served by a combination of risk reducing strategies (Calderon-Margalit and Paltiel, 2004).

1.2. Hormones, Growth factors and Endocrine therapy in breast cancer.

Breast cancer is one of the most widely studied solid tissue tumours. It is because of this that its molecular biology, in terms of the affect hormones have on this cancer, is relatively well understood. This chapter aims to give an overview of the important hormone receptors found in breast cancers and the different treatment modalities targeted at these receptors.

1.2.2 Estrogen and the Estrogen Receptor (ER.)

Estrogens (oestrogens) are a group of steroid based hormones. Though present in both men and women their role is predominately in the regulation of the ovulatory cycle in women. Produced by the developing follicles and corpus luteum in the ovary and by the placenta their production is under the control of follicle stimulating hormone (FSH) and luteinisng hormone (LH). Other secondary sources of estrogen production include the liver, adrenal glands adipose tissue and breasts, these secondary sources are important in postmenopausal women. The theca internal cells of the ovaries synthesise androstenedione from cholesterol, this is then converted either directly or indirectly via testosterone into oestrone or oestradiol. This final step is catalysed by the enzyme aromatase.

The role of estrogen in the pathogenesis of breast cancer has been recognised for over 100 years. Although the initial treatment of inoperable breast cancer by performing an oopherectomy is commonly attributed to Sir George Beatson in 1896 (Beatson, 1896), this treatment modality was originally described by Albert Schinzinger in 1889, in a

case series of 96 cases of breast cancer treated with oopherectomy (Schinzinger, 1889). He noticed that the 23 patients who were still menstruating showed most benefit with regards to tumour control. He suggested that tumour growth in these younger patients was driven by the ovaries and hence recommended oopherectomy for these patients. One of the first attempted randomised control trials conducted in breast cancers in the Christie Hospital in 1948 was aimed at determining if ovarian ablation using radiation at the time of a radical mastectomy (the treatment group) was superior to ovarian ablation at the time of first relapse (the control group). Though the initial results were not significant, analysis at 15 years did show a significant increase in the length of time to relapse in the treatment group ($p=<0.05$) (Stewart, 1991). This, along with many other early trials into the use of ovarian ablation, noticed that response to treatment occurs in approximately 2/3 of patients, suggesting that there was a yet to be discovered difference between the tumours that responded to this treatment and those which did not. In the early 1960's it was first noticed that estrogens when radioactively labelled concentrated in target organs. It was also noticed that they became especially concentrated in breast cancers (Folca et al., 1961, Jensen and Jacobson, 1962). This work led to the belief of the existence of an estrogen receptor (ER.).

It is now well known that ER is over expressed in approximately 70-80% of invasive breast cancers at the point of diagnosis. The importance of the ER status of a breast cancer was quickly realised. It has been shown that ER positive cancers are of a lower grade, a smaller size, more likely to be node negative and have better survival outcome than ER negative cancers (Aaltomaa et al., 1991, Fisher et al., 1988, Grann et al., 2005).

ER is part of the nuclear hormone receptor (NHR) super family. It belongs to sub-group III which also includes the progesterone and androgen receptors (Laudet, 1997). All of these intracellular hormone receptor are proteins which have the ability to bind to DNA and regulate transcription of associated genes. These NHRs have 5 common domains A/B,C,D,E,F with variations within these domains determining function.

Fig. 1.13: Diagrammatic representation of the common domains of the NHR.

ERα has been localised to chromosome 6. A second ER subtype (ERß) which is smaller than classical ERα has been localised to chromosome 14. Differences between these sub-types in the ligand binding domain, and the smaller size of ERß suggests that they have different physiological properties (Enmark, 1997). It has been show that ERα has a much higher affinity for 17α-estradiol compared with ERß (Kupier, 1997). ERα and ERß are primarily confined to the nucleus with a small proportion of receptors expressed within the cytoplasm and a 36KDa splice variant which is membrane bound (Wang et al., 2006). ERα and ERß have been shown to have a wide variation in the tissue type and proportions in which they are expressed. These tissues include myocardial cells, major arteries, smooth muscle cells of the lungs, glandular tissue in the GI tract, the pituitary and thyroid gland, the female reproductive organs and of course breast tissue.

In its unbound state ER is a monomer found predominantly within the cytoplasm where it is associated with a heat shock protein, HSP90. Heat shock proteins, of which there are many sub types, are virtually ubiquitous in all living organisms. They act as a chaperone to many other proteins including the steroid receptors. They have a regulatory functioning on protein interactions including folding and binding.

Upon activation with estrogen ER undergoes a confirmation change which involves the loss of the HSP. This loss of HSP and conformational change exposes two sites of dimerisation. The ligand binding thus allows the formation of stable ER dimers which may be homodimers or heterodimers depending on the sub-types involved (Smith and Toft, 1993). Gene transcription is then induced when these dimers are shuttled from the cytoplasm to the nucleus where they interact both directly and indirectly with the DNA. ER activity may be up-regulated by its interactions with many receptor co-factors.

We are now beginning to understand the complexity of ER signalling and its actions. The complexity of this ER signalling allows fine regulation of transcription of many ER dependant and ER independent genes. ER can act in a genomic and non-genomic mechanism.

1.2.2.1 Genomic action of ER

ER's genomic actions can be divided into estrogen response element (ERE) dependant and ERE independent actions. The ERE dependant or "classical mechanism" of ER action involves the shuttling of the ER dimer to the nucleus where

due conformational changes it can bind directly to the genomic ERE, recruit co-factors and induce gene transcription (Nilsson et al., 2001).

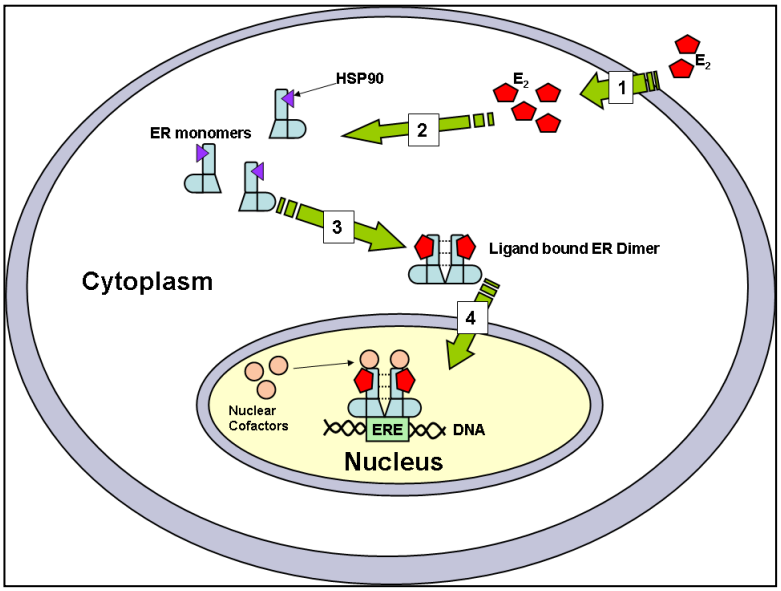

Fig. 1.14: The classical mechanism of ER action: 1. E2 enters cell. 2. Ligand binds to ER. 3. Dissociated HSP allows formation of ER dimer. 4. ER dimer shuttled to nucleus where it binds directly to ERE inducing transcription. (Courtesy of Dr A Davison, Department of Surgery, Newcastle University.)

ER may exert is genomic affects without binding directly with DNA. It has been shown that 1/3 of the genes up-regulated by ER do not contain an ERE. ER can up-regulate gene transcription by protein-protein interactions within the nucleus with other transcription factors (O'Lone et al., 2004). It has been shown the ER can directly bind to, phosphorylate and stabilise the AP1 genes transcription factors c-fos and c-jun (Duan et al., 1999). Other transcription factors that have be shown to be related to ER in this way include Sp1 at the GC-rich promoter sequences (Porter et al., 1997).

1.2.2.2 The None-Genomic action of ER.

Cross talk is a process where there are interactions between different signalling pathways, it is a mechanism that allows a specific ligand associated receptor to utilise a different signalling pathway. ER can cross talk with other cell signalling pathways at the non-genomic level. Studies have shown that ER exists both as a membrane and cytoplasmic associated receptor. They have been shown to be associated with the cytoplasmic scaffold protein caveolin-1 (Chambliss et al., 2000, Kim et al., 1999). At these locations ER may exert their non-genomic effects (Razandi et al., 2002). It is now recognised that estradiol stimulates signal transduction via plasma membrane associated ER that are G protein coupled to EGFR or insulin-like growth factor-1 receptors (Razandi et al., 2003). This leads to initiation of the downstream cell signalling pathways. These cell signal pathways include an increase in the Src kinase cascade and hence activation of MAP-kinase signalling pathway (Wong et al., 2002a). It is also important to note that at this level the cross-talk is bidirectional in nature with ER its self being phosphorylated and activated by MAPK (Kato et al., 1995). This gives a possible mechanism where, using the up regulation of protein kinase cascades due to cross talk at a receptor level, ER can increase its own activation via phosphorylation by MAPK. It has been shown that up-regulation of growth factor signalling is associated with endocrine therapy resistance in ER +ve breast cancers (Gee et al., 2001). There is also an increased dependence on EGFR/MAPK mediated signalling in ER +ve tamoxifen resistant MCF-7 cell lines (McClelland et al., 2001).

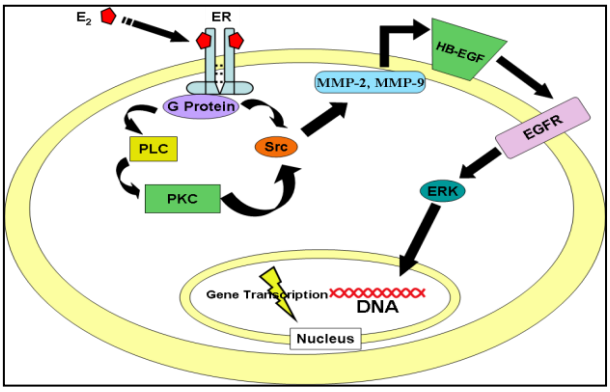

Fig.1.15: Diagram representing membrane protein associated alternative ER signalling pathway.

(Courtesy of Dr A Davison, Department of Surgery, Newcastle University.)

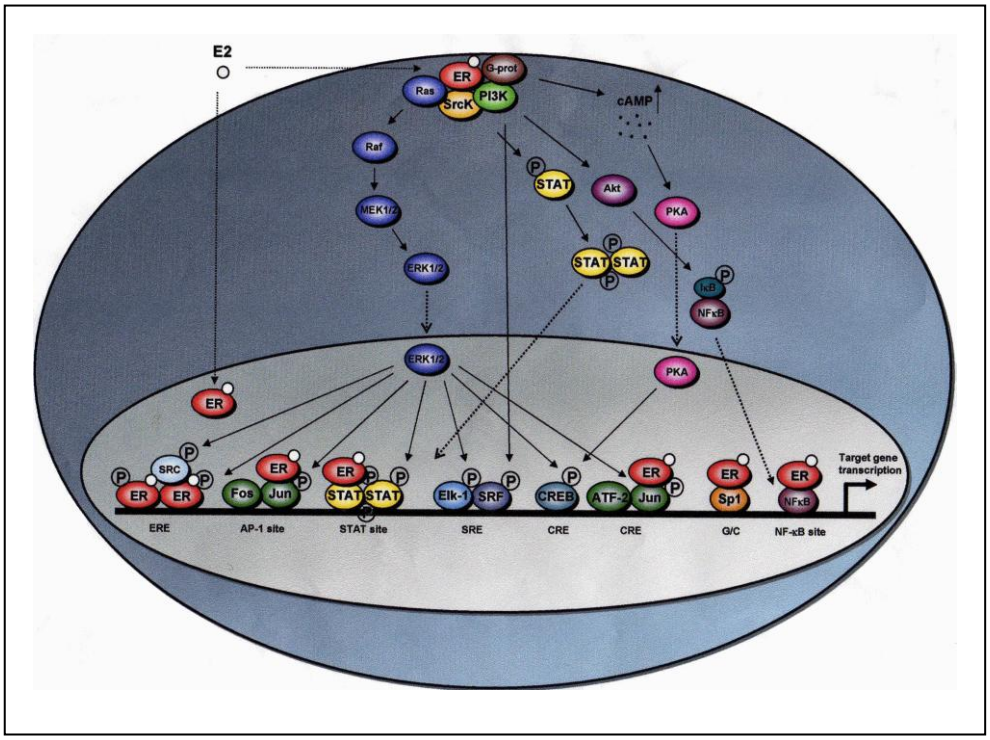

Fig.1.16: Overview of genomic and none genomic ER cross-talk. (Courtesy of Dr A Davison, Department of Surgery, Newcastle University.)

1.2.2.3 Estrogen manipulation in breast cancer.

Endocrine manipulation is an important treatment modality in the treatment of breast cancer. It is used as a primary or neo-adjuvant treatment, as an adjuvant treatment in early cancers and as a therapy for recurrent or metastatic disease. Until recently the most widely used drug of this type is tamoxifen.

Tamoxifen is a selective estrogen receptor modulator (SERM). It has both an anti-estrogenic and estrogenic action and depending on the target organ can be considered as a partial agonist. On binding to ER it alters the receptor's conformational form affecting its interaction with nuclear transcriptional co-activators. This allows tamoxifen to have different actions on different tissues i.e. estrogenic action in bone, increasing bone density and uterine tissue, increasing the risk of endometrial malignancy. Tamoxifen has an anti-estrogenic action in the breast which, for ER positive cancers, has a cytostatic rather than cytotoxic effect (Jordan, 1998, Pace et al., 1997).

Tamoxifen was introduced in 1969 where in advanced disease it introduced remission in some patients (Cole et al., 1971). Tamoxifen is used in advanced metastatic breast cancers, as a primary endocrine therapy and as an adjuvant therapy in early breast cancers. In metastatic breast cancers 50% of patients with previously untreated ER +ve cancers achieve an objective response or tumour stabilisation with tamoxifen (Jaiyesimi et al., 1995). As an adjuvant therapy in early ER positive breast cancer tamoxifen improves overall survival by 26% with a 47% reduction in disease recurrence and a 47% reduction in contralateral breast cancers. There is a four times increase in the incidence of endometrial cancers but in absolute numbers this is half

the number of the prevented cases of contralateral breast cancer (Early et al., 1998). Despite these benefits of tamoxifen treatment almost all the patients with metastatic disease and as many as 40% of patients receiving adjuvant therapy eventually relapse and die from their disease (Ring and Dowsett, 2004). On relapse these tumours are usually found to be resistant to tamoxifen and many of these recurrences remain ER positive on IHC testing (MacFarlane R et al., 2008).

Aromatase inhibitors, now the first choice treatment for post menopausal patients, have an indirect action on breast cancers. As their name suggest their mechanism is to inhibit the conversion of androstenedione into estrone and testosterone in to estradiol. There are two classes of aromatase inhibitors, type I are irreversible steroidal inhibitors (such as exemestane) which form a permanent bond with the aromatase enzyme and type II non-steroidal inhibitors (such as anastrozole and letrozole) which work via reversible competition. Though they have an action on the aromatase in the breast reducing local estrogens they also act in the liver and peripheral tissue. In the pre-menopausal patient the inhibition of this enzyme is “over ridden” by an increase in FSH and LH release increasing the amount of estrogen produced from the ovaries. Initial studies demonstrated that aromatase inhibitors were superior to tamoxifen in inducing remission in patient with advanced disease and in patients with tumour progression whilst on tamoxifen (Milla-Santos et al., 2000). In the adjuvant setting large randomised control trials have demonstrated a reduced recurrence rate compared to tamoxifen alone (ATAC et al., 2002) or following 2 or five year of tamoxifen when compared with tamoxifen alone for five years (Coombes et al., 2004, Goss et al., 2003). These drugs to have side effects including arthralgia and myalgia and the increase of osteoporosis related fractures.

1.2.2.4 Anti-estrogen resistance.

Tamoxifen resistance appears to be complex and multifactorial. There are two main phenotypes of tamoxifen resistance, primary resistance, where a tumour never responds to tamoxifen and secondary or acquired resistance. Acquired resistance is when an initially responsive tumour escapes tamoxifen control. Currently prediction of primary response to tamoxifen is based upon the expression of estrogen and progesterone receptors. Up to 75% of ER+/PR+ tumours will respond to tamoxifen (Clarke et al., 2003) showing that there is a considerable number of ER +ve tumours which demonstrate primary resistance. Of the ER +ve tumours which initially responded to tamoxifen, on relapse less than 25% had lost ER expression (Kuukasjarvi et al., 1996) and many of which would still be classified as ER +ve on IHC (Encarnacion et al., 1993). These results show that there is a population of tumours which whilst expressing ER do not respond as predicted to tamoxifen therapy.

A number of mechanisms have been proposed as to the mode in which an ER +ve tumours becomes resistant to tamoxifen. These include the loss or alteration of ER expression and function (Speirs et al., 1999), the expression of the sub type of estrogen receptor ERβ (Speirs, 1999), alterations in pharmacological properties and tolerance of tamoxifen (Ring and Dowsett, 2004) and alterations in the action of co-regulatory proteins involved in the interaction between ER and the ERE within the target genes (Ring and Dowsett, 2004). It has to be remembered that the tumour is a heterogeneous mass of cells, some of these cell may be ER positive and some may have lost their ER expression. The use of anti-estrogen treatments may well initially

cause a regression and suppression of the disease but over time it may select for the ER –ve cell population. A further mechanism of estrogen resistance has been suggested which involves the interaction or "cross-talk" of ER with other cell signalling pathways. It may be this cross talk between ER and other cell signalling pathways, via the non-classical mechanism discussed above which gives the most complete mechanism for tamoxifen resistance. With the advent of novel therapies concerned with blocking these alternate signalling pathways an understanding of this mechanism may improve the prediction of disease outcome and identify patients who may benefit from these novel therapies.

1.2.3 The Progesterone Receptor.

Progesterone is a steroid hormone that is important in normal development and reproduction. Like all steroid hormones it is synthesised from cholesterol predominantly from the ovaries (the corpus luteum) and from the placenta during pregnancy. Its action is mediated via the progesterone receptor (PR).

PR is a nuclear receptor and there are 2 forms of human PR, PR-α and PR-ß. They are both products of the same gene loci 11q22 (Grigrande et al., 1997). Transcription of this gene is induced by the action of estrogen and ER (Schultz et al., 2003b). Binding of the hormone to the carboxy-terminal hormone binding domain induces conformational changes to the receptor. This causes co-repressor dissociation, co-activator recruitment and binding to the DNA recognition sequences. The use of knockout mice has shown that a lack of PR causes reduced mammary development, uterine hyperplasia and failure to ovulate (Connely et al., 2001).

Both forms of PR are expressed in breast cancers, PR-α expression predominates over PR-ß expression. There is a strong correlation between ER-α expression and PR-α expression in breast cancers with only 19% of tumour positive for only one of these receptors (Reiner et al., 1990). There is evidence that PR expression is associated with well differentiated tumours with a better overall survival (Reiner et al., 1990). Most evidence regarding the prognostic role of PR is based upon the assumption that PR expression indicates a functioning ER pathway (Ravdin et al., 1992). It therefore has been shown that PR positive ER positive tumours have a better response to endocrine therapy than ER positive PR negative cancers (Payne et al., 2008).

Beyond the role of PR in the prediction of response to endocrine therapy, the progesterone receptor has yet to be shown to be of any further prognostic or therapeutic value in breast cancer. Recent guidelines now suggest that PR expression is no longer required to be routinely assessed and only ER status is required in the minimum histopathological dataset as described by the Royal College of Pathologists.

1.2.4 Human Epidermal Growth Factor 2 (Her2/neu).

HER2, also known as ErbB2-neu, is a member of the epidermal growth factor receptor family. This trans-membrane growth factor receptor is a product of the oncogene c-erbB-2. This oncogene is located at 17q21 and amplification of this oncogene results in over-expression of HER2 on the cell membrane (Schecter et al., 1984, Zoll et al., 1992).

HER2 is a "ligandless" or "orphan" receptor, it has no natural ligand. HER2 is activated when it forms a dimer with one of the other epidermal growth factor receptors HER-1, HER-3 or HER-4. On activation HER2 initiates a cascade of signalling via the MAPK, PI3K and phospholipid C pathways, this induces cell proliferation and resistance to apoptosis (Ciocca et al., 2006). The over expression of HER2 is reported to occur in 10-30% of invasive breast cancers (Ciocca et al., 1992). Over expression of HER2 is associated with higher grade tumours (Sjorgen et al., 1998), ER negative tumours (Gago et al., 2006) and poorer overall survival (Yamauchi et al., 2001).

HER2 over expression in breast cancer is a target for systemic biological therapy. The monoclonal antibody trastuzumab (Herceptin; Genetech, South San Francisco, CA, USA.) is directed against the HER2 receptor. Large trials have shown this drug's effectiveness both in the metastatic and in the adjuvant setting in disease response and improved recurrence rates (Slamon et al., 2001, Joensuu et al., 2006, Romond et al., 2005, Smith et al., 2007). The National Institute of Clinical Excellent has now approved the use of trastuzumab in both the adjuvant and metastatic setting.

1.2.5 EGFR in breast cancer.

Epidermal growth factor EGF was one of the first growth factors isolated when it was discovered in new born mice by Stanley Cohen in 1962 (Cohen, 1962). It was then later in 1975 that it was isolated in humans (Cohen and Carpenter, 1975, Gregory, 1975) and it was not until 1980 that the EGF receptor (EGFR) was isolated (Cohen et al., 1980).

EGFR is a receptor in the ErbB family of membrane bound receptor tyrosine kinases. There are four members of this family, EGFR (HER-1/ErbB1), HER-2 (ErbB2 or neu) HER-3 (ErbB3) and HER-4 (ErbB4). These receptors, apart from HER2, become activated by ligand binding. On activation these receptors form homo or hetero dimers with other receptors in the same class. The dimerisation causes subsequent tyrosine phosphorylation initiating a cascade of signalling pathways shown in fig.1.17 (Lo et al., 2006). Ligands which activate EGFR include EGF, Transforming growth factor α (TGFα) and Heparin-binding EGF-like growth factor (HB-EGF). It has been proposed that EGFR has a second direct pathway of signalling when the receptor translocates to the nucleus where it acts as a transcriptional co-factor (Lin et al., 2001). The action of EGFR activation is both proliferative and anti-apoptotic.

The role of EGFR in breast cancer has been widely studied. It has been reported that the rate of expression of EGFR in breast cancers varies widely depending on the study from 14% to 91% (Klijn et al., 1992). This variation is due to the different methods of analysing the tumours for expression of EGFR and the variation in patient tumour samples studied.

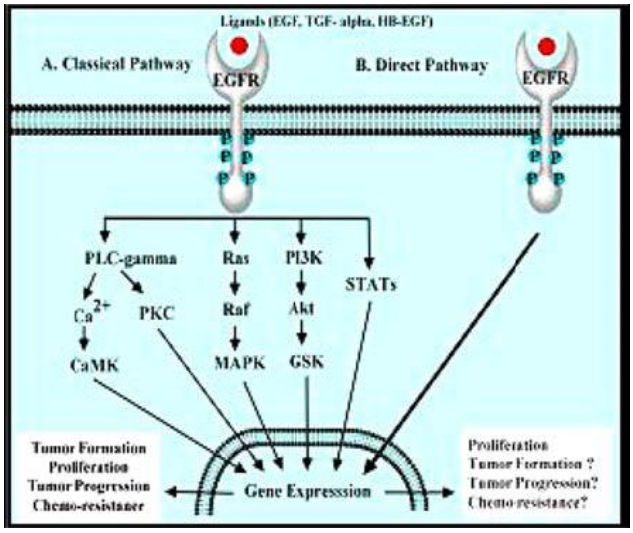

Fig.1.17: The EGF signalling pathway; A the traditional pathway involving transduction cascades and B the direct pathway involving translocation of activated EGFR to the nucleus (Lo et al., 2006).

It is suggested that EGFR expression is linked to higher grade, more aggressive tumours but not to lymph node involvement (Zeillinger et al., 1993). EGFR expression is associated with ER and PR negative breast cancers (Zeillinger et al., 1993, Klijn et al., 1994). EGFR expression in breast cancer has also been linked to poorer disease outcome in respect to both disease recurrence and overall survival (Pawlowski et al., 2000).

As EGFR plays an important role is breast cancer aetiology it is an obvious target for anti-cancer therapy. Gefitinib (Iressa; Astra-Zeneca) is a selective inhibitor of the tyrosine kinase portion of the EGF receptor. Initially licensed for the treatment of advanced non small cell lung cancer it has been trialled in the treatment of breast cancer.

1.3. Transcription factors.

1.3.1 Introduction to transcription factors.

Cells alter their behaviour by responding to extra cellular signals. These signals may be in the form of biological molecules such as hormones. The way a cell recognises these signals or hormones may involve receptors for these signals both on and in the cell itself. Once the signal has been recognised a common mechanism for that signal to be translated to an affect is via the up or down regulation of transcription, transcription being the mechanism in which DNA is copied into mRNA prior to the synthesis of proteins.

This transcription may be directly regulated by a receptor complex, such as the ERE rich genes controlled by classical ER signalling, or, in some occasions this regulation is carried out via an intermediary protein, a transcription factor. These transcription factors are small proteins which recognise and interact with specific DNA sequences located adjacent to the gene under its control (Latchman, 1997). They act by either promoting or repressing the transcription of DNA to RNA by recruiting the enzyme DNA polymerase. All transcription factors have at least one specific DNA binding domain (DBD) (Ptashne and Gann, 1997).

It is thought that there are approximately 2600 coded proteins in the human genomes with DBD which may act as transcription factors (Babu et al., 2004). Some genes require a combination of transcription factors to regulate them this gives these 2600 transcription factors the ability to uniquely regulate the large number of genes in the

human genome. This massive group of transcription factors are divided into 5 super family sub groups depending on the functional similarity of the factor.

Transcription factors, like all proteins are a product of gene transcription themselves, and there are occasion when a transcription factor can control, usually inhibiting, its own transcription. As transcription factors play such a fundamental role in the control of gene transcription and hence cell behaviour it is not unreasonable to suggest that abnormalities in their function may result in pathological changes. As these pathological changes include the up regulation of pro-proliferative genes (oncogenes) or the down regulation of anti-proliferative genes (tumour suppressor genes) it is clear that some alteration in transcription factor expression or function may result in the development of malignancies.

For the purpose of this study 3 transcription factors in different family groups have been selected, the reason for the selection of these 3 transcription factors is because of suggested properties which may make them of some importance in the biology of breast cancer. The transcription factors selected are; STAT3, SP1 and NFkB. The rational for the choice of these three transcription factors is expended on in the next section of the thesis but briefly, STAT3 has been studied in solid cancers and has been shown to act as an oncoprotein, it controls the transcription of genes important to cell cycle regulation and in breast cancer is associated with poor prognostic tumours. SP1 is important to the transcription of the estrogen receptor and ER-SP1 complexes are involved with the expression of other cell proliferation receptors, ER-SP1 complexes may also have a role in Tamoxifen resistant cancers. NFκβ have a role in proliferation in ER negative tumours and in metastatic behaviour of breast cancer cell lines. Other transcription factors were considered such as c-fos and c-jun but previous work

conducted at the QEH had demonstrated difficulties in showing any difference in levels of expression of these transcription factors in breast cancers when using IHC. It was felt that these 3 transcription factors were diverse enough to explore the different mechanism of tumour behaviour whilst also having previously been shown to be relevant to breast cancers.

1.3.2 STAT3

Signal Transducers and Activators of Transcription (STAT) are a diverse family of transcription factors involved in cell cycle regulation. They are cytoplasmic proteins that are essential for the normal cellular responses to cytokines and growth factors. STATs are activated by receptor associated kinase phosphorylation (Clevenger, 2004) and move to the nucleus where they act as transcription factors. There are functionally 2 different groups of STATs, firstly STAT 2, 4 and 6 which are activated by cytokines and play an important role in the development of T-cells and IFNγ, secondly STAT 1, 3 and 5 which are activated by various growth factor and are involved in mammary gland development. It is this second group which are considered to be of importance in breast cancer biology (Calo et al., 2003). STAT1 expression in breast cancer has been demonstrated to be a favourable prognostic indicator relating to its role in growth arrest and pro-apoptotic signalling pathways (Widschwendter et al., 2002).

STAT3 is classified as an oncoprotein because constitutively active STAT3 can mediate oncogenic transformation in cultured cells and tumorigenesis in nude mice (Song et al., 2004). The inactivation of STAT3 is lethal during embryological development of mice (Takeda et al., 1997). Blockage of STAT3 signalling results in growth inhibition and apoptosis of STAT3 +ve cells in vivo and in vitro (Turkson and Jove, 2000, Burke et al., 2001). STAT3 is activated by phosphorylation at the tyrosine residue 705 (tyr705). Activation induces the formation of tyrosine phosphorylated STAT3 dimers which trans-locate to the nucleus. In the nucleus it binds to specific STAT3 DNA response elements directly controlling gene expression (Bromberg and Darnell, 2000, Levy and Darnell, 2002). Modulation of and maximal transcriptional activity of STAT3 may occur via secondary phosphorylation at a serine residue

(ser727) (Wen et al., 1995). This is usually a rapid well controlled and transient process. STAT3 activation is predominantly via the Scr and Janus Kinase (JAK) pathways which occur downstream of cell membrane receptors, especially the EGFR and IL6/gp130 receptors (Berishaj et al., 2007, Garcia et al., 2001).

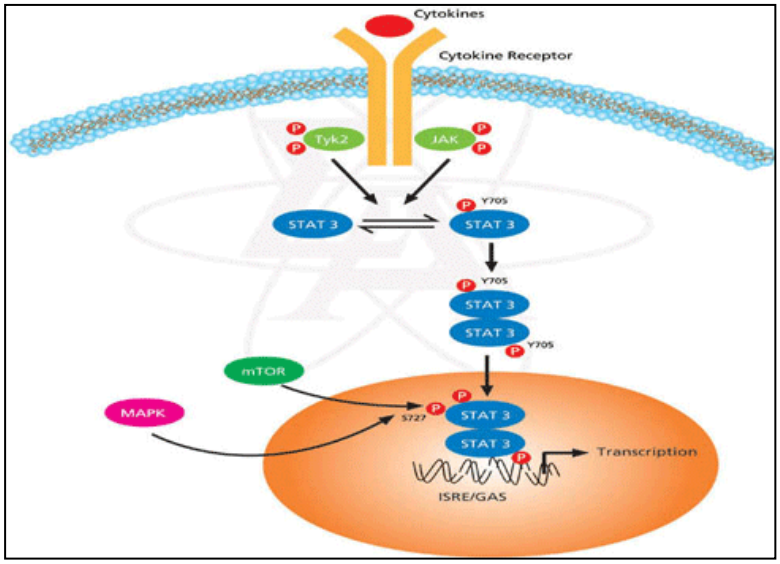

Fig. 1.18: Diagram representing the activation of STAT3 via cytokine induced phosphorylation. (courtesy of Sigma Aldrich signalling)

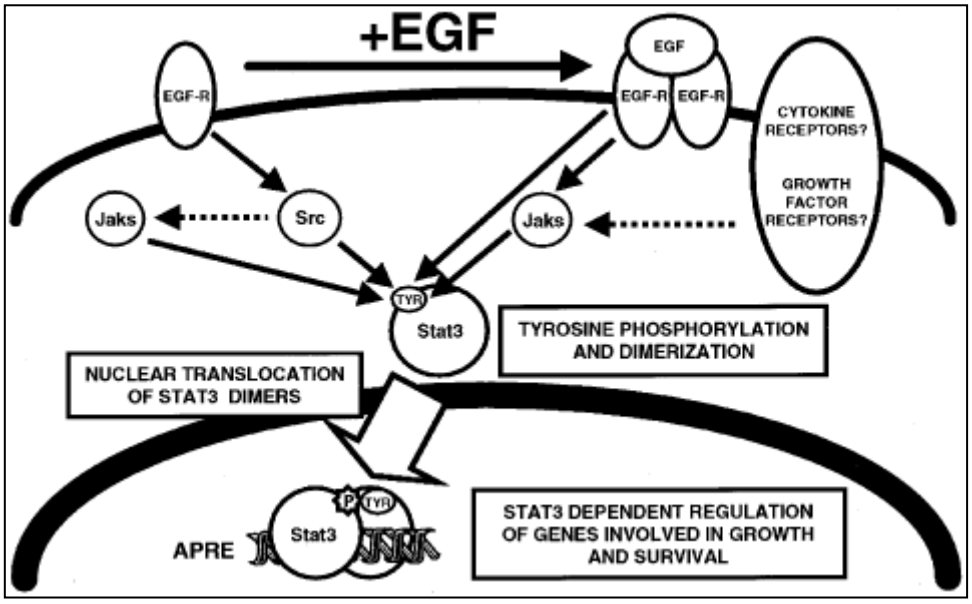

Fig. 1.19: Diagram showing mechanism of EGF induction of STAT3 activation. (Garcia et al., 2001)

Genes under the influence of STAT3 transcription include cell cycle progression and apoptosis control genes such as Cyclin D1, Bcl-xL and c-Myc (Bromberg et al., 1999). STAT3 has been implicated in many of the components of malignancy (Aggarwal et al., 2009). STAT3 activation has a role in mediating inflammation; it has been shown to be an acute phase protein (the area of its initial discovery,) (Pfitzner E et al., 2004), it is activated by pro-inflammatory agents such as IL-6 (Zhong et al., 1994) and has been shown to interact with NFκβ which is another inflammatory mediating transcription factor (Hoentjen et al., 2005). The transformation of cells by viruses and various oncogenes accompanies STAT3 activation, this is seen with the Hep-C virus (Yoshida T et al., 2002) and with the T-cell lymphotropic virus (Migone et al., 1995). As mention STAT3 activation can suppress apoptosis, conditional inactivation of STAT3 has a pro-apoptotic action in mammary gland involution (Chapman R et al., 1999), the suppression of apoptosis is via the products of genes including bcl-xl, bcl-2, survivn Mcl-1 and cIAP2 (Kanda et al., 2004). Activation of STAT3 can lead to cell proliferation due to the induction of cyclin D1(Masuda et al., 2002), it has also been show to upregulate the expression of other growth promoting genes such as myc (Kiuchi N et al., 1999). STAT3 activation can also mediate cellular invasion due to the regulation of matrix metalloproteinase (MMP1 and MMP2) which mediate cell invasion (Xie et al.). There has been shown to be a link between STAT3 activation and VEGF expression mediating angiogenesis and down regulation of STAT3 suppresses VEGF and down regulates angiogenesis (Li et al., 2006). Persistent or uncontrolled activation of STAT3 has been seen in solid tissue malignancies including myosarcomas, prostate, ovary and head and neck cancers (Song and Grandis, 2000, Yu and Jove, 2004).

Specifically in breast cancer STAT3 activation has also been shown to be involved in breast cancer proliferation (Li and Shaw, 2002). Persistent activation of STAT3 in breast cancer cell lines has been shown to induce survivin gene expression and confer resistance to apoptosis (Gritsko et al., 2006a). It has been shown that phosphorylation can be mediated by estradiol and ER via cross-talk with MAPK, Src-kinase and PI3-kinase cascades.(Yamamoto et al., 2000b, Bjornstrom and Sjoberg, 2004). STAT3 activation is also stimulated by EGFR and this activation contributes to growth and survival of breast cancer cells in culture (Garcia et al., 2001, Berclaz et al., 2001). In breast cancer STAT3 ser727 phosphorylation has been shown to be associated with ER negative tumours (Yeh et al., 2006a). STAT3 activation, in high risk breast cancers, is also associated with poor response to chemotherapy (Diaz et al., 2006b).

There is limited evidence of the use of STAT3 as a prognostic marker with one study demonstrating a correlation of activated Phospho-STAT3 with improved survival in node negative breast cancer patients (Dolled-Filhart et al., 2003a). There are no studies which have assessed the correlation of STAT3 expression with tamoxifen resistance in ER +ve breast cancers and no large studies in unselected breast cancers aimed at ascertaining its role as a prognostic marker.

1.3.3 Sp1

Sp1 is a sequence specific transcription factor that recognises GC rich promoter sequences. It is a product of the Sp1 gene which maps to chromosome 12q13(Dynan and Tjian, 1983, Kadonaga et al., 1987). Sp1 is a member of a family of Specificity Proteins along with Kruppel-like factors which all bind to the GC rich promoter genes. They are catagorised by their similar modular structure SP1-SP4 form one sub-group with SP5-SP8 are a structurally similar but truncated group. This family of transcription factors playes a critical role in the normal development of tissues and organs (Safe and Abdelrahim, 2005). Expression of SP1 varies between tissues by at least 100 fold (Saffer et al., 1991). As with STAT3, Sp1 is an essential transcription factor controlling the expression of genes related to cell growth, survival and angiogenesis. On binding to these GC boxes in the promoter regions it is activated by phosphorylation (Jackson et al., 1990). Genes containing these regions can be regulated by interactions of Sp1 with ERs (Porter et al., 1997) and the number of genes known to be regulated by estradiol via these ER-Sp1 complexes is increasing. They include cyclin D1 (Castro-Rivera et al., 2001), c-fos (Duan et al., 1998), bcl2, IGF binding protein and DNA polymerase α (Khan et al., 2003). There is also evidence that these interactions between ER and Sp1 are important for the expression of TGFα, EGFR and progesterone receptor (Khan et al., 2003, Schultz et al., 2003a). It has also been shown that the Sp1 is essential for the transcription of the ERα gene (deGraffenried et al., 2002). It is known that ER-Sp1 complex activation does take place in the presence of SERMs (tamoxifen) (Saville et al., 2000). With regards to angiogenesis it has been shown that in some cancer cell lines Sp1 expression regulated the transcription of the VEGF receptor and that angiogenesis was mediated

by Sp1 via this induction of transcription (Shi et al., 2001). It appears that the transcriptional action of Sp1 is dependent on its interaction with multiple co-factors. These co-factors may be other transcription factors, NFκβ or c-jun, enzyme dependent modulation by phosphokinases etc or chemical induced modulation.

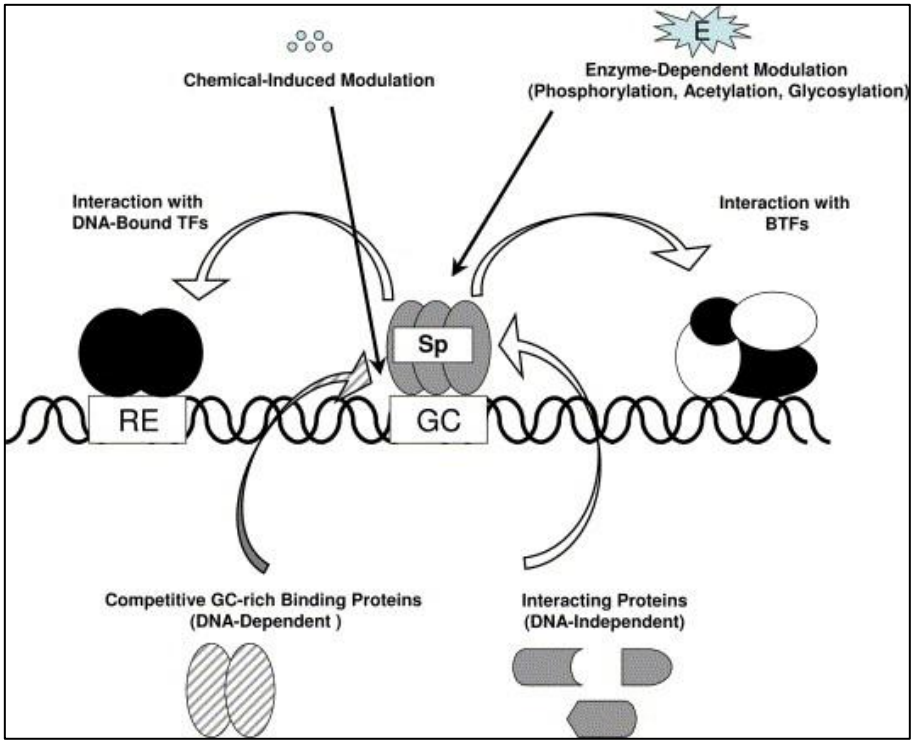

Fig. 1.19: Proposed model for regulating Sp1 dependent transactivation and pathways that modulate this response (Safe and Abdelrahim, 2005)

There is little published research into the over expression of SP1 in breast cancer samples, in one study 11 out of 14 breast cancers expressed SP1 compared with 1 out of 5 benign breast lesions (Zannetti et al., 2000). Other solid tissue tumours do however appear to over express Sp1 to varying degrees including 54% of gastric cancers (Zhang et al., 2005b), 31% of colon cancers (Maurer et al., 2007) and 45% of pancreatic cancers (Jiang et al., 2008). Due to its role in the transcription of the ERα

gene Sp1 will play an important role in the behaviour of breast cancers and may provide a target for future therapies. Despite this few studies have investigated its use as a potential prognostic marker in breast cancer. In gastric cancers there is an association of decreased survival in patients over expression Sp1 compared to patients with weak Sp1 expression (Wang et al., 2003) and it has been suggested that this may be an important prognostic marker. The inclusion of this prognostic marker to this study will allow the exploration of the use of Sp1 as a prognostic marker in breast cancer.

1.3.4 Nuclear Factor Kappa β (NFκß)

NFκβ is a transcription factor first identified in 1986 named because of its binding to the immunoglobulin kappa light chains in B cells (Aggarwal, 2004). It was initially thought only to be present in B cell, later it was found to be ubiquitous in all cells. NFκß is a transcription factor activated in a variety of cell survival settings (Pahl, 1999). NFκβ has many actions it is important in the inflammatory response after cell injury but in malignancy its predominate role is the activation of anti-apoptotic gene expression, it also plays a role in cell proliferation, adhesion and angiogenesis (Karin et al., 2002). Prior to activation NFκß exists as a hetero-dimer in the cytoplasm. It is bound to inhibitory sub-units known as Iκβs. The Iκβs bind to the nuclear localisation sequences causing its retention in the cytoplasm. It is activated via phosphorylation of the Iκβ and its subsequent degradation allowing translocation of NFκβ to the nucleus (Romashkova and Makarov, 1999). Activation of NFκβ is a tightly controlled event upon stimulation by various factors. Known activators of NFκβ include pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) interlukin-1β (IL-1β), epidermal growth factor (EGF) T and B cell mitogens, viral proteins and physical and chemical stresses such as ionising radiation and chemotherapeutical agents. It is suggested that in a neoplastic setting molecular alterations may lead to loss of this tight control and the presence of constitutively active NFκβ (Sethi et al., 2008).

Again like the other transcription factors NFκβ has been implicated in carcinogenesis due to its key role in cell survival, cell adhesion, inflammation differentiation and cell growth. NFκβ mediated the genes of growth factors including TNFα, IL-6 and IL-1β. These have been shown to be important in the growth of tumours such as

glioblastomas (TNFα) (Aggarwal et al., 1996) and head and neck cancers (IL-6) (Kato et al., 2000). Along with growth factors NFκβ also is important in the regulation of cyclin D1 a cell cycle regulatory protein (controls progression of cells from G1 into S phase) (Foehr et al., 2000). NFκβ activation has been shown to have anti-apoptotic properties in T-cell lymphomas, melanomas, pancreatic cancers (Aggarwal, 2004). It has also been implicated in a role of invasion of tumour cells, angiogenesis and cancer metastasis. See fig 1.20

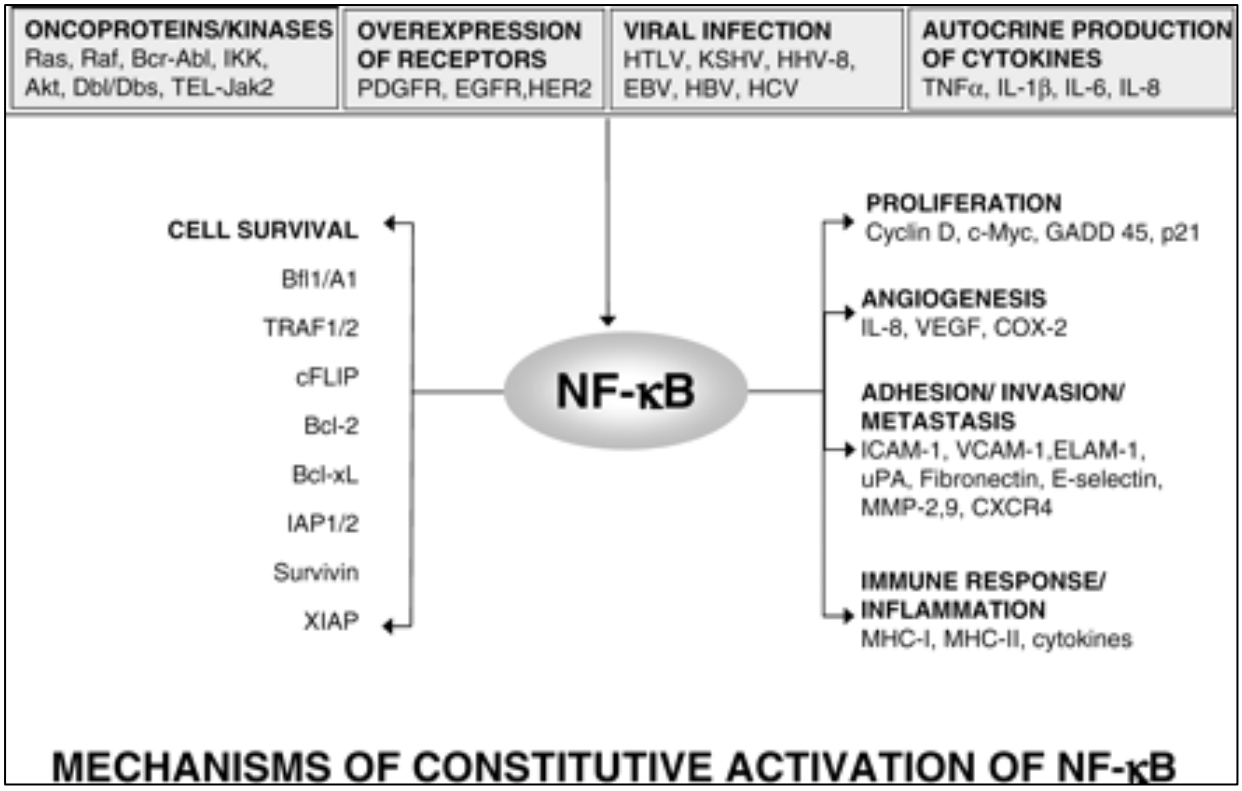

Fig 1.20 diagram showing both the multiple factors activating NKκβ and the effect of constitutive action leading to malignancy(Sethi et al., 2008) .

Transcripts of NFκß regulated genes are found to be elevated in breast tumours when compared to the surrounding tissue indicating functional NFκß activity (Cogswell et al., 2000). Estradiol activation of Akt signalling leads to an increase in gene

expression containing NFκß binding sites (Kawagoe et al., 2003).NFκß expression is associated with the over expression of Bcl-2 in tumours with poor prognosis and may represent an important mechanism for the promotion of malignant behaviour in MCF7 cells (Ricca et al., 2000). Activation of NFκß up-regulates the expression of matrix metalloproteinases, urokinase-type plasminogen activator and cytokines in highly metastatic breast cancer cell lines and may be implicated in the metatasis of breast cancers in vitro (Helbig et al., 2003). NFκß has been shown to have a major role in cell proliferation in ER –ve breast cancers (Nakshatri et al., 1997), with certain breast cancer cells relying on this pathway for proliferation and avoidance of apoptosis (Biswas et al., 2004). EGFR +ve breast cancer cells also demonstrate an increase basal activity of NFκß (Biswas et al., 2000). There is some evidence that inhibition of NFκß can restore anti-estrogen induced apoptosis in anti-estrogen resistant breast cancer cells (Riggins et al., 2005). NFκß has been considered as a prognostic marker of disease outcome in breast cancer, though it does appear to be important in the prediction of response to chemotherapy (Montagut et al., 2006b) there are few papers describing its role in overall disease survival.

2. Aims of this Study.

As described in the introduction and background, breast cancer in comparison with other solid tissue tumours, has been extensively studied at the molecular biological level. Despite the knowledge accumulated of the years there are still patients who appear to have relatively good pathological prognostic features at presentation whom do poorly and vice versa. This is an indication that the current understanding and application of prognostic pathological and molecular markers, though useful, is not complete.

In earlier studies, the transcription factors STAT3, Sp1 and NFκß, though quite different in nature, have all been shown to have a possible role in breast cancer pathology. It may be hypothesised that as these transcription factors are so fundamental to the behaviour of both the normal and malignant cell they may well provide valuable information on the outcome of disease in breast cancer patients.

There are two parts to this study; a clinical study and a cell model study.

The aim of the clinical study is to identify the level of expression of the 3 transcription factors in a cohort of unselected breast cancer patients who are representative of the full range of breast cancer as a disease. It is then proposed that the expression of these transcription factors will be correlated to the pathological features of the tumours.

The tumours will be assessed for their expression of the hormone receptors; ER, PR, HER2 and EGFR. This will allow correlation of the expression of these receptors to that of the transcription factors. The culmination of this part of the study will be the correlation of the expression of these transcription factors with the outcome of patients with respect to disease free interval and overall survival.

It is proposed that the second part of this study will aim to ascertain the change in expression of STAT3 in breast cancers cell lines under the influence of estrogen and epidermal growth factor (EGF) stimulation. To do this 3 different cell models will be used to test the hypothesis that Estrogen and EGF not only induce STAT3 phosphorylation but that this action may be interlinked.

It if felt that it is important to understand the action of estrogen and EGF on STAT3 activation as both of these hormones are currently targets for endocrine manipulation in the treatment of breast cancers. Without greater understanding of the action of these hormones on STAT3 activation cell behaviour may be inadvertently influenced via STAT3 when we use endocrine therapy in these tumours. Some of these changes in cell behaviour via STAT3 may adversely affect the disease outcome.

To summarise the overall hypothesis for this study is;

Transcription Factors STAT3, Sp1 and NFκβ are important prognostic factors in breast cancer survival and that STAT3 induction may change depending on stimulation with estrogen and EGF.

3 The Clinical Study

The aim of the clinical study is to firstly ascertain the expression of the 3 transcription factors, STAT3 Sp1 and NFκβ, in an unselected group of breast cancers. Then it is to go on and correlate this expression with known prognostic factors and also to disease recurrence and overall survival.

The hypothesis is that increased expression of STAT3, Sp1 and NFκβ is associated with endocrine resistance, disease recurrence and poorer survival.

This section of the study was conducted with ethical approval for the retrospective testing of breast cancer samples with novel transcription factors. It was approved with an amendment to prior ethical approval via the Local Regional Ethical Committee. LREC ref: 52/02.

3.1 Methodology of Clinical Study.

3.1.1 Selection of the patient group.

The breast unit at the Queen Elizabeth Hospital serves a population of 200,000 people in the South Tyne region. It is also a regional breast screening centre. Annually the team of 2 consultant surgeons, breast radiologists, pathologists, oncologists and breast care nurses treat approximately 250 cases of screen detected and symptomatic invasive breast cancers. The group of patients treated represents a sample of both symptomatic and screen detected cancers which would be expected at any large unit in the UK. The treatment of these patients follows nationally agreed protocols and guidelines. The application of these protocols is tailored to the individual patient following case discussion at the weekly multi-disciplinary team meetings.

To allow the collection of adequate follow-up data it was decided that a retrospective patient sample group should be derived from the patients diagnosed with invasive breast cancer in 1999. At the time of this study this would allow 7 years of survival data to be collected.

The pathology database at the Queen Elizabeth Hospital was interrogated to generate a list of every sample of breast tissue received in 1999. From this list the pathology report for each sample was retrieved, from these reports it was possible to identify all the patients with a diagnosis of invasive breast cancer. As the aim of this study involved the retrospective testing of breast cancer samples, the patients who were managed non-operatively were excluded from this group due to inadequate tissue volume. The H&E case slides and paraffin embedded blocks of tissue were then

retrieved from storage. All the case slides were then reviewed by a breast pathologist to confirm the diagnosis of invasive breast carcinoma and select representative blocks for study.

3.1.2 Collection of patient data.

Patient data was obtained by a review of the hospital notes for all the patients identified in the study group. The following data was obtained for each patient;

- Sex, date of birth, age at diagnosis, presentation (screening vs. symptomatic).
- Consultant, date of operation, nature of surgery.
- Histological type of cancer, tumour size, tumour grade, measured clearance margins, presence of vessel space invasion, nodal status.
- Neo-adjuvant treatment, adjuvant chemotherapy regime, adjuvant radiotherapy, endocrine therapy, duration of endocrine therapy, switching of endocrine therapy.
- Disease outcome, date of disease recurrence, site of recurrence, treatment for recurrence.
- Survival, date of death, cause of death, length of follow up, discharge date.

There were a number of patients whom were lost to hospital follow up at the Queen Elizabeth Hospital. Every effort was made to complete this data collection, this included contacting the last known general practitioners, writing to other hospitals when a patient had moved out of the area and contacting responsible consultants when a patient had been cared for in the private sector.

3.1.3 Principles of immunohistochemistry.

Immunohistochemistry (IHC) is based on the principle of the specific binding of antibodies to antigens, usually proteins, within a tissue sample. The antibodies can be classified as monoclonal, recognising only one epitope of the antigen of interest, or polyclonal which may bind to more than one epitope. The antibodies themselves are not visible using light microscopy therefore they have to be attached to a dye or enzyme that allows visualisation. Antibodies may be primary conjugated i.e. already attached to a dye or enzyme (the direct method), or require a secondary antibody to be applied which will recognised the specific primary antibody and is attached to the desired dye or enzyme (the indirect method). The indirect method may be more sensitive than the direct method due the amplification of the reaction by more than one secondary antibody molecule binding to the primary antibody.

In this study the IHC was performed using the indirect method. Secondary biotinylated antibodies couples with the enzyme streptavidin-horseradish peroxidase were applied as a second layer after the primary antibody. 3,3-diaminobenzidine (DAB) was then added which reacts with this enzyme producing a brown stain easily seen on light microscopy.

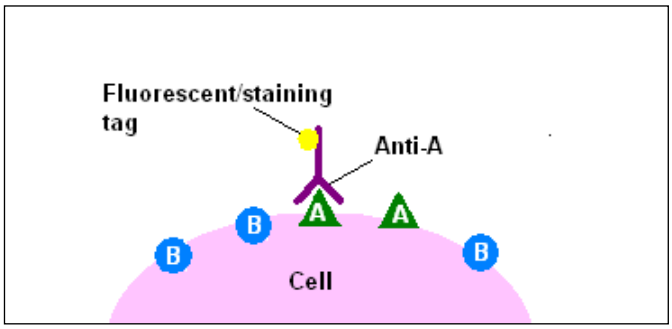

Fig.3.1: Direct immunohistochemistry; the anti-A antibody is directly conjugated to the dye or enzyme.

Courtesy of www.wikepedia.org

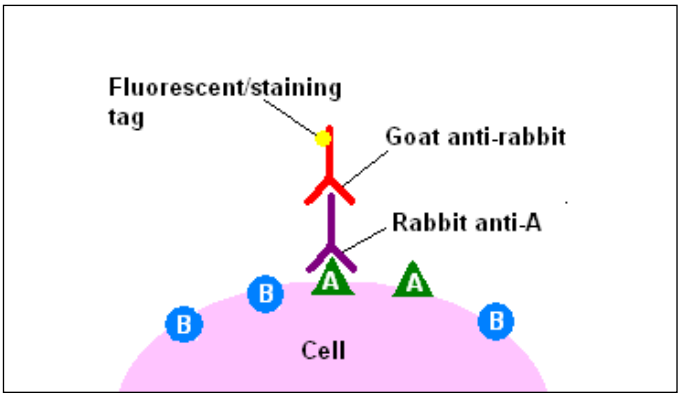

Fig. 3.2: Indirect Immunohistochemistry; the primary anti-A antibody is recognised by a secondary antibody attached to the desired dye or enzyme. Courtesy of www.wikepedia.org

In this study IHC was to be carried out on archival paraffin embedded samples of breast tissue. In paraffin embedded blocks the antigens of interest are hidden and must be exposed by a process of sample retrieval. Techniques for sample retrieval included the use of enzyme solutions, heat treatments and microwave treatment.

The optimum techniques for IHC protocols were determined by the process of working up an antibody using different retrieval techniques, titrations of antibodies and staining methods against known positive controls expressing the proteins of interest. Once stained the slides were then assessed with a light microscope and the expression of the antigen of interest scored and recorded.

For this study the IHC work was performed in the Department of Immunohistochemistry at the Queen Elizabeth Hospital Gateshead under the supervision of Dr D. Hemming, Consultant cellular Pathologist. The Slides were cut and stained with assistance from Miss E. Scott as part of her work towards a MSc degree .

3.1.4 Preparation of samples.

Following retrieval from the tissue store, paraffin embedded blocks of representative tumour for each case was identified. The samples were then cut with a microtome (LEICA RM2255 Microtome) to a thickness of 4µm. The sections were placed upon vecta-bonded coated glass slides and placed in an oven for 60 min at 60^0C. Each section preparation was then checked with light microscope (Leitz Diaplan microscope). The sections were deparaffinised and rehydrated in graded ETOH. Following this preparation the samples were then treated with the appropriate retrieval method and then processed on a Techmate Horizon immunostainer (Dako Cytomation, Ely, Cambridgeshire UK) or a Bond Max immunostainer (Vision bio systems)

Consumables used in processing IHC;

Buffers - CheMate Buffer Kit No. K5006 (Dako.)

Hydrogen Peroxide – NO.H-1009 (Sigma Aldrich Company Limited Dorset UK)

Envision- CheMate Envision Detection Kit No.K5007 (Dako.)

Haematoxylin – CheMate Haematoxylin No.S2020 (Dako.)

Retrieval solution – Dakocytomation Target Retrieval Solution No. S2367 (Dako.)

For the Bond Max the following reagent were used;

Primary Antibody diluted – Bond Primary Antibody Diluent Cat No. AR932 (Vision BioSystems.)

Polymer refines Detection – Bond Polymer Refine Detection Cat No. AR9800 Anti-NF-kB p65 CT (rabbit polyclonal IgG) Code db033 Delta biolabs, Cambridge Biosciences (Vision BioSystems.)

Bond Enzyme – Bond Enzyme Pre-treatment Kit Cat No. AR9551 (Vision BioSystems.)

DAB Enhancer – Bond DAB Enhancer Cat No. AR9432 (Vision BioSystems.)

Epitope Retrieval 1 – Bond Epitope Retrieval Solution 1 Cat No. AR9961 (Vision BioSystems.)

Epitope Retrieval 2 – Bond Epitope Retrieval Solution 2 Cat No. AR9640

3.1.5 Individual protocols used in the clinical study.

As the antibodies for ER, PR and HER2 are routinely used in this lab the protocols for staining are well established. The pathology lab at the Queen Elizabeth participates in ICC NEQAS (an external quality assessment program). The study antibodies (EGFR, STAT3, SP1 and NFκß) had to be worked up and the test concentrations were identified after a process of optimisation. The concentrations of antibodies used were those which gave a consistent staining result of the positive control with the least background staining (i.e. clean staining).

3.1.5.1 Assessment of Estrogen Receptor α (ERα) status.

Retrieval: Dako target retrieval solution high pH, pressure cooker @121^{0}C for a total of 2 hours 20min.

Processor: Techmate ^{TM} Horizon Automated immunostaining Machine.

Antibody: Dako ERα monoclonal mouse anti-human antibody M7047, concentration 1/50

Controls: Breast cancer ER quick score 3, 12, 18 supplied with antibody.

3.1.5.2 Assessment of Progesterone receptor (PR) status.

Retrieval: Dako target retrieval solution high pH, pressure cooker @121^{0}C for a total of 2 hours 20min.

Processor: Techmate ^{TM} Horizon Automated immunostaining Machine.

Antibody: Dako M3569 Monoclonal mouse anti human PR, concentration 1/60.

Controls: Breast cancer PR quick score 3, 12, 18 supplied.

3.1.5.3 Assessment of CerB-2 (HER2) status.

Retrieval: Water bath 40min @95^{0}C in Dako Epitope retrieval solution pH 9 then left to stand for 20 min.

Processor: Techmate ^{TM} Horizon Automated immunostaining Machine.

Antibody: CerbB2- Rabbit Anti-Human c-erbB-2 Oncoprotein code No. A 0485(Dako Cytomation, Ely Cambridgeshire, UK) concentration: 1/2500

Control: Breast cancer controls (supplied with Hercep Test kit DAKO, Ely Cambridgeshire, England.)

3.1.5.4 Assessment of EGFR.

Retrieval: Dako target retrieval solution high pH, pressure cooker @121^{0}C for a total of 2 hours 20min.

Processor: Techmate ^{TM} Horizon Automated immunostaining Machine.

Antibody: Epidermal growth factor receptor (EGFR) –Clone EGFR.25 No. NCL-EGFR-384 (Novocastra Laboratories Limited, Newcastle – Upon –Tyne, UK) concentration: 1/100.

Control: Placenta.

3.1.5.5 Assessment of STAT3

Retrieval: 30 mins @ 95^{0}C(Bond Max Machine) Bond Epitope Retrieval Solution 1 Cat No. AR9961 (Vision BioSystems.)

Processor: Bond Max Automated Immunostainer.

Antibody: Anti- STAT3 (rabbit polyclonal IgG) Code Number. 06-596 (Upstate Cell signalling Solutions, Hampshire, UK) concentration: 1/800

Control: Placenta.

3.1.5.6 Assessment of Sp1.

Retrieval: 30 mins @ 95^0C(Bond Max Machine) Bond Epitope Retrieval Solution 1 Cat No. AR9961 (Vision BioSystems.)

Processor: Bond Max Automated Immunostainer.

Antibody: Anti- SP1 (rabbit polyclonal IgG) Code Number. 07-645

(Upstate Cell signalling Solutions, Hampshire, UK) concentration: 1/2000

Control: colon.

3.1.5.7 Assessment of NFkB.

Retrieval: Dako target retrieval solution high pH, pressure cooker @121^0C for a total of 2 hours 20min.

Processor: Techmate ^{TM} Horizon Automated immunostaining Machine.

Antibody: NFkB p65 (C20) Code Number. DB033 (Delta Biolabs, Gilroy, CA 95020) concentration: 1/5000

Control: diffuse large B cell lymphoma.

3.1.6 Scoring of results

The slides once stained were scored jointly by Dr D Hemming (Consultant Breast Pathologist) and the author. The scoring systems used varied depending on the receptor or protein of interest being examined. The details of the scoring systems used are described below. During the scoring of the slides Dr Hemming and the author were blind to any other details of the patient. The images shown below were taken with a Leica photo system in the Department of Pathology Queen Elizabeth Hospital.

3.1.6.1 Scoring of ER and PR

There are a number of systems in use for the scoring of estrogen and progesterone receptor in breast cancer, these include the Allred score (Allred et al., 1998) and the Quick score (Detre et al., 1995). Both of these methods are similar in the fact that they combine the proportion of stained nuclei with the intensity of the staining.

Score for proportion stained	Score for intensity of staining
0 = no nuclear staining	0 = no stain
1 = <1% nuclei staining	1 = weak stain
2 = 1-10% nuclei staining	2 = moderate staining
3 = 11-33 % nuclei staining	3 = strong staining
4 = 34-66% nuclei staining	
5 = 67-100% nuclei staining	

Table 3.1: The Allred score(Allred et al., 1998).

For the Allred score the 2 values are added to give a possible score of 0-8. The cut off suggested for the treatment of a tumour as ER or PR positive is >3 (Leake et al., 2000, Harvey et al., 1999) as tumours with a score below this value were shown to be less likely to respond to anti-estrogen treatment.

In the Quick score these 2 values are multiplied to give a score of 0-18. When the quick score value was compared to the quantitative Abbott enzyme immunoassay using a semi-quantitative H-score it was suggested that a score of 3 or greater was indicative of ER positive tumour (Detre et al., 1995).

Score for proportion stained	Score for intensity of staining
0 = no nuclear staining	0 = no stain
1 = 0-4% nuclei staining	1 = weak stain
2 = 5-19% nuclei staining	2 = intermediate staining
3 = 20-39 % nuclei staining	3 = strong staining
4 = 40-59% nuclei staining	
5 = 60-79% nuclei staining	
6 = 80-100% nuclei staining	

Table 3.2: The Quick Score(Detre et al., 1995).

The histoscsore is a semiquantitative evaluation of immunocytochemical staining. It is performed using a histoscore which relates to the tumour ER content. Based on the intensity of nuclear staining a subjective integer score of 0 to 4 is given with 0 indicating no evidence of staining. To assess the average degree of staining within a tumour, various regions of the tumour section are analyzed. The second parameter

was the estimated proportion of stained cancer cells in percentage. The formula for the histoscore is:

Histoscore = 2 (i + 1) x P/, where i = intensity of nuclear staining (0-4) and P, = percentage of stained cancer cells.

In the initial studies a histoscore of >100 was considered positive for ER (Jonat et al., 1986). Further studies showed that a histoscore of >75 correlated well with a cytosol analysis of 20 fmol in predicting ER sensitivity(Kinsel et al., 1989) and that a histoscore is equivocal if not superior to a direct cytosol binging assay in predicting ER response (Helle et al., 1989).

These semi-quantative analyses all introduce an element of intra and inter observer variation. Many studies have been conducted into how to minimise that variation this includes the standardisation of techniques in staining, the methods of scoring and the use of QA programs within the pathology labs (Diaz et al., 2004). The pathology department here at the Queen Elizabeth Hospital is actively involved in an ongoing QA program as part of its role in the cancer network.

In the Queen Elizabeth hospital the Quick score was routinely used for the scoring of ER and PR hence it is this method that was used in this study. Since this study the Allred scoring system has been adopted by the department. The Allred score is used by the majority of breast pathology units.

Illustrated below are examples of slides assessed for ER status;

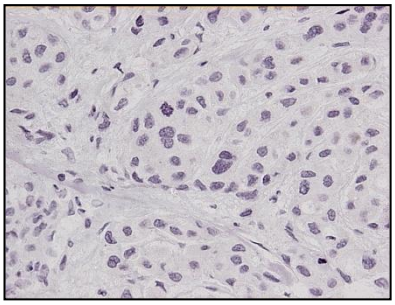

Fig. 3.3: Ductal carcinoma of the breast stained for ERα quick score 3. (x400)

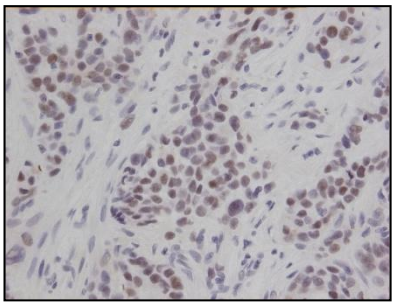

Fig. 3.4: Ductal carcinoma of the breast stained for ERα quick score 9. (x400)

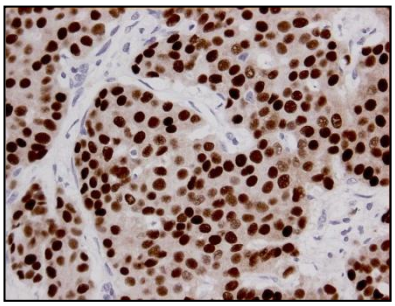

Fig. 3.5: Ductal carcinoma of the breast stained for ERα quick score 18. (x400)

Examples of staining and scoring of PR:

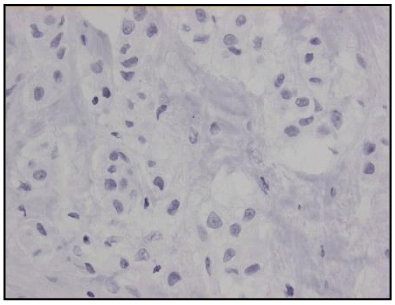

Fig. 3.6: Ductal carcinoma of the breast stained for PR quick score 3. (x400)

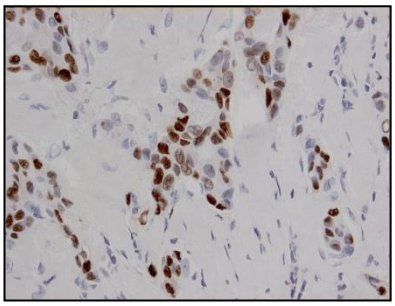

Fig. 3.7: Ductal carcinoma of the breast stained for PR quick score 12. (x400)

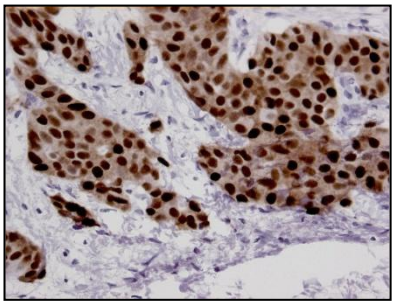

Fig. 3.8: Ductal carcinoma of the breast stained for PR quick score 18. (x400)

3.1.6.2 Scoring of HER2.

HER2 is a membrane associated receptor therefore positive results are found in invasive cancers with membrane staining. Membranous staining should not be seen in normal breast tissue. As HER2 assessment in breast cancers is now vitally important in planning adjuvant treatment with Trastuzumab there are widely published guidelines on the scoring of IHC samples. These include the American Society of Clinical Oncology / College of American Pathologists who recently published a scoring system (Wolff et al., 2007) which has been endorsed in the latest UK HER2 testing recommendations (Walker, 2008). These guidelines score the samples negative if none or <10% of cell membranes are stained; 1+ (negative) if there is faint membrane staining in >10% of cells, 2+ (equivocal) is weak to moderate complete membrane staining in >10% of cells or <30% of cells with strong membrane staining and 3+ (positive) if >30% strong membrane staining (Walker, 2008). HER2 over expression can also be ascertained by the use of fluorescent in-situ hybridisation (FISH). This process is directed at the detection of the over amplification of the HER-2/neu gene rather than the protein detected by IHC. The advantage of the use of ICH over FISH is that it is faster, more economic and easier to provide as a routine diagnostic IHC service (Rhodes et al., 2002). In clinical practice in the UK the use of FISH is limited to the confirmation of the status of the 2+ (equivocal) IHC results. Since this study the FISH has been superseded by the process of silver enhanced insitu hybridisation (SISH). This process is quicker and cheaper than FISH whilst still providing unequivocal results (Dietel et al., 2007). SISH has now been adopted by the Queen Elizabeth for the testing of its HER2 2+ ICH results. In this study the use of FISH was not available for these equivocal cases and a decision was made based upon the data in the department that the majority (80%) of IHC 2+ are FISH negative to

class all IHC 2+ tumours as HER2 negative. The published FISH positivity of IHC 2+ cancers varies from 17% (Yaziji et al., 2004) to 24% (Tubbs et al., 2007). The reason for the preclusion of FISH testing was limited financial funding. This decision was not taken without some careful consideration as it is a possible source of error within this part of the study. It was ascertained the proportion of cancers which were scored at 2+ was small, 25/213 cancers and if 80% of these cases were HER2 negative then 20% would be wrongly classified as HER2 negative when actually being HER 2 positive, this would equate to 5 out of 213 (2.3%) cases placed in the wrong group. It was felt that this was an acceptable error margin

Examples of HER2 scoring.

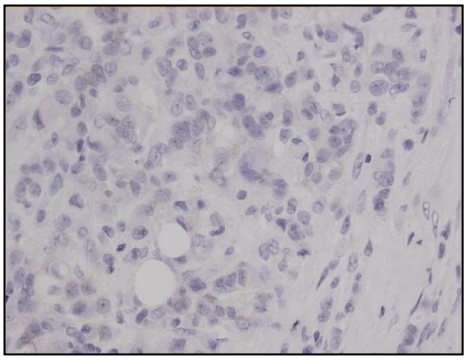

Fig. 3.9: HER2 score 0 (negative) (x400)

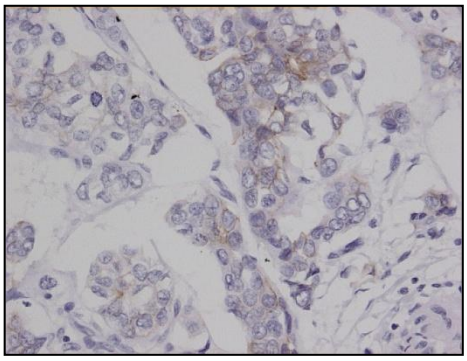

Fig. 3.10: HER2 score 1+ (negative) (x400)

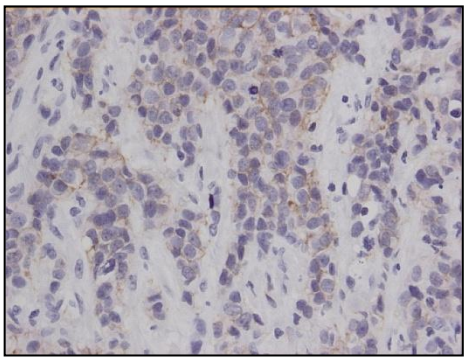

Fig. 3.11: HER2 2+ (equivocal) (x400)

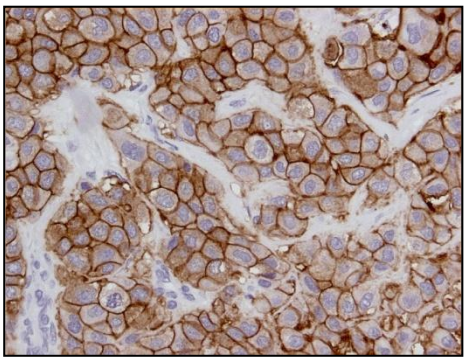

Fig. 3.12: HER2 3+ (positive) (x400).

3.1.6.3 Scoring of EGFR.

As with HER2, EGFR is a membrane receptor and staining where positive is membranous. Due to the fact that EGFR is not routinely assessed on breast cancer samples, unlike HER2, there are no set evidenced based scoring protocols. Publications have included a 0-3 score similar to HER2, an H-score test or positive if any staining present (Walker, 2008). The studies which have used a 0-3 scoring system were assessing EGFR expression in high grade locally advanced cancers with a high overall EGFR expression(Nieto, 2007b). Our study includes cancer samples of all grades and stages, the overall EGFR positive cancer population was expected to be low. We therefore considered any membranous staining to be indicative of EGFR positive cancers. This scoring has been used previously in groups with lower overall expression (Gee et al., 2005).

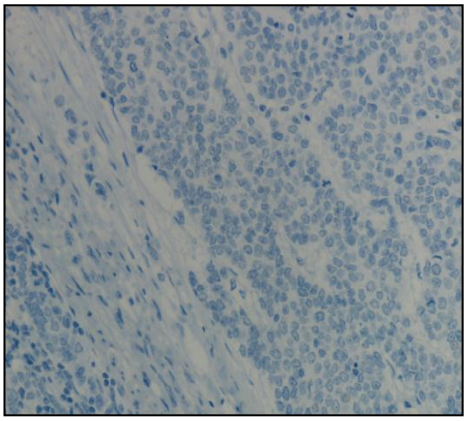

Fig. 3.13: EGFR negative, no plasma membrane staining,(x400)

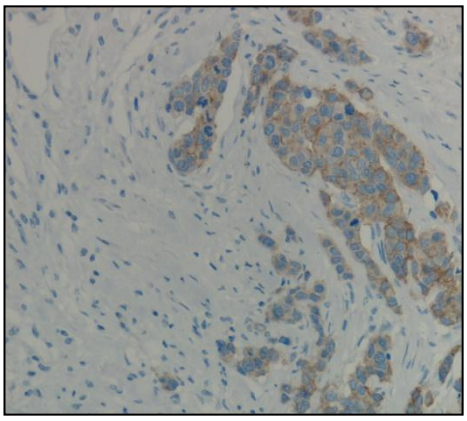

Fig. 3.14: EGFR positive, minimal plasma membrane staining. (x400)

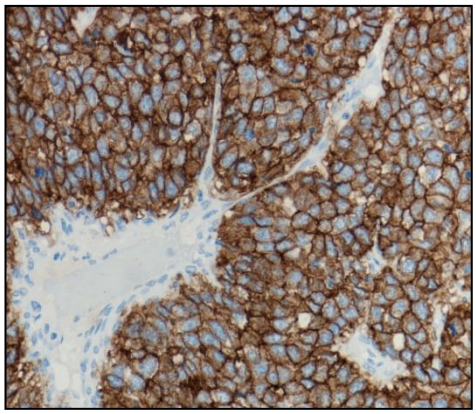

Fig. 3.15: EGFR positive, florid membrane staining. (x400)

3.1.6.4 Scoring of STAT3.

There have been a number of studies where IHC has been used to asses STAT3 expression in breast cancers. The results of tumour staining have demonstrated that some cancer samples do not exhibit staining for STAT3, some show cytoplasmic staining and some strong nuclear staining. These previous studies have considered the nuclear staining of STAT3 to be evidence of over or up-regulated expression (Diaz et al., 2006b, Yeh et al., 2006a) Studies have also been published which considered the specificity of using nuclear staining of anti-STAT3 anti-body as a marker of activated STAT3 by comparing its staining to that of anti-Phospho-STAT3 antibodies in matched samples. These studies have shown a correlation between nuclear STAT3 and Phospho-STAT3 staining (Yeh et al., 2006a, Dolled-Filhart et al., 2003a). It is based upon this published work that we graded our tissue samples as 0= no staining, 1= cytoplasmic staining and 2= nuclear staining and considered the nuclear staining to be indicative of STAT3 activation.

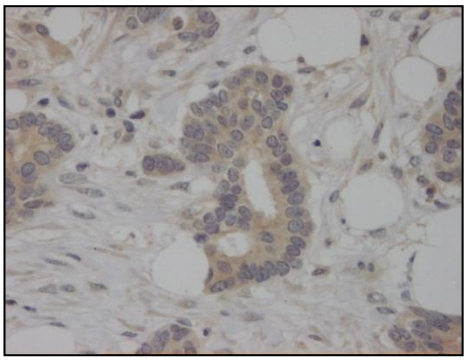

Fig. 3.16: Cytoplasmic staining of STAT3 in invasive breast cancer. (x400)

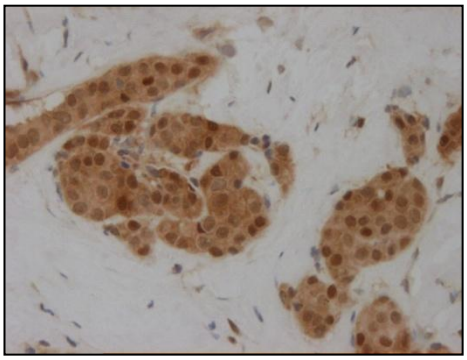

Fig. 3.17: Nuclear and cytoplasmic staining of STAT3 in invasive breast cancer. (x400)

3.1.6.5 Scoring of SP1.

There are few studies published using IHC analysis of SP1 and none could be found specifically in breast cancer. The scoring of the SP1 slides in this study therefore was based upon methods previously described in studies looking at immunohistochemistry of SP1 expression in gastric carcinomas. As discussed with STAT3, nuclear staining is considered significant. As it appeared that the majority of samples were found to exhibit a degree of nuclear SP1 staining an assessment of the degree of staining has to be made. Based upon these previous studies the slides were assessed upon the percentage of the population and the intensity of the nuclear staining. Using this method the slides were graded into 3 groups; 0 = negative, 1= weak, and 2=strongly positive. For the data analysis the strongly positive group were considered to have over-expression of nuclear SP1 (Zhang et al., 2005b, Wang et al., 2005).

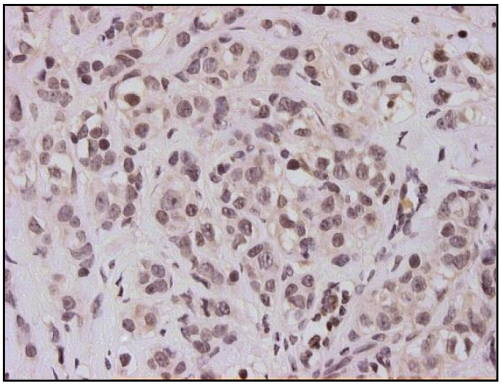

Fig. 3.18: SP1 negative, minimal cytoplasmic staining only. (x400)

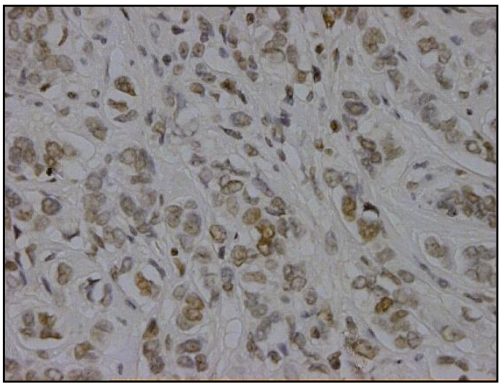

Fig. 3.19: SP1 weak positive score 1 (25-50% cell, moderate intensity) (x400)

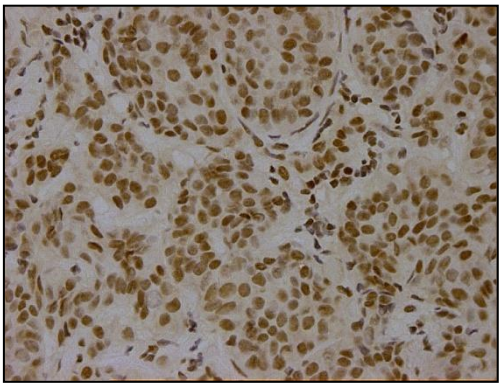

Fig. 3.20: SP1 strong positive score 2 (>50% cells moderate intensity) (x400)

3.1.6.6 Scoring of NFκß.

NFκß is transcriptionally active when it has translocated from the cytoplasm to the nucleus. The scoring system for this antibody is dependent on the location of the stain in the cell. In this study we determined if staining was present in the sample and then the location of the staining. If the staining was absent or located only in the cytoplasm then these samples were considered to be nuclear NFκß negative. If nuclear staining was present irrespective of the presence of cytoplasmic staining then the sample was considered nuclear NFκß positive. There have been a number of published studies which have used a similar IHC scoring protocol which was then validated with either cDNA micro-arrrays looking for NFκß dependent gene products (Van Laere et al., 2007b) or electrophoretic mobility-shift assay (Biswas et al., 2004). The assumption that nuclear NFκß staining indicates the transcriptions factor in its active state is now widely held (Biswas et al., 2004, Montagut et al., 2006b, Ghosh and Karin, 2002).

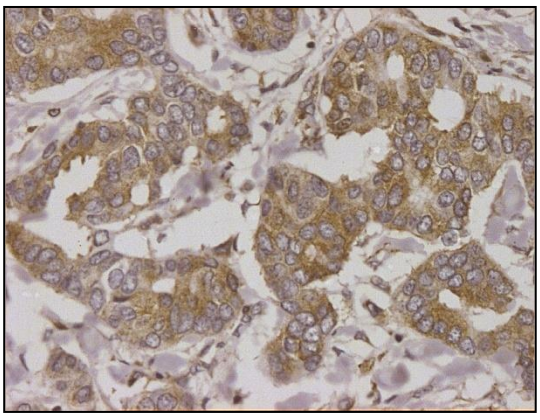

Fig. 3.21: Cytoplasmic staining of NFκβ with no nuclear staining. (x400)

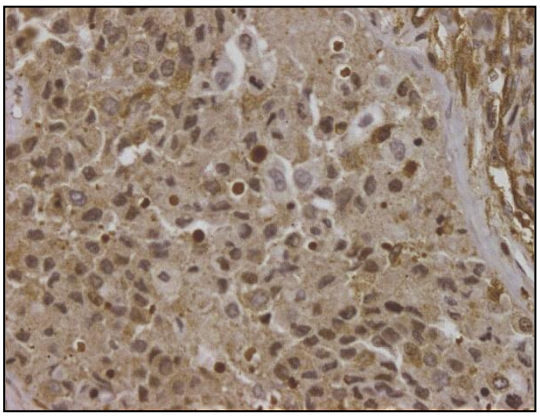

Fig. 3.22. Nuclear and cytoplasmic NFκβ staining (x400)

3.1.7 Power of the Study

Without knowing the prevalence of expression or effect of expression of the selected transcription factors it was not possible to calculate the sample size necessary to adequately power the study directly regarding these transcription factors. We did consider the sample size required to power the study with 80% power to detect the 25% survival difference with an α type 1 error rate of 0.05 when considering ERα expression. This calculation determined that a total sample size of 140 was adequate. This calculation was performed using Epi Info 2000. (EpiInfo was developed by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA.) with the kind help of Dr T Chadwick, University of Newcastle upon Tyne. (see appendix)

3.1.8 Statistical analysis.

The association between ICH results and pathological features were assessed using the X^2 and Fisher exact test where indicated. Survival was plotted using the Kaplan-Meier method. The log-rank method was used to study the correlation of possible prognostic variables with survival times. Multi-variant analysis of survival was performed using the Cox-regression model including the identified significant variables of tumour grade, size and lymph node involvement. All the statistical tests were conducted at the two-sided tailed 0.05 level of significance. Statistical analysis was performed with the SPSS Statistical Software, 15.0 version. (SPSS, Inc, Chicago, IL, USA.)

3.2 Results of Clinical Study.

3.2.1 Patient group demographics.

During 1999, 213 patients were diagnosed with invasive breast cancer and received surgical treatment in the Queen Elizabeth Breast unit. Of these patients 204 were suitable for use in the study in that there was sufficient tumour tissue and full survival data. This group of 204 patients presented with 206 cancers i.e. 2 patients had bilateral cancers. There were 2 cases of male breast cancer within the group. The age range of the patients was 27 - 87 years old with a mean age of 57 (fig 3.23 & 3.24). 72 (35%) of the patients presented with asymptomatic breast cancers via the N.H.S. breast screening program. All the patients were discussed at the multi-disciplinary team meeting at the time of diagnosis. The patients' management was directed by the guidelines at the time with regards to the surgical intervention and any neo-adjuvant or adjuvant therapy.

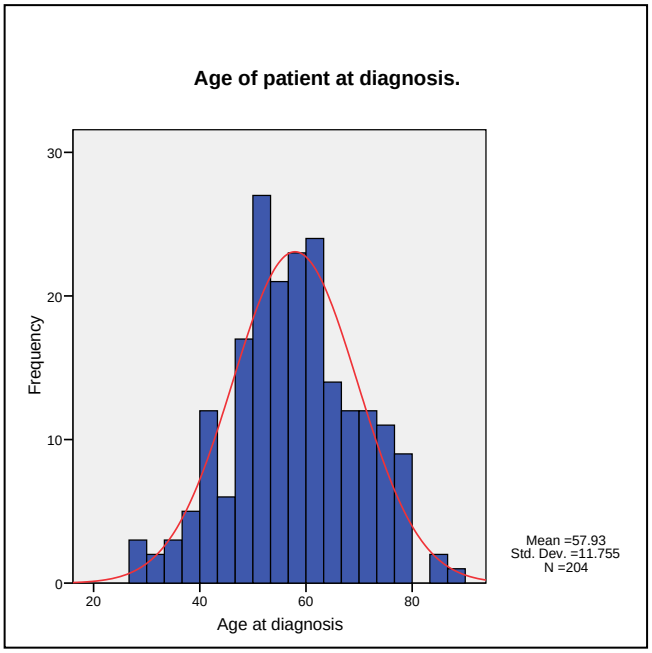

Fig. 3.23: Bar chart showing the age at the time of diagnosis of the 204 patients in the study group.

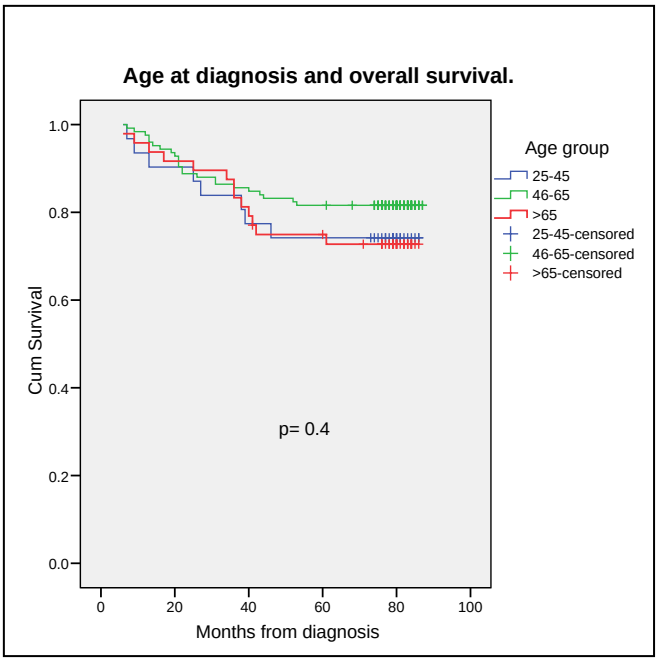

Fig.3.24: Survival curve showing the effect of age group at the time of diagnosis on overall survival.

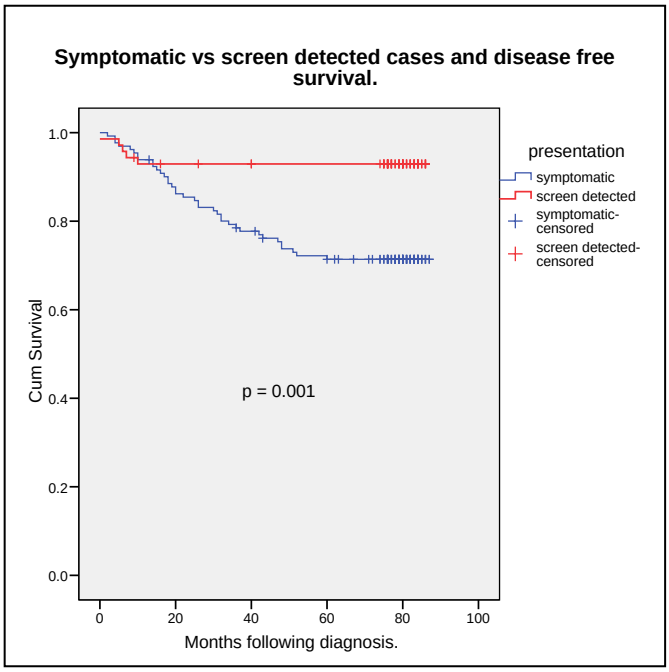

Fig. 3.25: Survival curve showing the significant affect of mode of presentation (screening vs symptomatic) on overall survival.

3.2.2 Pathological features of study group.

3.2.2.1 Histological type of breast cancer.

The breast cancer histological type was ascertained from the initial histological report. Any samples without a defined histological type were re-examined by the breast pathologist.

Type	Number (%)
Ductal	174 (85%)
Lobular	21 (10%)
Tubular	7 (3%)
Mixed	2 (1%)
Mucinous	1 (0.5%)
Medullary	1 (0.5%)
Total	206 (100%)

Table 3.3: Table showing the histological type of breast cancers diagnosed within the study group.

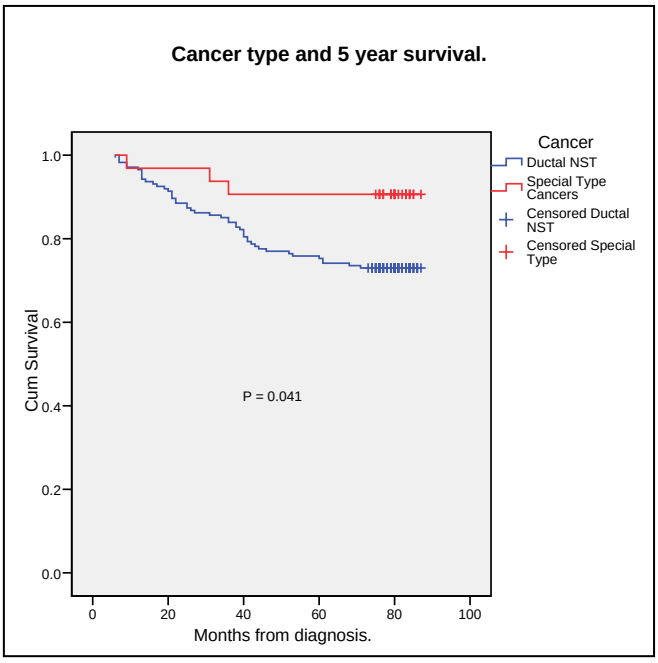

Fig. 3.26: Survival curve showing the significant affect of the histological type of breast cancer (special type vs. NST) on overall survival.

3.2.2.2 Tumour Grade.

The cancers were graded according to the Elston and Ellis modification of the Bloom and Richardson system. Again if the initial grading was unclear the samples were re-examined.

Grade	Number (%)
1	58 (28%)
2	98 (48%)
3	50 (24%)
Total	206

Table 3.4:Table showing the grade of cancers diagnosed in the study groups.

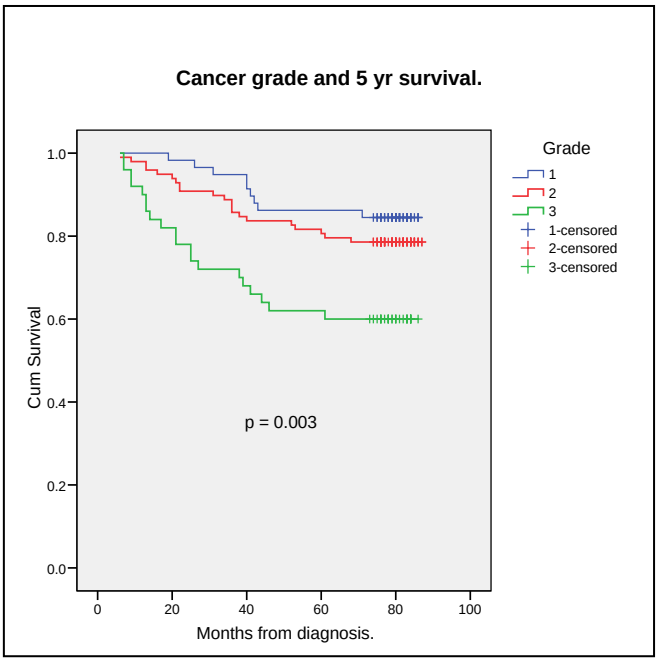

Fig. 3.27: Survival curves showing the significant affect of tumour grade on overall survival.

3.2.2.3 Tumour size.

The tumour size was recorded from the initial pathology report. The range of size was 1mm to 190mm with a mean of 20.81mm. Median tumour size 18mm. The tumour sizes were divided into the staging groups of T1 <20mm, T2 20-50mm, T3 >50mm.

T size Gp.	Number (%)
T1 <20mm	118 (57%)
T2 20-50mm	80 (39%)
T3 >50mm	8 (4%)

Table 3.5: Table showing the distribution of tumour sizes diagnosed in the study group.

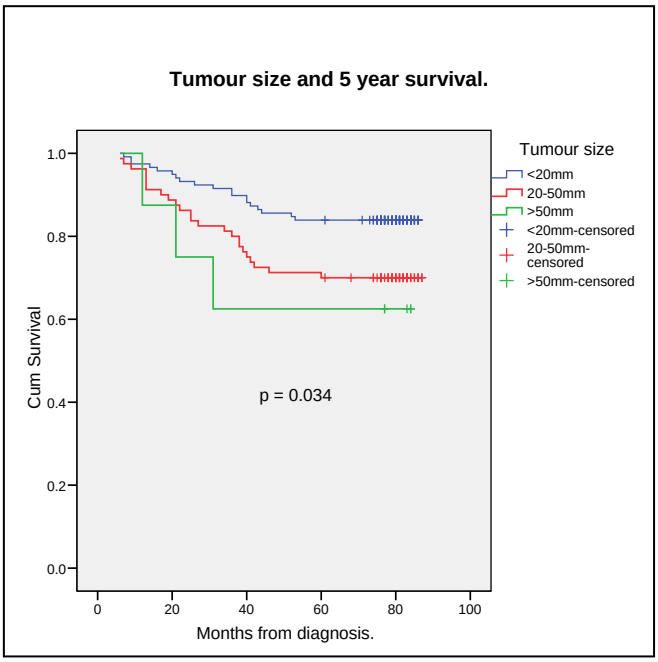

Fig. 3.28: Survival curves showing the significant affect of tumour size upon over all survival.

3.2.2.4 Vessel space invasion.

The presence or absence of vessel space invasion was reported in all 206 cancer samples. Vessel space invasion was present in 53 (26%) of the 206 samples.

Vessel space invasion	Number (%)
No	153 (74%)
Yes	53 (26%)
Total	206

Table 3.6 Table showing the presence of vessel space invasion in the tumour samples.

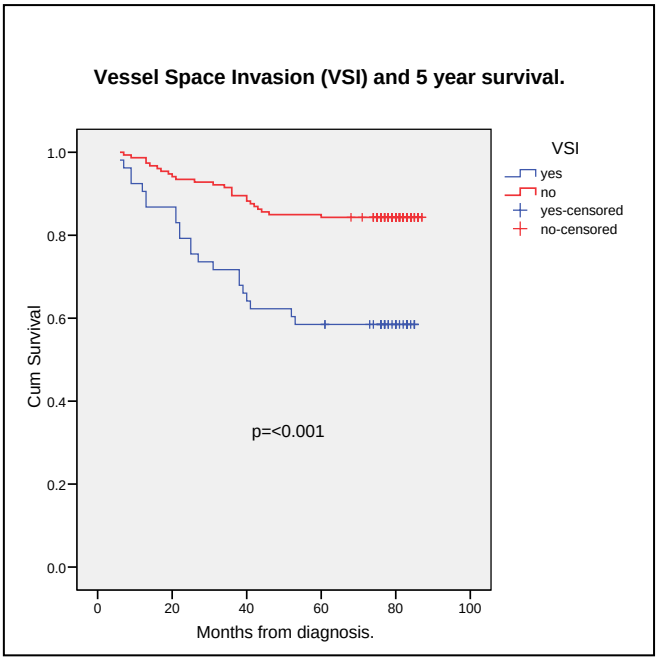

Fig. 3.29: Survival curves showing the significant affect of vessel space invasion on overall survival.

3.2.2.4 Lymph node status.

The lymph node status was available on 205 out of the 206 cancers operated on. The missing case was an elderly lady considered not fit for axilliary surgery.

Nodal status	Cases	5yr OS
Negative	130	86%
1-3 nodes involved	59	64%
>3 nodes involved	16	44%
Total	205	

Table 3.7: Table showing the lymph node involvement of the study group, the results have been split into the 3 prognostic groups used in calculating NPI. 5 yr overall survival rates are also shown.

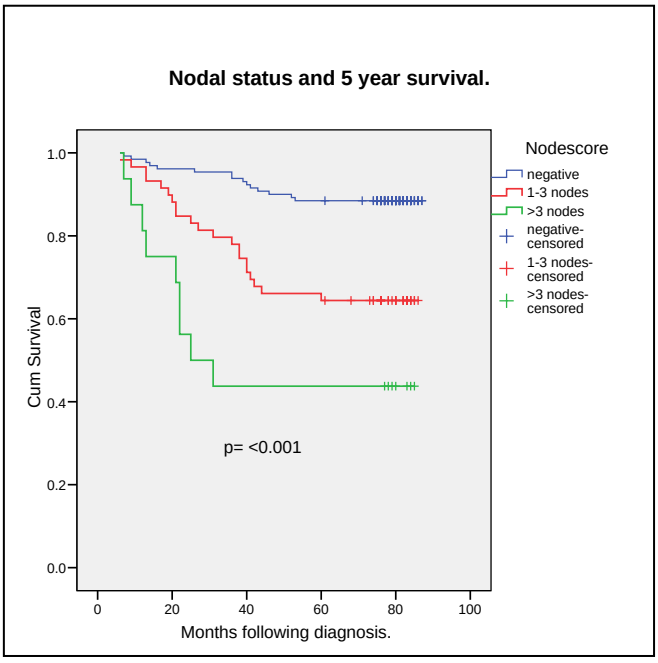

Fig. 3.30: Survival curves showing the significant affect of lymph node metastases on overall survival.

3.2.2.5 Nottingham Prognostic Index (NPI).

The NPI was calculated for the 205 patients that we had the nodal status available.

NPI score	Prognostic Gp.	Number	5yr OS
<2.4	Excellent	44	94%
2.5 - 3.3	Good	41	88%
3.4 – 5.4	Moderate	95	77%
> 5.4	Poor	25	42%

Table 3.8: Table showing the distribution of cases in the study group when NPI was calculated, 5 yr survival is also shown.

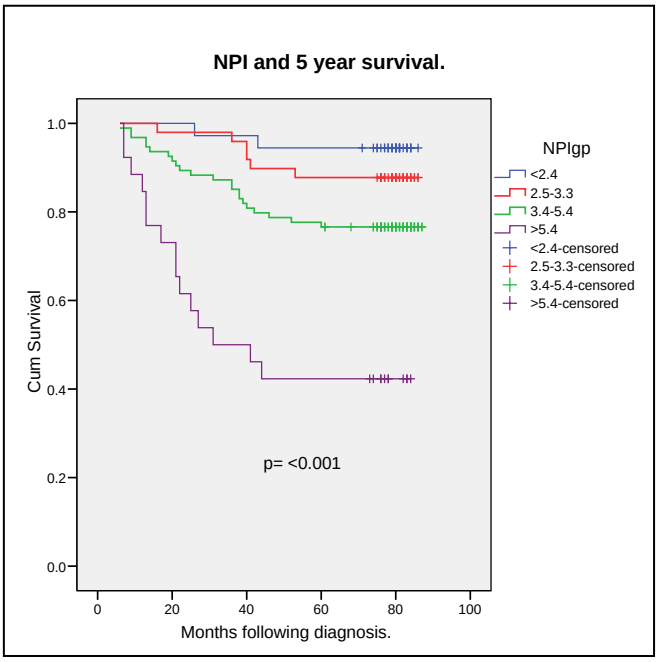

Fig. 3.31: Survival curve showing the significant affect of NPI group on overall survival.

3.2.2.6 Type of surgery performed.

The patients' operation type was defined as breast conserving surgery including wide local excisions, lumpectomies, quadrentectomies or as a total mastectomy. If a patient initially underwent a breast conserving procedure which following pathology results proceeded to a mastectomy they were placed in the mastectomy group.

Type of surgery	Number (%)
Breast conserving surgery	99 (48%)
Mastectomy	107 (52%)
Total	206

Table 3.9: Table showing the type of surgery performed.

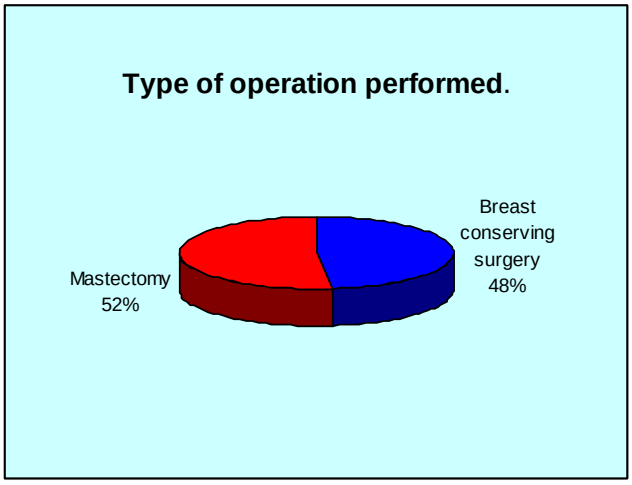

Fig. 3.32: A pie chart representing the mastectomy rate of 52%.

3.2.2.7 Disease Recurrences:

In the sample group there were recurrences of metastatic breast cancer in 50 cases. A number of cases had multiple sites of recurrence of the disease. The recurrences were recorded under the first site of recurrence.

Site	Number (%)
Local	17 (34)
Bone	14 (28)
Lung	8 (16)
Liver	7 (14)
Brain	3 (6)
Choroid	1 (2)
Total	50

Table 3.10: Table showing the site of first recurrence in the 50 cases in the study group.

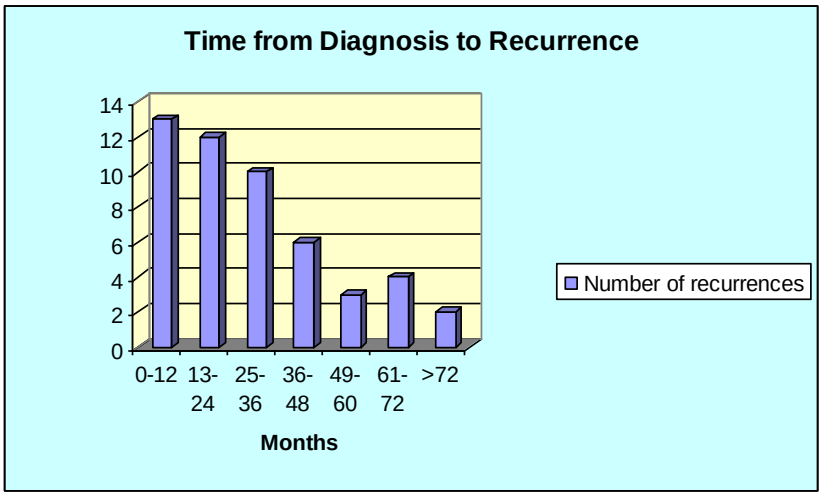

Fig.3.33: Bar Chart to show time from diagnosis to first recurrence of disease.

3.2.2.8 Correlation of Pathological features.

When the pathological features had been collected the relationship between each of the pathological features was compared along with 5 year survival.

		Grade	Tsizegp	VSI	Nodescore	NPIgp	RIP5yr
Grade	Chi-square	.	24.631	20.402	19.150	179.771	10.005
	Df	.	4	2	4	6	2
	Sig.	.(a)	>.001(*)	>.001(*)	.001(*)	>.001(*)	.007(*)
Tsizegp	Chi-square	24.631	.	17.864	71.102	94.007	6.414
	Df	4	.	2	4	6	2
	Sig.	>.001(*)	.(a)	>.001(*)	>.001(*)	>.001(*,)	.040(*)
VSI	Chi-square	20.402	17.864	.	56.906	36.221	15.135
	Df	2	2	.	2	3	1
	Sig.	>.001(*)	>.001(*)	.(a)	>.001(*)	>.001(*)	>.001(*)
Nodescore	Chi-square	19.150	71.102	56.906	.	113.555	25.622
	Df	4	4	2	.	6	2
	Sig.	.001(*)	>.001(*)	>.001(*)	.(a)	>.001(*)	>.001(*)
NPIgp	Chi-square	179.771	94.007	36.221	113.555	.	27.845
	Df	6	6	3	6	.	3
	Sig.	>.001(*)	>.001(*)	>.001(*)	>.001(*,b)	.(a)	>.001(*)
RIP5yr	Chi-square	10.005	6.414	15.135	25.622	27.845	.
	Df	2	2	1	2	3	.
	Sig.	.007(*)	.040(*)	>.001(*)	>.001(*)	>.001(*)	.(a)

Table 3.11: Summary table showing the significant correlation between the pathological features and 5 year survival (RIP5yr). The Chi-square statistic is significant at the 0.05 level. a: The Chi-square test is not performed for this sub-table because row and column variables are identical. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)*

A short comment on the above table; it has to be appreciated when testing multiple variant against each other of the increased probability of finding a spurious correlation between these factors. This is due to the numbers of times the test is performed and the small numbers in some of the groups. To ascertain if there is a significant correlation each factor should be subjected to a multivariate analysis, this would control for the influence of the other factors on the outcome and therefore demonstrate if the correlation is meaningful.

In the following section there are a number of discrepancies in the numbers of positive and negative receptor in the tables, this is due to the fact that in some of the cancers not all the receptor status was known i.e. one cancer may have and ER or EGFR known but not a PR status.

3.2.3 Hormone receptor expression.

3.2.3.1 The Estrogen Receptor:

The ER status was determined on 196 cancer samples. There were a number of samples in which it was not possible to stain for ER due to technical problems e.g. insufficient tumour samples left in the blocks.

ER status	Number (%)
ER +ve	139 (71%)
ER –ve	57 (29%)

Table 3.12: Table of ER status results.

		ER	
		neg	pos
		Count	Count
Grade	grade 1	4	53
	grade 2	21	83
	grade 3	34	15
Tsizegp	<20mm	26	97
	20-50mm	31	49
	>50mm	2	5
VSI	yes	26	29
	no	33	122
Nodescore	negative	28	104
	1-3 nodes	23	39
	>3 nodes	8	7
NPIgp	<2.4	0	34
	2.5-3.3	9	43
	3.4-5.4	34	63
	>5.4	16	10
PR	neg	55	14
	pos	4	136
HER2	neg	36	118
	pos	22	31
EGFRsc	negative	39	143
	positive	17	2
RIP5yr	yes	26	19
	no	32	122

Table 3.13: Table showing the distribution of ER status in for each pathological feature and for 5 year survival(Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

Pearson Chi-Square Tests

		ER
Grade	Chi-square	57.107
	df	2
	Sig.	.000*
Tsizegp	Chi-square	7.443
	df	2
	Sig.	.024*
VSI	Chi-square	13.566
	df	1
	Sig.	.000*
Nodescore	Chi-square	10.280
	df	2
	Sig.	.006*
NPIgp	Chi-square	32.901
	df	3
	Sig.	.000*
PR	Chi-square	134.742
	df	1
	Sig.	.000*
HER2	Chi-square	6.428
	df	1
	Sig.	.011*
EGFRsc	Chi-square	39.633
	df	1
	Sig.	.000*
RIP5yr	Chi-square	23.084
	df	1
	Sig.	.000*

Results are based on nonempty rows and columns in each innermost subtable.

*. The Chi-square statistic is significant at the 0.05 level.

Table 3.14: Table showing the correlation between ER status and pathological features, 5 year survival included. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

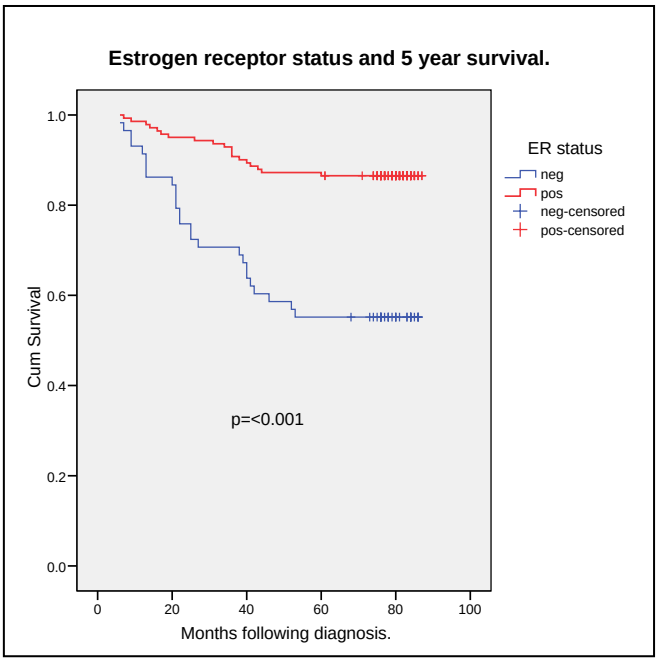

Fig. 3.34: Survival curves showing the significant affect of ER status on overall survival.

3.2.3.2 Progesterone Receptor.

The progesterone receptor status was ascertained in 195 cases within the study group. As with the ER status there were a number of samples where the block cut out and no tumour could be retrieved.

PR status	Number (%)
PR +ve	132 (68%)
PR –ve	63 (32%)

Table 3.15: Table showing PR status of cases.

		PR	
		neg	pos
		Count	Count
Grade	grade 1	4	53
	grade 2	30	74
	grade 3	35	13
Tsizegp	<20mm	34	88
	20-50mm	33	47
	>50mm	2	5
VSI	yes	27	28
	no	42	112
Nodescore	negative	38	94
	1-3 nodes	23	38
	>3 nodes	8	7
NPIgp	<2.4	2	32
	2.5-3.3	12	40
	3.4-5.4	38	59
	>5.4	17	8
ER	neg	55	4
	pos	14	136
HER2	neg	39	114
	pos	29	24
EGFRsc	negative	49	133
	positive	17	2
RIP5yr	yes	23	21
	no	42	112

Table 3.16: Table showing the distribution of PR status in for each pathological feature and for 5 year survival. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

Pearson Chi-Square Tests

		PR
Grade	Chi-square	52.795
	df	2
	Sig.	.000*
Tsizegp	Chi-square	3.977
	df	2
	Sig.	.137[a]
VSI	Chi-square	8.723
	df	1
	Sig.	.003*
Nodescore	Chi-square	4.460
	df	2
	Sig.	.108
NPIgp	Chi-square	29.068
	df	3
	Sig.	.000*
ER	Chi-square	134.742
	df	1
	Sig.	.000*
HER2	Chi-square	15.206
	df	1
	Sig.	.000*
EGFRsc	Chi-square	30.522
	df	1
	Sig.	.000*
RIP5yr	Chi-square	9.700
	df	1
	Sig.	.002*

Results are based on nonempty rows and columns in each innermost subtable.

*. The Chi-square statistic is significant at the 0.05 level.

a. More than 20% of cells in this subtable have expected cell counts less than 5. Chi-square results may be invalid.

Table 3.17: Table showing the correlation between PR status and pathological features, 5 year survival included. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

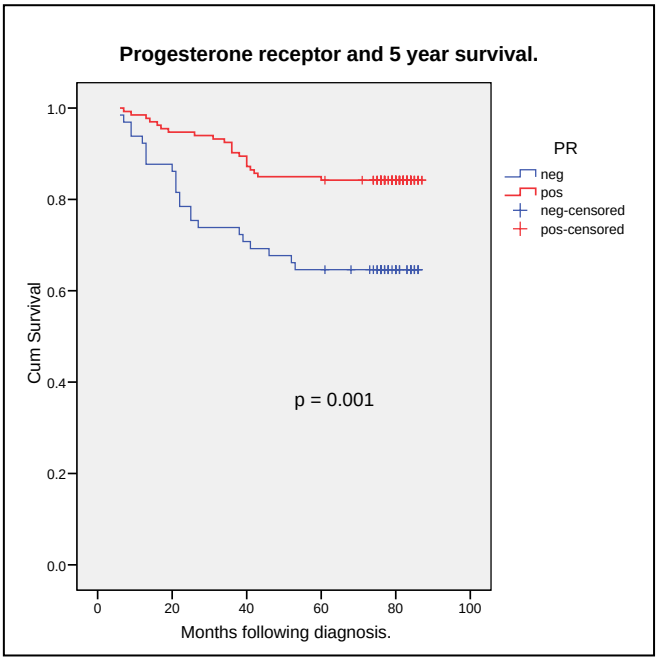

Fig. 3.35: Survival curves showing the significant affect of PR status on overall survival

1.2.5 EGFR in breast cancer.

The ER/PR combined expression demonstrated the following results in 194 cases.

ER/PR status	Number (%)
ER+ve / PR+ve	128 (66%)
ER+ve / PR-ve	10 (5.1%)
ER-ve / PR+ve	3 (1.6%)
ER-ve / PR-ve	53 (27.3%)

Table 3.18: Table showing the co-expression of ER and PR.

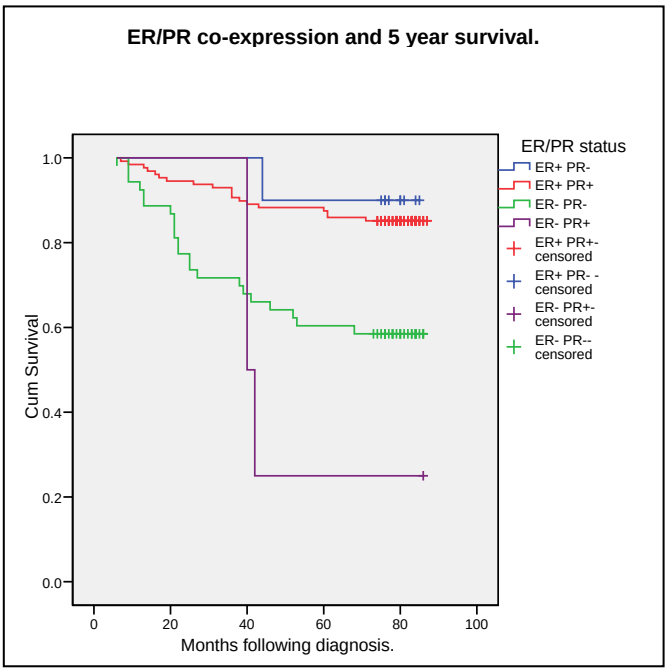

Fig. 3.36: The survival curves were plotted but the significance is limited due to the small numbers in 2 of the groups (ER+ve/PR-ve and ER-ve/PR+ve).

3.2.3.4 HER2 receptor.

HER2 receptor status was successfully tested for in 200 of the sample cases. As discussed earlier the IHC result 2+ was not reassessed with FISH and in this study is considered HER2 negative. When the HER2 0, 1+ and 2+ groups were compared there was no statistically significant difference between the groups and pathological feature and survival.

HER2 score	Number (%)
0	90 (44%)
1+	33 (17%)
2+	25 (13%)
3+	55 (26%)

Table 3.19: Table showing the results of the HER2 for the study group.

		HER2	
		neg	pos
		Count	Count
Grade	grade 1	53	4
	grade 2	71	34
	grade 3	33	17
Tsizegp	<20mm	98	26
	20-50mm	55	25
	>50mm	4	4
VSI	yes	35	20
	no	122	35
Nodescore	negative	104	30
	1-3 nodes	41	21
	>3 nodes	12	4
NPIgp	<2.4	33	2
	2.5-3.3	38	14
	3.4-5.4	72	26
	>5.4	14	13
ER	neg	36	22
	pos	118	31
PR	neg	39	29
	pos	114	24
EGFRsc	negative	136	47
	positive	12	7
RIP5yr	yes	28	15
	no	122	36

Table 3.20: Table showing the distribution of HER2 status in for each pathological feature and for 5 year survival. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

Pearson Chi-Square Tests

		HER2
Grade	Chi-square	14.581
	df	2
	Sig.	.001*
Tsizegp	Chi-square	5.180
	df	2
	Sig.	.075
VSI	Chi-square	4.197
	df	1
	Sig.	.040*
Nodescore	Chi-square	2.917
	df	2
	Sig.	.233
NPIgp	Chi-square	14.427
	df	3
	Sig.	.002*
ER	Chi-square	6.428
	df	1
	Sig.	.011*
PR	Chi-square	15.206
	df	1
	Sig.	.000*
EGFRsc	Chi-square	1.094
	df	1
	Sig.	.296
RIP5yr	Chi-square	2.613
	df	1
	Sig.	.106

Results are based on nonempty rows and columns in each innermost subtable.

*. The Chi-square statistic is significant at the 0.05 level.

Table 3.21: Table showing the correlation between HER2 status and pathological features, 5 year survival included (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

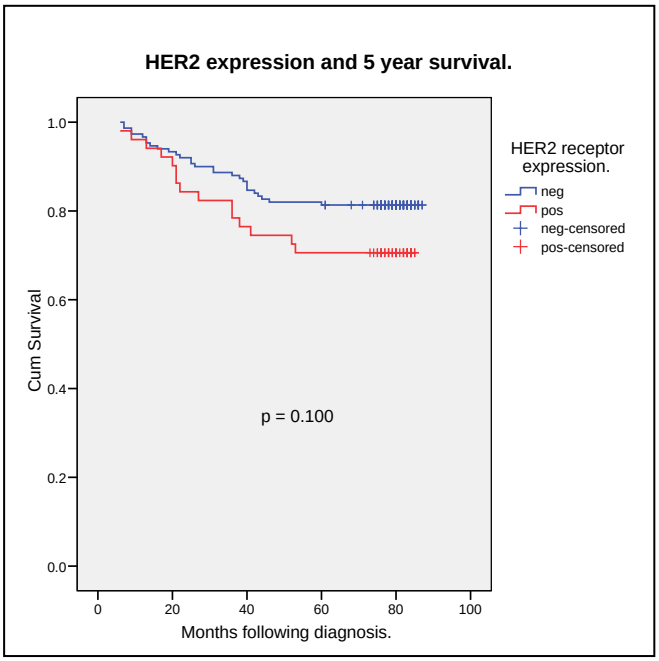

Fig. 3.37: Survival curves showing a non significant trend towards poorer survival in the HER+ group When this was re-plotted for cancer specific deaths the p value was significant at 0.009.

3.2.3.5 HER2 and ER Status

Following the IHC results for HER2 the co-expression between ER and HER2 could then be investigated. .

ER/HER2 status	Number.
ER+ve/HER2+ve	27 (14%)
ER+ve/HER2-ve	111 (57%)
ER-ve/HER2+ve	23 (12%)
ER-ve/HER2-ve	34 (17%)

Table 3.22: Table showing the co-expression of ER and HER2 in the study group.

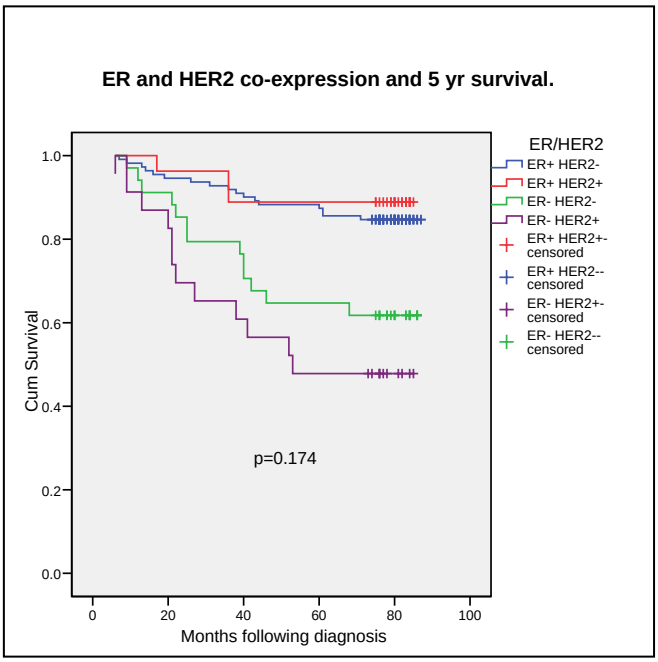

Fig.3.38: Survival curves of each of the ER/HER2 groups showing little additional impact of the HER2 status over the ER status.

3.2.3.6 EGF Receptor.

EGF receptor status was determined in 203 cases of invasive breast cancers.

EGFR status	Number (%)
EGFR +ve	19 (9.4%)
EGFR –ve	184 (90.6%)

Table 3.23: Table showing the results of the EGFR status in the study group.

		EGFRsc	
		negative	positive
		Count	Count
Grade	grade 1	53	1
	grade 2	93	8
	grade 3	38	10
Tsizegp	<20mm	115	5
	20-50mm	63	13
	>50mm	6	1
VSI	yes	47	8
	no	137	11
Nodescore	negative	119	9
	1-3 nodes	52	8
	>3 nodes	13	2
NPIgp	<2.4	32	0
	2.5-3.3	50	2
	3.4-5.4	81	14
	>5.4	21	3
ER	neg	39	17
	pos	143	2
PR	neg	49	17
	pos	133	2
HER2	neg	136	12
	pos	47	7
RIP5yr	yes	31	10
	no	142	9

Table 3.24: Table showing the distribution of EGFR status in for each pathological feature and for 5 year survival. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

Pearson Chi-Square Tests

		EGFRsc
Grade	Chi-square	11.283
	df	2
	Sig.	.004*
Tsizegp	Chi-square	9.389
	df	2
	Sig.	.009*,a
VSI	Chi-square	2.391
	df	1
	Sig.	.122
Nodescore	Chi-square	2.214
	df	2
	Sig.	.331
NPIgp	Chi-square	8.684
	df	3
	Sig.	.034*,b
ER	Chi-square	39.633
	df	1
	Sig.	.000*
PR	Chi-square	30.522
	df	1
	Sig.	.000*
HER2	Chi-square	1.094
	df	1
	Sig.	.296
RIP5yr	Chi-square	12.283
	df	1
	Sig.	.000*,b

Results are based on nonempty rows and columns in each innermost subtable.

*. The Chi-square statistic is significant at the 0.05 level.

a. The minimum expected cell count in this subtable is less than one. Chi-square results may be invalid.

b. More than 20% of cells in this subtable have expected cell counts less than 5. Chi-square results may be invalid.

Table 3.25: Table showing the correlation between EGFR status and pathological features, 5 year survival included (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.

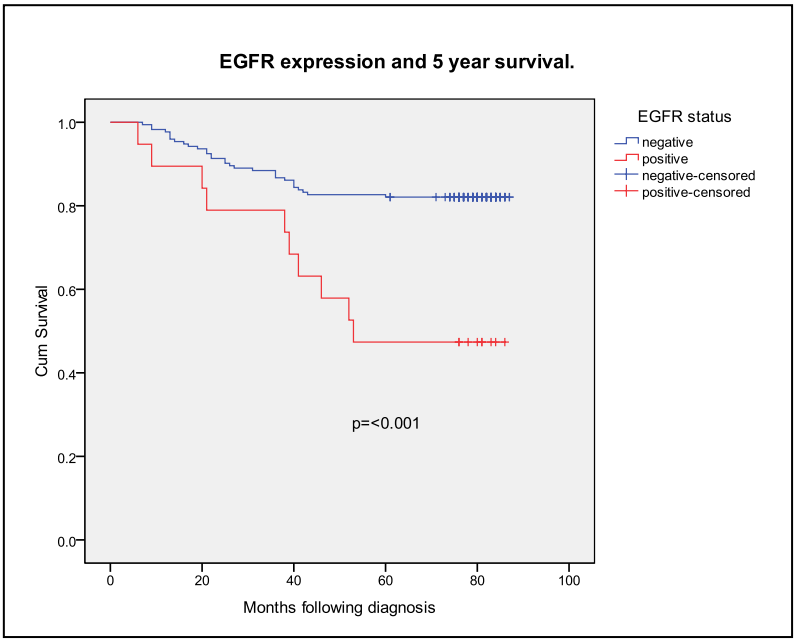

Fig. 3.39: Survival curves showing significant poorer outcome for the EGFR+ve cancers.

As described earlier EGFR is one of the growth factor receptors that when activated form a heterodimer with the orphan receptor HER2. The results of the HER2 positive breast cancers split by their EGFR status were plotted below. Of the 54 HER2 positive cancers 7 of these were EGFR positive. The survival curves for this group were plotted below.

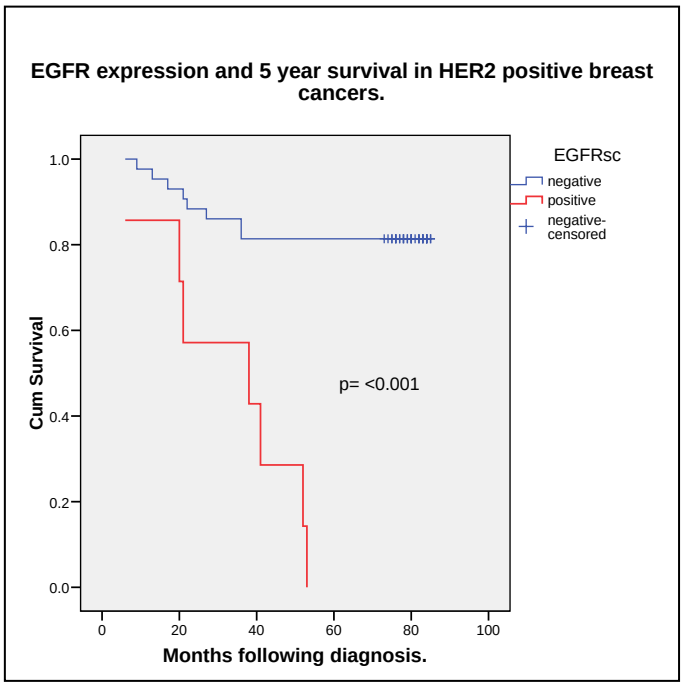

Fig. 3.40: Survival curve showing the significant effect on EGFR status on HER2 positive cancers.

3.2.4 Transcription factor results.

The overall staining results for the 3 transcription factors in the clinical study are shown in the appendix.

3.2.4.1 STAT3 expression.

209 cancer samples were successfully assessed for stat3. Overall 56 (26.8%) of the tumours demonstrated nuclear staining for STAT3 and were graded as STAT3 positive.

STAT3 status	Number (%)
0 : negative	9 (4.3%)
1 : faint	80 (38.3%)
2 : cytoplasmic	64 (30.6%)
3 : nuclear	56 (26.8%)

Table 3.26: Table showing the STAT3 staining characteristics of all of the 209 patients.

		STAT3sc	
		neg	pos
		Count	Count
Grade	grade 1	53	4
	grade 2	74	29
	grade 3	26	23
Tsizegp	<20mm	99	22
	20-50mm	49	31
	>50mm	5	3
VSI	yes	35	21
	no	118	35
Nodescore	negative	104	27
	1-3 nodes	37	25
	>3 nodes	12	4
NPIgp	<2.4	30	4
	2.5-3.3	49	4
	3.4-5.4	57	38
	>5.4	17	10
ER	neg	33	24
	pos	116	32
PR	neg	40	27
	pos	109	28
HER2	neg	115	39
	pos	37	17
EGFRsc	negative	138	43
	positive	9	10
RIP5yr	yes	29	14
	no	120	35

Table 3.27: Table showing the distribution of STAT3 results for other pathological features and hormone receptor status. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

Pearson Chi-Square Tests

		STAT3sc
Grade	Chi-square	21.600
	df	2
	Sig.	.000*
Tsizegp	Chi-square	10.873
	df	2
	Sig.	.004*
VSI	Chi-square	4.470
	df	1
	Sig.	.034*
Nodescore	Chi-square	8.365
	df	2
	Sig.	.015*
NPIgp	Chi-square	23.815
	df	3
	Sig.	.000*
ER	Chi-square	8.696
	df	1
	Sig.	.003*
PR	Chi-square	9.013
	df	1
	Sig.	.003*
HER2	Chi-square	.770
	df	1
	Sig.	.380
EGFRsc	Chi-square	7.360
	df	1
	Sig.	.007*
RIP5yr	Chi-square	1.799
	df	1
	Sig.	.180

Results are based on nonempty rows and columns in each innermost subtable.

*. The Chi-square statistic is significant at the 0.05 level.

Table 3.28: Table showing the correlation between STAT3 positivity and other pathological features and hormone receptor status. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

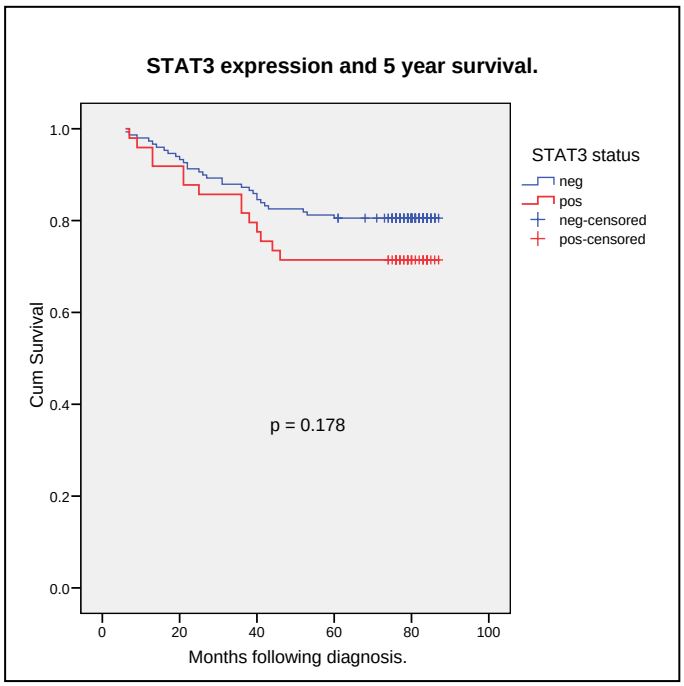

Fig 3.41: Survival curves showing a trend towards poorer survival in STAT3 positive cancers.

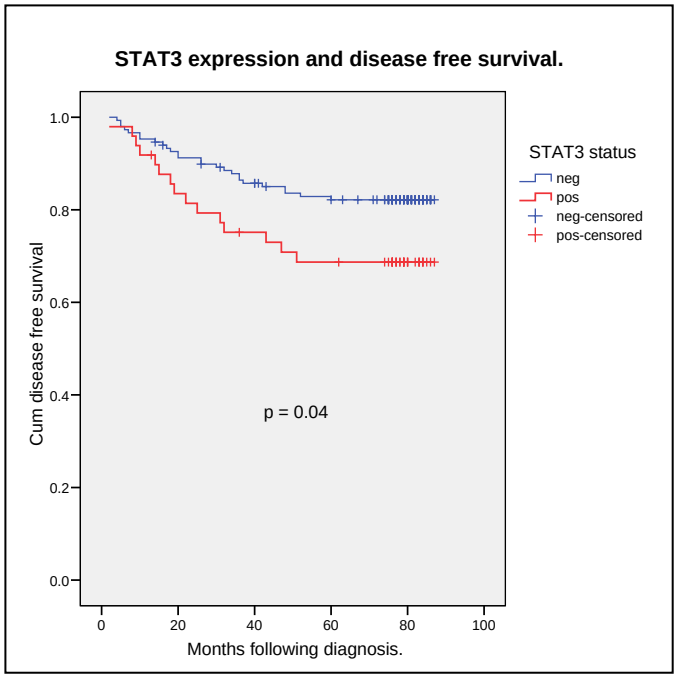

Fig. 3.42: Survival curves demonstrating the significant relationship between STAT3 positivity and disease recurrence.

3.2.4.2 Stat3 and HER2 expression.

As discussed earlier STAT3 has been identified as one of the end points of HER2 related intracellular signalling. It therefore is reasonable to assess the impact of STAT3 status depending on HER2 expression.

Total cases	HER2 negative	HER2 positive
Stat3 negative	115	37
Stat3 positive	39	27

Table 3.29: Table showing the distribution of STAT3 and HER2 results.

% 5yr survival	HER2 negative	HER2 positive
Stat3 negative	88%	77%
Stat3 positive	81%	53%

Table 3.30: Table showing the 5 year survival (%) dependent on STAT3 and HER2 expression.

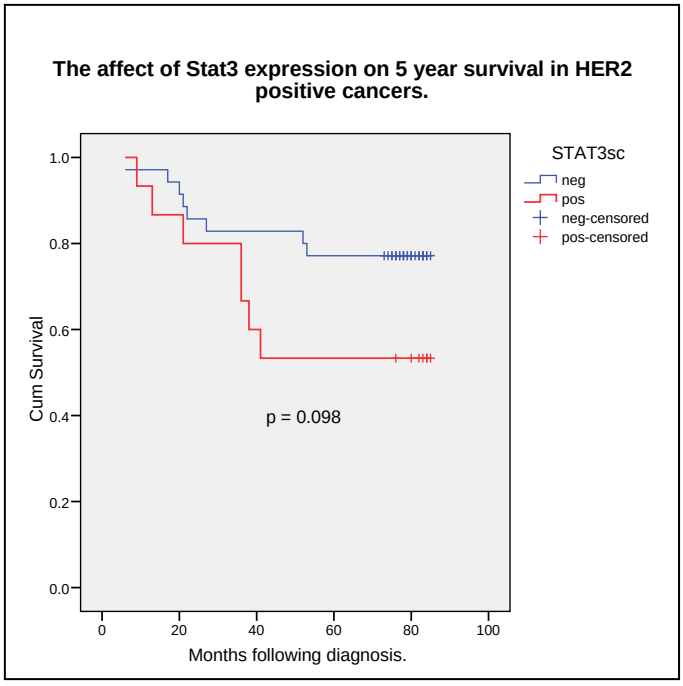

Fig. 3.43: Survival curves showing the trend towards poorer survival in the STAT3 positive HER2 positive cancers.

3.2.4.3 STAT3 expression in node negative breast cancers.

One of the aims of this study was to identify the group of low risk breast cancers (those whom we expect good prognosis) who do poorly. To this end we analysed the affect of STAT3 expression in lymph node negative breast cancers.

Node negative Cancers	Number	5yr DFS %
Stat3 negative	100	94%
Stat3 positive	23	78.3%

Table 3.31: Table showing the distribution of STAT3 positivity in node negative breast cancers and the 5 year survival of each group (%).

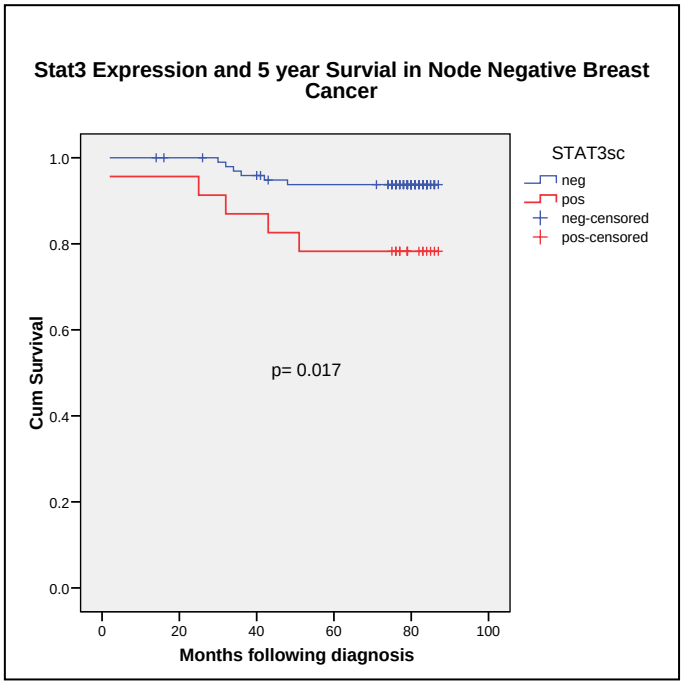

Fig. 3.44: Survival curve showing the significant relationship between STAT3 positivity and poor survival in node negative breast cancers.

3.2.4.3 SP1 expression.

SP1 expression was determined in a total of 208 samples in the study group. 68 (32.7%) of these cancers demonstrated nuclear staining and were determined as SP1 positive.

SP1 Status	Number (%)
Non – nuclear (negative)	140 (67.3%)
Nuclear (positive)	68 (32.7%)

Table 3.32: Table showing the results of the Sp1 staining.

		SP1sc	
		SP1 negative	SP1 positive
		Count	Count
Grade	grade 1	35	23
	grade 2	68	34
	grade 3	37	11
Tsizegp	<20mm	75	46
	20-50mm	59	20
	>50mm	6	2
VSI	yes	39	15
	no	101	53
Nodescore	negative	85	47
	1-3 nodes	43	17
	>3 nodes	12	4
NPIgp	<2.4	19	16
	2.5-3.3	37	13
	3.4-5.4	65	32
	>5.4	19	7
ER	neg	46	11
	pos	92	54
PR	neg	48	18
	pos	90	47
HER2	neg	101	51
	pos	39	16
EGFRsc	negative	119	61
	positive	15	4
RIP5yr	yes	35	8
	no	103	52

Table 3.33: Table showing the distribution of SP1 results for other pathological features and hormone receptor status. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.

Pearson Chi-Square Tests

		SP1sc
Grade	Chi-square	3.382
	df	2
	Sig.	.184
Tsizegp	Chi-square	3.727
	df	2
	Sig.	.155
VSI	Chi-square	.801
	df	1
	Sig.	.371
Nodescore	Chi-square	1.458
	df	2
	Sig.	.482
NPIgp	Chi-square	4.112
	df	3
	Sig.	.250
ER	Chi-square	5.892
	df	1
	Sig.	.015*
PR	Chi-square	1.012
	df	1
	Sig.	.314
HER2	Chi-square	.367
	df	1
	Sig.	.544
EGFRsc	Chi-square	1.287
	df	1
	Sig.	.257
RIP5yr	Chi-square	3.559
	df	1
	Sig.	.059

Results are based on nonempty rows and columns in each innermost subtable.

*. The Chi-square statistic is significant at the 0.05 level.

Table 3.34: Table showing the correlation between Sp1 and other pathological features and hormone receptor status. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

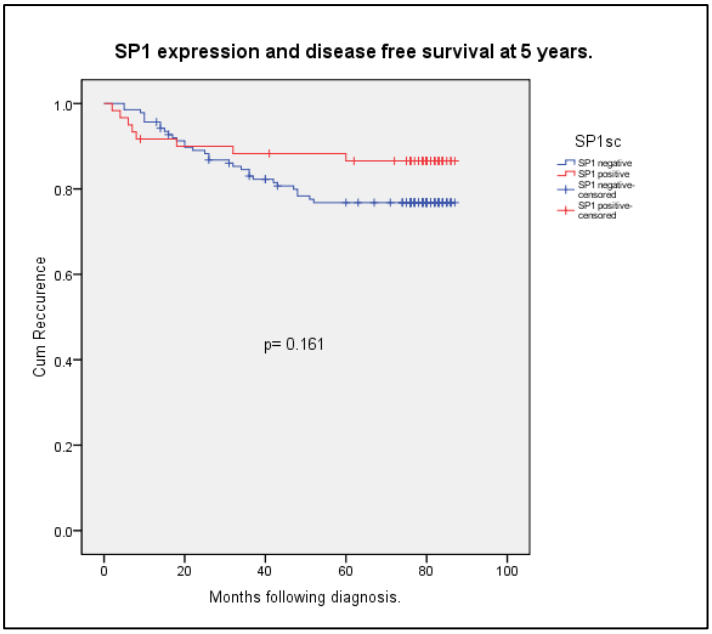

Fig 3.45 Survival curves showing improved disease free survival in the SP1 positive group.

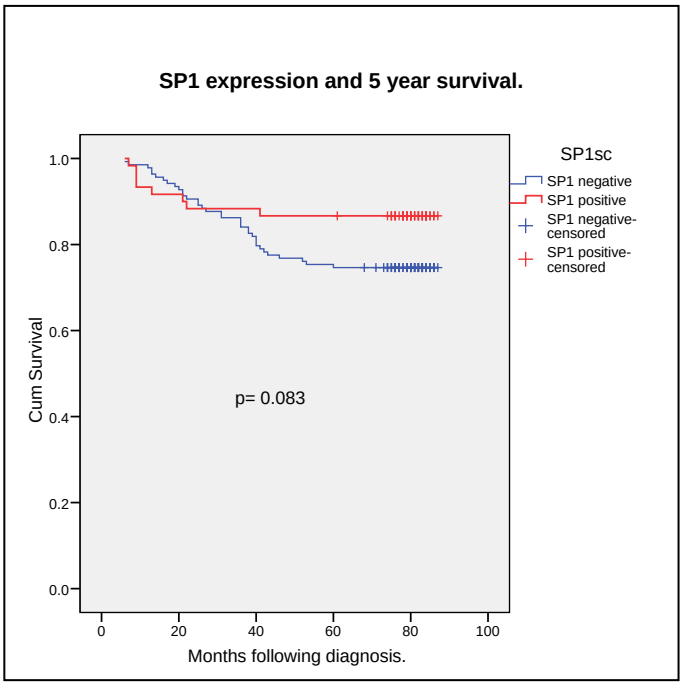

Fig. 3.46: Survival curves showing the trend towards improved survival in the Sp1 positive group.

The overall survival curves (fig. 3.45) appear to show a divergence of the lines after 2 years of follow up. To assess the significance of this we selected all the patients who had survived 24 months after diagnosis and re-plotted that groups survival dependent of Sp1 expression.

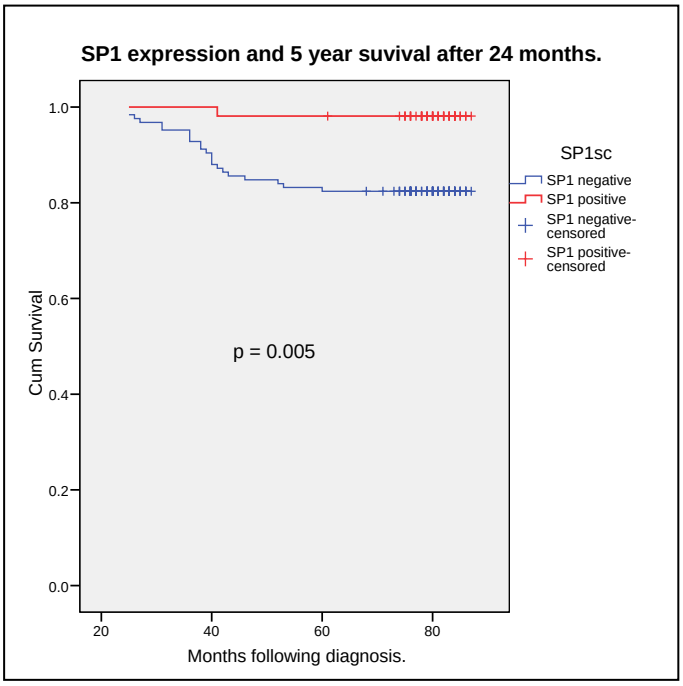

Fig 3.47: Survival curves showing the improved outcome in the Sp1 positive group after 24 months of follow up.

3.2.4.4 NFκß.

208 samples were successfully analysed for NFκß expression. Of these samples 31 (14.9%) of the cancers demonstrated nuclear staining and were determined to be NFκß positive.

NFκß status	Number (%)
No stain	93 (44.7%)
Cytoplasmic	84 (40.4%)
Nuclear	31 (14.9%)

Table 3.35: Table showing the distribution of NFκß staining in the 208 samples.

		NFkBnuc	
		negative	positive
		Count	Count
Grade	grade 1	52	5
	grade 2	92	11
	grade 3	33	15
Tsizegp	<20mm	110	11
	20-50mm	60	19
	>50mm	7	1
VSI	yes	46	9
	no	131	22
Nodescore	negative	111	20
	1-3 nodes	52	9
	>3 nodes	14	2
NPIgp	<2.4	30	4
	2.5-3.3	50	2
	3.4-5.4	76	21
	>5.4	21	4
ER	neg	42	15
	pos	131	16
PR	neg	48	18
	pos	124	13
HER2	neg	134	19
	pos	41	12
EGFRsc	negative	157	24
	positive	12	7
RIP5yr	yes	35	7
	no	132	23

Table 3.36: Table showing the distribution of Nfκß for other pathological features and hormone receptor status. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

Pearson Chi-Square Tests

		NFkBnuc
Grade	Chi-square	13.252
	df	2
	Sig.	.001*
Tsizegp	Chi-square	8.472
	df	2
	Sig.	.014*
VSI	Chi-square	.126
	df	1
	Sig.	.723
Nodescore	Chi-square	.088
	df	2
	Sig.	.957
NPIgp	Chi-square	8.781
	df	3
	Sig.	.032*
ER	Chi-square	7.590
	df	1
	Sig.	.006*
PR	Chi-square	10.887
	df	1
	Sig.	.001*
HER2	Chi-square	3.218
	df	1
	Sig.	.073
EGFRsc	Chi-square	7.301
	df	1
	Sig.	.007*,a
RIP5yr	Chi-square	.086
	df	1
	Sig.	.770

Results are based on nonempty rows and columns in each innermost subtable.

*. The Chi-square statistic is significant at the 0.05 level.

a. More than 20% of cells in this subtable have expected cell counts less than 5. Chi-square results may be invalid.

Table 3.37: Table showing the correlation between NFκβ positivity and other pathological features including hormone receptors status. (Tsizegp= tumour size group, VSI= vessel space involvement, Nodescore=axillary lymph node stage, NPIgp= NPI prognostic group, RIP5yr= overall 5 yr survival.)

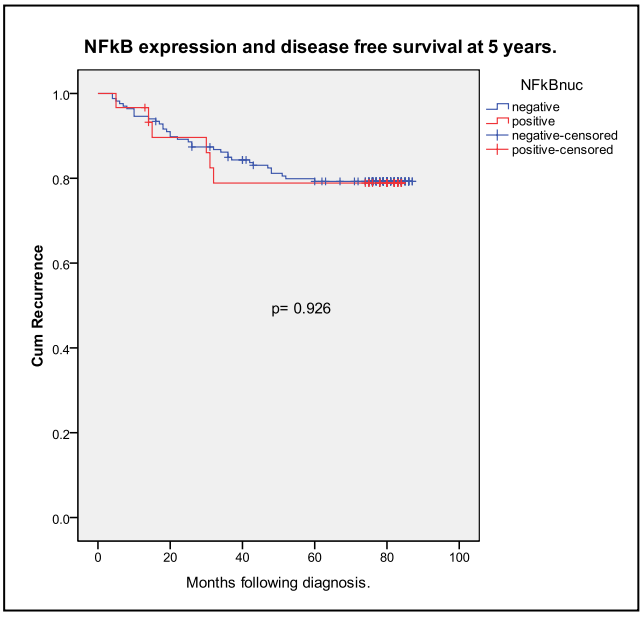

Fig. 3.48: Survival curves demonstrating no correlation between NFkB expression and recurrence.

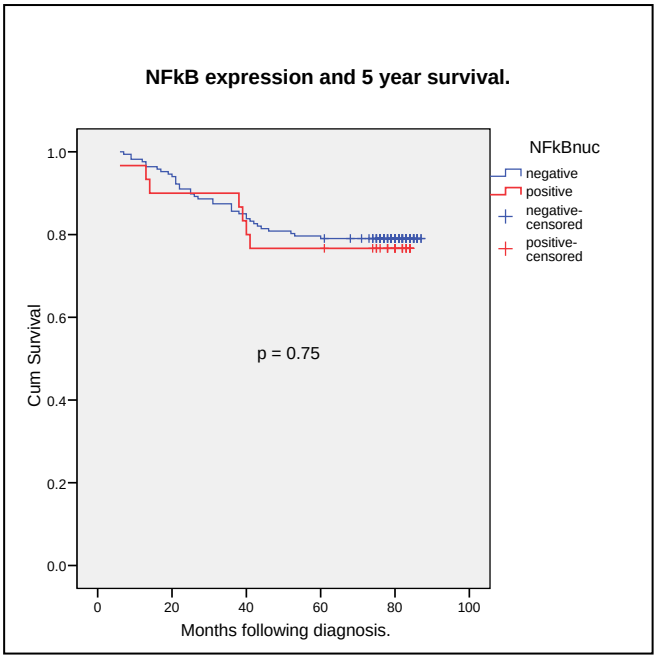

Fig. 3.49: Survival curves demonstrating that NFκß status has no effect on overall outcome.

3.3 Discussion of the Clinical Study Results.

3.3.1 The patient group demographics.

The demographics recorded show that the patients in the study group were largely representative of the overall population of breast cancer patients. The age range and distribution correlates with the published data from 2005(Office of National Statistics, 2008). There was a peak in the age of diagnosis in the early 50's which corresponded with the commencing of screening of patients in that age group (fig 3.23). There was no statistically significant affect of age of diagnosis on overall survival, there was a trend towards an improved outcome in the 45 – 65 year old population (fig 3.24). This may be accounted for by the increased proportion of screen detected cancers within this group. The poorer survival of the younger patients is well recognised. The poorer outcome in overall survival of the older patients may be explained by the non-cancer related deaths in this group however when analysed for disease recurrences, this group also had a trend towards a poorer outcome. This group will contain a higher proportion of symptomatic rather than screen detected cancers. The NHS breast screening program (NHSBSP) detected 14,000 (30%) of the 46,000 breast cancers diagnosed nationwide in 2005(Office of National Statistics, 2008). Local provision of the NHSBSP with the Queen Elizabeth Breast Unit providing screening services for its own and neighbouring primary care trusts has increased this proportion of screen detected cancers in this study group to 35%. The significant positive impact of the screening program can be seen in the effect that the mode of diagnosis has on disease free survival (fig 3.25). The screen detected cancers had a five year disease free survival of 93% compared to 72% for the symptomatic cancers ($p=0.001$). This improved outcome is due to the fact that 53% of cancers detected by the NHSBSP are

15mm or less and may not have been detected by clinical examination(The Information Centre, 2008).

3.3.2 Pathological Features.

The histological types of breast cancers in the study group (table 3.3) was representative of the tumour types expected with 85% of the cancer being non-special type ductal carcinomas. The improved survival outcome of special type cancers in comparison to non-special type ductal cancers is well documented (Ellis I.O., 1992) and this has also been shown in this study group, 91% vs. 73% respectively ($p = 0.041$) (fig 3.26)

The majority of breast cancers diagnosed were grade 2 cancers (48%) with grade 1 and grade 3 cancers representing 28% and 14% of the cases (table 3.4). Grade of cancer is an important prognostic factor and is used as one of the components of the Nottingham Prognostic Index (NPI). The significant effect of grade on survival is shown in the survival curves plotted (fig 3.27).

The tumour size was recorded in each case and there was a wide range of size found from 1mm to 190mm. The cancers were divided into size groups according to the TNM staging system (table 3.5). The majority of the tumours in the group were T1 cancers (<20mm). This group includes the small screen detected cancers. Again the size of the tumour is known to be an important independent prognostic factor and this was demonstrated in this study group with the larger T3 tumours having a significantly poorer outcome compared to the smaller T1 tumours. (fig. 3.28)

Though not used in the NPI, lympho-vascular vessel space invasion (VSI) is recognised as an independent prognostic indicator. The presence of VSI is important when making decisions regarding adjuvant therapy. The results of this study show that VSI was seen in 26% of the cancers (table 3.6). The presence of VSI had a significant detrimental effect on survival, the 5 year survival of patients with VSI in the tumour was 59% compared with 84% in the group without (p=<0.001) (fig. 3.29).

Lymph node status is established as the single most significant prognostic factors in invasive breast cancers (Carter C.L., 1989). The lymph node status was available on 205 of the cancers in the group. As expected the presence of lymph node metastases had a significant effect on survival. The 5 year survival decreased as the burden of nodal disease increased. Node negative patients had a 5 yr survival of 89% compared with 64% with 1-3 nodes involved and 44% in those with more than 3 nodes involved (table 3.7). The survival curves (fig. 3.30) illustrate the significant relationship of node status and recurrence.

The Nottingham Prognostic Index (NPI) was plotted for all of the cases where the components (tumour grade, size and node status) were available. The results show the expected distribution of cases between the NPI groups (table 3.8) and the predictive value of NPI group on overall survival (fig 3.31).

The type of surgery performed was recorded in all cases; the operation recorded was the definitive surgical procedure, i.e. the final operation performed. There are a number of patients whom may initially have undergone breast conserving surgery but due to involved margins, mammographically invisible disease or multi-focal disease

went on to have a completion mastectomy. There has always been a wide variation in the rates of breast conserving surgery compared to mastectomies between different breast units. This is not necessarily related to the pathological features of the cancers presenting to the unit (Caldon et al., 2005), but more related to patient choice and communication with patients. The mastectomy rate at the QEH breast unit in 1999 was 52% (table 3.9, Fig 3.32).

During the follow-up period any incidences of disease recurrence were recorded irrespective as to whether the recurrences lead to a patient death. There were 50 patients who suffered a recurrence of their disease (table 3.10). The most common site of recurrence was local to the primary tumour 34%. This group included recurrence in the same breast (if breast conserving surgery had been the initial treatment), chest wall recurrence and local lymphatic recurrence. It did not include cancers of the contralateral breast which were recorded as a second primary breast cancer. Bone metastases 28% was the next most common site followed by lung 16%, liver 14%, brain 6% and 1 case of a choroid metastases. The time between diagnosis and recurrence of disease was also recorded (fig. 3.33) and showed a diminishing incidence of recurrence as the time from diagnosis increases. This does not quite follow the bimodal curve of recurrence well established by previous large studies (Romano D., 2008). In the bimodal model there is a peak of incidence of recurrence in the after 24 months with a second broader peak after 60 months. In this study there was a high incidence of recurrence in year 1 compared with year 2 but the numbers are small in each group (13 cf 11 respectively). With greater numbers the group may become representative of the bimodal model.

In summary the pathological feature of this study group conformed to expectations and standards set by previous studies in breast cancer. The correlations of these pathological features are shown in table 3.11. This table shows that there is a significant relationship between all the pathological feature listed and 5 year survival.

3.3.3 The Receptor Status.

3.3.3.1 Estrogen receptor.

We determined the ER status of 196 cancers using IHC. There were problems with some very small cancers cutting out of the blocks reducing the numbers of results available. In this study group 71% of the patients had ER positive tumours (table 3.12). It has been long established that ER status is an important factor in breast cancer correlating to pathological features and overall survival. In this study group ER positive tumours were significantly related to low grade tumours (p=<0.001), smaller tumours (p=0.024), negative VSI (p=<0.001), node negative tumours (p=0.006) (table 3.14) and increased overall survival (p=<0.001) (fig. 3.34). These results all agree with previously published work (Fisher et al., 1988, Grann et al., 2005, Aaltomaa et al., 1991) and it is now widely agreed that ER status in an independent prognostic marker of disease outcome. Our results also demonstrated a significant relationship between ER positive and PR positive cancers (p=<0.001), this is not surprising as progesterone receptors expression is induced by estrogen (Schultz et al., 2003a). This study showed an inverse correlation between ER positive and HER2 positive tumours (p= 0.011). This is well recognised and described in previous studies, (Ciocca, 1992, Witton, 2003) though it is suggested that this relationship or predictive power of ER status on Her2 status is more apparent in the elderly patients and less significant in those younger patients (Huang, 2005). Along with an inverse correlation with HER2 the ER results also showed a similar relationship with EGFR (p=<0.001). This agrees with previous studies demonstrating this correlation. (Zeillinger, 1993, Klijn, 1994)

3.3.3.2 Progesterone receptor.

PR status was determined in 195 of the sample cases. Of these cases 132 (68%) of the cancers were PR positive (table 3.15). As described above there is a strong correlation between ER expression and PR expression. As the PR gene transcription is regulated by oestrogen PR expression is considered to be a marker of functioning ER (Horwitz, 1978).

PR expression is known to be strongly associated with ER expression and other positive predictive markers this study has shown like wise; PR expression is significantly associated with low grade (p=<0.001), negative VSI (p=0.003), lower NPI group (p=<0.001) (table 3.17), and an increased 5 year survival (p= 0.001) (fig 3.35). When considering PR correlation with other receptors it is clear that it is significantly associated with ER (p=<0.001) and like ER it is also negatively correlated to HER-2 (p=< 0.001) and EGFR (p=<0.001) (table 3.17).

3.3.3.3 Combined ER and PR

In this study it was shown that the majority of the tumours were ER/PR double positive (66%) or ER/PR double negative (27%) (table 3.18). The impact of this on survival is clearly seen on the survival curves with the double negative tumours doing poorly (fig. 3.36). The other 2 groups ER+ve/PR-ve (5.1%) and ER-ve/PR+ve (1.6%) are too small to attach and significance to. In larger studies it has been suggested that PR positive ER positive cancers are more responsive to hormonal therapy and hence have a better outcome when compared to PR negative ER positive cancers. This again is based on the theory of the PR representing a functioning ER signalling system.(Bardou, 2003)

3.3.3.4 HER-2.

In this study we have shown that HER2 is over expressed in 26% of the cases it was technically possible to assess (table 3.19). The literature published on this is varied suggesting that over expression of HER2 occurs in 10-30% of invasive breast cancers (Ciocca, 2006). Though the assumption that we made regarding the samples scoring 2 (assigning them as HER2 negative,) may have reduced slightly the proportion of cancer we classified as HER2 positive, we still have reported an over expression rate greater that that seen in some other studies. There now has been a revision of the expected rates of HER expression in an unselected group of cancers bases on UK audit data (Walker et al., 2008). See below;

	2006 audit (15 689 cases)	2007 audit (17 270 cases)
IHC score (% positivity):		
2+	18% (range 7 to 41%)	19% (range 1 to 38%)
3+	13% (range 7 to 22%)	11% (range 4 to 20%)
FISH positive	24% of 2+	19% of 2+
Total HER2 positive	17%	15%

Table 3.37: data of HER2 positivity in unselected cases (FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry)

Due to the historic variations in HER2 results between laboratories and the recognition of the importance of accurate reporting for the selection of patients for adjuvant Herceptin there are now a number of guidelines to reduce this variation. These include guidance on sectioning, staining and scoring as well as audit and quality assurance testing (Walker et al., 2008). Despite these efforts, it is accepted that there always will be a degree of regional variation in HER2 expression.

As with the previous receptors, we then assessed the correlation of HER2 expression with the other pathological features of the tumours (table 3.20 & 3.21). We have shown the over expression of HER2 was significantly associated with tumours of a higher grade (p=0.001) and vessel space invasion. (p=0.04), but not with tumour size (p=0.075), or nodal involvement (p=0.233), this is in agreement with previously published work (Huang, 2005, Sjorgen, 1998). As would be expected and because we had shown a significant correlation between HER2 over expression and grade we also demonstrated that HER2 over expression was also significantly related to tumours in a poorer prognostic group (p=0.002) (table 3.21).

It is widely reported that HER2 over expression is inversely associated with ER/PR expression.(Gago, 2006) Our study agrees with these results, we demonstrated a significant inverse relationship between HER2 expression with ER (p=0.011) and PR (p=<0.001) status. In our study we found no relationship between HER2 and the other type 1 growth factor receptor EGFR (table 3.21).

It is generally accepted that HER2 over expression is a marker of increased tumour aggressiveness (Pawlowski, 2000) and in many studies HER2 over-expression has been related to a poorer overall survival (Sjorgen, 1998). In this study however, though we showed a significant relationship between HER2 and markers of poorer outcome (high grade, high NPI and ER negative tumours), we have not shown a significant relationship between HER2 over expression and overall 5 yr survival. As seen in fig 3.37, the 2 survival curves do begin to diverge after 20 months and though there appears to be a trend towards poorer survival this is not significant. (p=0.1). We did however demonstrate a significant result between HER2 expression and disease

free survival at 5 yrs. (p=0.009). This disparity in results may be due to the length of time the patients were followed up. If the follow up time was extended to 7 yrs then we may possibly have seen a significant difference in overall survival.

It has been suggested by earlier studies that HER2 expression is important in the prediction in response to adjuvant treatment (Payne, 2008). Studies have shown that HER2 status expression favours response to certain anthracycline containing chemotherapy regimens (Pritchard, 2006). It has also been suggested that HER2 positive ER positive breast cancers may be resistant to endocrine (tamoxifen) treatment, (Gago, 2006) but it is accepted that currently these trials are insufficient to allow HER2 to influence choice of endocrine adjuvant therapy (Prowell, 2006). In our study group we compared the survival curves of the ER+/HER2+, ER+/HER2-, ER-/HER2+ and ER-/HER2- sub groups. Though the ER+ sub groups were of a reasonable size (HER2 +ve 27 patients, HER2 -ve 111 patients,) (table 3.22) we were unable to show a difference in survival between the groups (fig. 3.38). As all the ER+ patients would have received Tamoxifen based adjuvant therapy we would agree that the HER2 status appears not to affect their response. Though in the ER- group there does seem to be a trend towards poorer outcome in the HER+ compared to HER- group the numbers are too small to be significant. In one study recently published a lower rate of HER2 positivity was seen in the ER positive group (13.3%) compared to 19.5% in this study. Again a correlation was seen with high grade, nodal involvement and ER/PR negative tumours. A poorer survival (-7.5% at 5 years) was identified but this was confined to the lymph node positive sub-group (Purdie et al., 2010).

3.3.3.5 EGFR.

The assessment for the expression of EGFR was completed in 203 cases. We found in our study 19 (9.4%) of the cancers expressed EGFR (table 3.23). The published rate of EGRF expression seen using IHC in the literature varies widely from 14-91% (Klijn, 1994) The higher rates of expression quoted are predominantly from earlier studies where the proportion of the small low grade tumours was very low (1.8% grade I cancers) (Zeillinger, 1993). Our study had 28% grade I cancers and this inclusion of predominantly screen detected cancers may account for the lower rates of EGFR expression.

It is widely reported that EGRF expression is a marker of more aggressive disease in the in-vitro setting but many in vivo studies have reported contradictory results. Some of these studies confirm the relationship between EGFR receptor expression and aggressive disease and some are unable to demonstrate this link. (Klijn, 1992, Rampaul, 2005) In these studies it appears that the studies quoting the higher rates of expression on EGFR were less likely to show significant associations with poorer outcomes.

In this study we have demonstrated a significant association between EGFR expression and tumours of a high grade: 1 out of 53 grade I tumours compared to 10 out of 38 grade III tumours ($p=0.004$). We were also able to demonstrate an association between EGFR expression and increased tumour size ($p=0.009$). Some studies have managed to demonstrate this relationship with grade and size (Zeillinger, 1993), but others have not (Klijn, 1994). Though it appears that EGFR expression is associated with larger more poorly differentiated tumour we found that there was no

significant association between EGFR and lymph node involvement (p=0.331). This concurs with the majority of the published data (Nieto, 2007a). We were however able to show a significant relationship between EGFR expression and the NPI group. Only 2 of the 82 patients in the good and moderate prognostic groups expressed EGFR compared to 17 of the poorer prognostic group. (p=0.034) (tables 3.24 & 3.25).

When we compared EGFR expression with the expression of ER and PR we found that the majority (17/19) of tumours expressing EGFR were ER and PR negative (p=<0.001). There were only 2 cases where EGFR expression existed with ER and PR expression. Interesting both of these cases, compared to many of the other EGFR patients, did very well and were still disease free after 7 years. This relationship between positive EGFR expression and steroid receptor negative tumours has been previously demonstrated (Klijn, 1994, Zeillinger, 1993). As mentioned many studies have failed to show a link between EGFR expression and overall survival. In this study we found that the five year survival for the EGFR positive group of patients was 47% in comparison to 82% in the EGFR negative group. When the survival curves (fig. 3.39) were plotted this difference was highly significant (p=<0.001).

As EGFR plays a vital role in HER2 activation we analysed the sub group of HER2 positive patients (n54) and their EGFR status. In this group 47 (87%) of the patients were EGFR negative and 7 (13%) of the patients were EGFR positive. It was shown that there was no significant correlation between HER2 and EGFR expression. When the survival of these two groups of patients was analysed it was demonstrated that all 7 (100%) of the HER2 positive/EGFR positive patient died of disease recurrence within 5 years of diagnosis. This is compared to only 8 (17%) of the 47 HER2

positive/EGFR negative patients (p=<0.001). These results demonstrate, even in this small sample, the significance of co-expression of HER2 and EGFR on disease outcome (fig. 3.40). When considering these results it is logical to assume that dual therapy with anti-HER2 (Trastuzumab) and anti-EGFR (Gefitinib) would improve the outcome of these HER2 positive cancers. Unfortunately early phase I-II studies are not supporting this.(Arteaga, 2008). A new dual inhibitor of EGFR and HER2 tyrosine kinases (Lapatinib) is now undergoing trials with some encouraging results (Medina, 2008).

3.3.4 Transcription factor expression in the study group.

Where possible the transcription factor status of all of the tumours in the study group was ascertained. There were some technical problems with some of the samples predominantly being due to the lack of representative tissue in the blocks for some of the small tumours.

3.3.4.1 STAT3.

In this study 209 breast cancer samples were successfully analysed using IHC for the expression of STAT3. As described the samples were scored according to the cellular location of the STAT3 stain, nuclear vs. non-nuclear. Using this technique 56 (26.8%) of the cancers were positive for nuclear STAT3 (table 3.26). This rate of nuclear STAT3 is similar to the published rate seen using IHC of 23% (Clevenger, 2004).

As discussed in section 1.3.2, STAT3 expression is considered a marker of more aggressive cancer cell morphology and behaviour but there has been little published work in the clinical setting. The results of the correlation of STAT3 are seen in tables 3.27 and 3.28. In this study we have demonstrated a significant link between STAT3 expression and the grade of the breast cancer. The expression of STAT3 increases with the increased grade of cancer. Our results show that 47% of Grade III cancers express nuclear STAT3 compared with 28% of Grade II cancers and only 7% of Grade I cancers ($p=<0.001$). As demonstrated in cell models over expression of STAT3 increases cell proliferation and its inhibition induces the converse (Burke, 2001). In this study we have shown that STAT3 expression increases with increasing

tumour size, 18% of T1 (<20mm) tumours expressed nuclear STAT3 compared with 39% on T2 and T3 (>20mm) tumours (p=0.004).

The role of up-regulation of STAT3 and the invasive potential of breast cancer is not clear. Some studies have suggested that STAT3 inhibition decreases invasiveness in cell lines (Selander et al., 2004a). Other studied have show the opposite with a decrease in invasiveness and a negative correlation between STAT3 expression and lympho-vascular invasion (Dien et al., 2006).

In this showed that there is a there is a relationship between STAT3 and VSI and also STAT3 and lymph node involvement. 38% of the breast cancers demonstrating VSI were STAT3 positive compared with 22% of cancers without VSI (p=0.034). Of the cases with lymph node involvement 37% were STAT3 positive compared with only 20% of lymph node negative cases (p=0.015). These results are in agreement that Stat3 expression increases the tumours ability to metastasise. There is no current published work on the correlation of STAT3 with NPI group. It is logical to assume that as a significant relationship between STAT3, tumour grade, size and nodal status, which constitute NPI score was shown, we will see this relationship continue with NPI. This study did indeed demonstrate this relationship. Our results showed that 9% of tumours in the excellent and good (NPI<3.3) prognostic groups were STAT3 positive compared to 40% of tumours in the moderate and poor (NPI>3.3) prognostic groups (p=<0.001).

As described earlier STAT3 phosphorylation provides possible pathway for non-classical ER signalling. It is therefore important to ascertain if there is a relationship

between ER and STAT3 expression in breast cancers. Our results show that there is a significant relationship between ER and STAT3 expression. When we analysed the ER positive cancers 22% of them were STAT3 positive this compares with 42% of ER negative cancers (p=0.003). It is not surprising that there is an increase in nuclear STAT3 in the ER negative cancers. It is likely that an ER positive cancer is being regulated by the classical ER pathway and therefore STAT3 will not be elevated. ER negative cancers however will grow through other stimulatory pathways such as EGFR, HER2 and IGF receptors. All of these pathways induce the phosphorylation of STAT3 and its shuttling to the nucleus (Gritsko et al., 2006b), hence we see an increase in nuclear STAT3 in these ER negative cancers. These results agree with other studies which described this relationship between ER and STAT3 (Yeh et al., 2006b). Though STAT3 is more common in ER negative cancer we were interested to ascertain if it was important in ER positive cancers. As discussed earlier ER positive cancers which become resistant to anti-estrogen treatment may escape this inhibition by utilising non-classical pathways. The survival of the ER positive cancers split by the STAT3 expression was analysed, there was no difference in survival between the 2 groups analysed. STAT3 expression does not help identify the ER positive cancers which become resistant to tamoxifen and relapse. These results infer that if a cancer is proliferating via a STAT3 mediated non-classical ER pathway its outcome is no worse than those proliferating via the classical pathway.

There is little published data on the relationship between STAT3 expression and PR expression. It can however be assumed that because of the very significant relationship between ER and PR co-expression we would see a similar relationship between PR and STAT3 as we did with ER and STAT3. This study data showed that

when analysed 20% of PR positive cancers were positive for STAT3 compared to 40% of PR negative cancers (p=0.003). This findings do contradict that of another study but in that study there was a massive discrepancy between ER expression and PR expression (Yeh et al., 2006b).

There were 2 indications for the analysis of HER2 and its relationship with STAT3, firstly HER2 signalling activity involves STAT3 phosphorylation (Fernandes et al., 1999) and STAT3 activity may up-regulate the expression of HER2 (Qian et al., 2006). In this study group we determined the combined HER2 and STAT3 status of 208 cancers. Of the HER2 negative cancers (n154) 25% were STAT3 positive and in the HER2 positive group (n54) 31% on the tumours were STAT3 positive (table 3.29). There was no statistical difference between these two groups (p=0.38). This shows that, though an in-vivo mechanism has been shown for the up-regulation of HER2 via activation of STAT3, this is not apparent in this clinical study.

As STAT3 is an important component of HER2 signalling, we were interested to see if the presence of STAT3 in the HER2 positive group would affect outcome for the patients. The 5 year overall survival of the HER2 positive patients in this study group was 70%. We assess the co-expression of HER2 and STAT3 in 218 patients. Of the 64 HER2 positive cases 27 (42%) co-expressed STAT3 (table 3.29). The 5 year survival of this group was 53%, this is compared to 77% in the HER2 positive STAT3 negative group (table 3.30). The survival curves show a trend to poorer survival but the data did not reach statistical significance (p=0.098) (fig 3.43). This failure to reach significance may be due to the smaller numbers in this sub group. If these results on further investigation are found to be significant then there would be a considerable

impact on the use of Herceptin in the management of these patients. These results suggest that anti HER2 therapy may only be effective in the STAT3 positive group of patients as it is these patients who are suffering the negative outcome of being HER2 positive.

It is known that EGFR is a potent activator of STAT3 phosphorylation (Real et al., 2002). It was therefore expected that a significant relationship between the EGFR positive cancers and STAT3 would be demonstrated, though other studies had failed to do so (Diaz et al., 2006a). This studies results showed that 52% (10/19) of the EGFR positive cancers were STAT3 positive compared to only 24% (43/181) of the EGFR negative cancers, this result was statistically significant (p=0.007). From this result EGRF expression does correlate with STAT3 activation in a clinical setting. The finding of STAT3 activation in EGFR negative cancers suggests that this phosphorylation is also under the control of other receptor activated tyrosine kinases. Unfortunately due to the small numbers in the EGFR positive sub group (n19) the survival curves for the STAT3 positive and STAT3 negative groups did not reach statistical significance.

The survival of the above sub-groups has already been discussed but one of the primary aims of this study was to ascertain if STAT3 expression affected overall patient outcome. There have been a number of studies which have looked at outcome following treatment for advanced or recurrent disease with adjuvant or palliative chemotherapy dependent on STAT3 expression. These studies suggest that patients with tumours over-expressing STAT3 have a poorer response to chemotherapy for recurrent disease (Diaz et al., 2006a). As mentioned in chapter 1.3.2 one study

suggested a survival advantage in the over expression of STAT3 in node negative tumours (Dolled-Filhart et al., 2003b). In this study group, when overall survival was analysed, a divergence of the survival curves after 2 years was noticed. At 5 year, though there was a trend towards a poorer survival in the STAT3 positive group, this result was not statistically significant (fig. 3.41). When the study group with regards to disease recurrence was analysed, it was found that there was a correlation between STAT3 positive cancers and recurrence of disease. The results showed that 35% of the STAT3 positive patients developed disease recurrence compared with 20% of STAT3 negative patients (p=0.049). When disease free survival was considered, an earlier split in the curves was shown this demonstrated that the STAT3 positive group were at a significantly higher risk of earlier disease recurrence (p=0.04) (fig 3.42). These results have not been shown before in a large unselected group of breast cancers.

As these results have shown that STAT3 may be related to disease outcome we were interested to see if STAT3 status was useful in identifying those breast cancer patients with good prognostic group tumour who do poorly. Previous studies have suggested that in a sub-group of node negative breast cancers nuclear STAT3 expression was associated with an increased disease free survival (Dolled-Filhart et al., 2003b). The survival impact of STAT3 status on node negative cancers was analysed. The node negative breast cancer patients usually fall into the better prognostic groups, many of whom are not offered adjuvant chemotherapy. In this group of 123 patients 23 (18.6%) were STAT3 positive (table 3.31). The STAT3 positive cancers had a reduction in 5 year survival compared to the STAT3 negative cancers (78.3% vs. 94%). When the survival curves were plotted this result was significant (p=0.017)

(fig. 3.44). These results show that STAT3 expression may be a marker of poorer prognosis in those patients with node negative tumours.

To see if the overall predictive value of STAT3 remained significant when the effect of grade and nodal status, the 2 most significant pathological predictive markers, were considered a Cox regression analysis was performed. In this analysis unfortunately the predictive value of STAT3 was not significant. Although we have shown that STAT3 status is significantly associated with disease recurrence it is not a significant independent predictor of disease free survival.

3.3.4.2 Sp1

The Sp1 expression was ascertained in 208 of the cancers. As discussed in chapter 1.3.3 Sp1 transcription factor is implicated in many of the important gene transcriptions associated with breast cancer, it was expected that results would show Sp1 expression to some degree in the majority of samples tested. What was discovered was the vast majority, (91%,) of the samples showed a degree of IHC staining for Sp1 i.e. only 8.7% of samples had no staining (score 0). The majority of positive samples could be divided into 3 groups, those with weak cytoplasmic staining (score 1) n39 (18.8%), those with strong cytoplasmic staining (score 2) n 83 (38.9%) and those with strong cytoplasmic and nuclear staining (score 3) n 68 (32.7%) (table 3.32). There is little published data relating to either the prevalence or cellular location of Sp1 in clinical breast cancer samples, therefore there are no comparisons to make with other studies. In the assessment of other solid tissue tumours the nuclear expression of Sp1 has been reported as 54% in gastric cancers (Zhang et al., 2005a), 31% in colorectal cancers (Maurer et al., 2007) and 45% of pancreatic cancers (Jiang et al., 2008). As Sp1 is consider to be functioning predominantly at the DNA level both in the transcription of ERα and ERα induced gene transcription (deGraffenried et al., 2002, Khan et al., 2007), we thought it reasonable that we should consider the samples with strong nuclear staining as having higher than usual Sp1 expression and activity i.e. Sp1 positive. Our over-expression / nuclear expression rate of 32.7% in breast cancer is therefore in a similar range to that of other solid tissue tumours.

Due to the important role of Sp1 with ERα in both its expression and activity we hypothesised that there would be a relationship between Sp1 positive tumours and "good" prognostic features such as low grade, node negative and smaller tumours

which are a feature of ER positive disease. These results were unable to show any relationship between these pathological features and Sp1 expression (tables 3.33 & 3.34). There was no correlation between Sp1 over-expression and tumour grade, tumour size, VSI, nodal status, or NPI group. In other solid tissue tumours over expression of Sp1 has been correlated with markers of poor prognosis such as tumour depth, TMN stage and rate of angiogenesis in gastric cancers (Zhang et al., 2005a, Wang et al., 2005) and high grade, stage and lymph node metastasis in pancreatic cancers (Jiang et al., 2008). It may be that in these cancers the SP1 transcription factor up-regulates pro-metastatic genes which are not as evident in the predominantly ER driven breast cancers.

The study's results showed that only 19% of ER negative cancers over expressed Sp1 compared with 37% of ER positive cancers ($p=0.015$). Though there is an association with ER and Sp1 positive cancers there are obviously a proportion of ER positive cancers which do not over-express Sp1. These tumours are a demonstration that though Sp1 is essential for ERα gene transcription (deGraffenried et al., 2002) normal levels of Sp1 are adequate for ER expression. When the tumour samples which did not demonstrate any Sp1 IHC staining (score 0), 8.7% of the study group were looked at, 10 of the 18 cancers were still ER positive. These tumours must still have a degree of functioning Sp1 which was not evident on our IHC assay.

It has been shown that PR expression is a measure of functioning ER stimulation. It is now know that the PR gene contains 2 regions that rely on Sp1 sites to confer this estrogen responsiveness (Schultz et al., 2003a). As an association between ER expression and Sp1 positive cancers had been demonstrated a similar relationship

between Sp1 and PR expression was expected. This study however was unable to demonstrate a significant relationship between these SP1 and PR. The lack of this significant relationship may be due to fact that the PR gene transcription does not rely on a single estrogen response element but on the co-operative interaction of numerous transcription factors and *cis* elements working together.

There is no published evidence that Sp1 action may play a role in the expression of either EGFR or HER2. It is then expected that our results failed to show a correlation between Sp1 and EGFR or HER2, ($p=0.544$ and $p=0.257$ respectively).

This study aimed to investigate if Sp1 transcription factor expression could be used as an indication of prognosis in invasive breast cancer. The successful treatment of ER positive breast cancer relies on both adequate surgery and also the use of adjuvant endocrine manipulation. The aim of the adjuvant endocrine therapy may be estrogen depravation (aromatase inhibitors) or alteration to ER response to estrogen (SERMs). It has been shown that on recurrence 25% of these ER positive tumours have lost ER expression(Kuukasjarvi et al., 1996).

It was therefore hypothesised that Sp1 over-expression may decrease the loss of ER expression and therefore improve survival in this group. In our analysis of overall survival there appeared to be a trend towards improved survival in the Sp1 positive group 86.6% c.f. 74.6% in the Sp1 negative group but this failed to reach statistical significance ($p=0.83$) (fig. 3.46). When looking at the survival curves this separation of the curves appeared only after 2 years of follow-up. When the sub-group of patients alive after 2 years was analysed, 98% of the Sp1 positive group were alive

after 5 years compared with 82% of the Sp1 negative group. This relationship was significant (p=0.005) (fig 3.47). If this improved outcome was due to the maintenance of estrogen responsiveness in the ER positive cancers then one would expect to find a more impressive difference when the survival of ER positive sub-group was analysed. This however was not evident in the sub-group analysis where Sp1 lost its significant affect on survival. The ER negative group did show a separation of the curves after 2 years but with a trend towards improved survival in the SP1 negative group but this did not reach statistical significance due to the loss on many ER negative patients in the first 24 months reducing the group numbers. It therefore appears that in this study the prognostic affect of Sp1 status after 2 years is independent of the ER status of the cancer.

It is important to consider the recurrences that occur after 2 years. The bi-phasic nature of breast cancer recurrence in well know with the majority of recurrences occurring in the first 24 months but then a second significant peak of recurrences between 36 and 60 months. With the introduction of aromatase inhibitors it was considered that the aromatase inhibitors would be more effective in reducing the second peak of later recurrences, this gave rise to the "switching" strategies. Switching is where a patient is given 24 months tamoxifen then switched to an aromatase inhibitor. Two large trials were conducted into this strategy the TEAM/IES trial comparing Tamoxifen and Exemestane both as five year mono-therapy and a switched arm, and the BIG 1-98 comparing Tamoxifen and Letrazole as a switched strategy. The IES study did show a reduced disease recurrence in the arm that switched to exemestane for tamoxifen after 2 years compared with 5 years of tamoxifen (Coombes et al., 2007) and this did translate into a modest survival benefit.

Again in the BIG 1-98 trial there was an advantage in switching from tamoxifen to Letrazole but neither of these studies showed an advantage of the switching over 5 years of AI treatment (Joerger and Thürlimann, 2009). There have also been studies into extending adjuvant anti-oestrogen beyond 5 years. The MA 17 study, which is a double blind randomised control study into the use of a further 2 years of letrazole following 5 years of tamoxifen, suggests that there is a significant reduction in recurrence and death in the node positive sub-group, and a trend towards significant improvement in outcome in all patients (Ingle et al., 2006).

Though this study is not designed to ascertain if SP1 expression could identify patents that would benefit from switching or extended therapy it would be interesting to consider this with extended follow up data.

The impact of Sp1 expression on survival in breast cancer has not been published previously and therefore these results cannot be corroborated by other studies. The previously discussed studies in gastric, pancreatic and colon cancers have all shown the converse association of Sp1 expression with prognosis (Zhang et al., 2005a, Jiang et al., 2008, Maurer et al., 2007).

3.3.4.3 NFκß

208 cancer specimens were successfully assessed for the expression of NFκß. As with STAT3 and Sp1 the IHC scoring of the samples was based upon the presence and location of the stain. Our results show that 44.7% of the samples did not show any staining for NFκß, 40.4% of the patients exhibited cytoplasmic staining for NFκß and 14.9% of the samples had nuclear staining (table 3.35). Previous studies have demonstrated that nuclear localisation is a marker of activation of NFκß (Biswas et al., 2004) therefore it was these cancers with positive nuclear stain that were considered as NFκß positive. The rate of nuclear expression demonstrated in this study, 14.9%, compares closely to the published rate of 13% seen in studies using a similar IHC technique (Buchholz et al., 2005).

NFκß expression was then compared to pathological markers (tables 3.36 & 3.37) and demonstrated a significant correlation between NFκß positive tumours and higher pathological grade. Our results show that only 8.7% of grade I tumours expressed nuclear NFκß compared with 31.3% of grade III tumours (p=0.001). There was a significant relationship between NFκβ and tumour size, 9% of tumours <20mm were NFκß positive compared with 33% of larger tumours (p=0.014). There was no correlation between node status of tumour or the presence of vessel space invasion and NFκß. Only 6.9% of the good prognostic tumours expression NFκß compared to 20.4% of the poorer prognostic tumours. This correlation with higher grade, larger and poorer prognostic tumours is not widely reported in the published literature with some studies unable to demonstrate a correlation between NFκß and clinical stage of the disease (Buchholz et al., 2005). When the pro-proliferative and anti-apoptotic action of NFκß is considered (Biswas et al., 2003), it is unsurprising that this

correlation with higher grade and larger tumours was found. As discussed later there is known association between NFκß and hormone receptor negative tumours and as already shown in this study these ER and PR negative tumours tend to be the higher grade and poorer prognostic cancers.

Our data agrees with the link between NFκß expression and ER negative cancers (Van Laere et al., 2007a, Biswas et al., 2000, Biswas et al., 2004). The results have shown that 10.9% of ER positive cancers are NFκß positive compared with 28% of ER negative cancers (p=0.006). It has also been suggested that the ER expression may be down regulated as an effect of EGFR activation of NFκß (Van Laere et al., 2007a). A significant relationship between nuclear NFκß and EGFR expression was shown with 36.8% of EGFR positive cancers expressing nuclear NFκß compared with only 13.2% of EGFR negative cancers (p=0.007).

Following the significant association between NFκß, grade, tumour size and ER negative tumours a relationship between NFκß and disease outcome was expected to be shown. In this study however we found no relationship between NFκß and disease outcome. The 5 year overall survival for the NFκß positive group was 76.7% compared to 79.1% in the NFκß negative group (p=0.77) (fig 7.25).

In-vitro studies have shown that NFκß activation is associated with resistance to anti-oestrogens (Zhou et al., 2007) and inhibition of NFκß enhances sensitivity of resistant tumours to Tamoxifen (deGraffenried et al., 2004). Previous patient studies have also suggested that NFκß status identifies a sub set of ER positive cancers that are at a high risk of disease recurrence (Zhou et al., 2005). When the subset of ER positive cancers,

who would have received tamoxifen as adjuvant treatment was analysed, NFκß status had no impact on disease recurrence or overall survival.

It has been suggested in some studies that activation of NFκß is linked to resistance to neo-adjuvant chemotherapy (Montagut et al., 2006a) and activation of the NFκß / bcl-2 pathway decreased pathological response to doxorubicin based chemotherapy (Buchholz et al., 2005). A sub group of patients with a high nodal burden which were likely to have received chemotherapy were selected, analysis of this group showed no difference in disease outcome related to the NFκß status (fig 3.48.)

Though some significant relationships between NFκß and various pathological features have been shown, its expression does not seem to have an impact on patient outcome.

3.3.5 Summary of the discussion.

This clinical study has yielded some significant results. It has show that STAT3 expression appears to correlate with poorer outcome of disease. It has shown that SP1 expression is associated with decreased disease recurrence after 2 years and it suggests that IHC expression of NFκβ has no relationship to disease outcome.

There have been many studies of varying quality into potential prognostic markers in cancer. Due to the issue with many of these initial studies failing to fulfil their suggested potential the National Cancer Institute – European Organisation for Research and Treatment of Cancer (NCI-EORTC) developed a set of criteria that

these studies should be judged against, the REMARK criteria (McShane et al., 2005) (see table 3.38).

INTRODUCTION

1. State the marker examined, the study objectives, and any pre-specified hypotheses.

MATERIALS AND METHODS

Patients

2. Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.

3. Describe treatments received and how chosen (e.g., randomized or rule-based).

Specimen characteristics

4. Describe type of biological material used (including control samples) and methods of preservation and storage.

Assay methods

5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantisation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.

Study design

6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.

7. Precisely define all clinical endpoints examined.

8. List all candidate variables initially examined or considered for inclusion in models.

9. Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.

Statistical analysis methods

10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.

11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cut point determination.

RESULTS

Data

12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.

13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumour marker, including numbers of missing values.

Analysis and presentation

14. Show the relation of the marker to standard prognostic variables.

15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumour marker on a time-to-event outcome, a Kaplan–Meier plot is recommended.

16. For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.

17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.

18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.

DISCUSSION

19. Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.

20. Discuss implications for future research and clinical value.

Table 3.38: The "REMARK" criteria for reporting tumour marker prognostic studies(McShane et al., 2005).

When considering this study and the remark criteria I would hope that it will satisfy most of the recommendations. In the introduction the rational for the chosen markers and hypothesis has been made clear. In the methods and materials the patient group has been defined the tumour material explained, the IHC methods described including the scoring systems, the end points defined and the power of the sample size calculated. In the results the demographics and other disease specific variable have been illustrated and correlation with known prognostic markers and disease end points made using recommended statistical tests. In the discussion the findings have been compared with other published works and the implications for further work discussed.

3.3.6 Critique of the methodology and suggestions for improvements to the clinical study.

I consider the basic methodology behind this study to be sound. The case selection is unbiased and the follow up data thorough. The protocols used for the standard receptor expression ER, PR, HER2 and EGFR are well validated. The development of the staining and scoring protocols for the novel transcription factors followed accepted methodology. An area of discussion within this study is the issue of inter and intra observer variation. In this study over 1400 slides were assessed and scored; this was conducted jointly by an experienced breast pathologist and me with discussion and agreement over borderline cases at the time of assessment. We were blinded to the other case details and reported results by case number alone. To improve the validity of this area of the study it would have been ideal to have the slides scored independently by 2 pathologists and where discrepancies arose a third pathologist

could be consulted. Slides could have also been re-assessed at a later time point by the same pathologists. This method would have allowed the intra and inter observer variability to have been assessed using a k-test. It may have also improved the consistency of the sample scoring.

To improve the efficiency of sample scoring the newer method of tissue micro arrays could have been considered. The construction of a micro array involves tissue cores of 0.6 mm in diameter from regions of interest in paraffin-embedded tissues such as clinical biopsies or tumour samples. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide and then analyzed by any method of standard histological analysis. Each microarray block can be cut into 100 – 500 sections, which can be subjected to independent tests. These tissues micro-arrays are cumbersome to produce but allow analysis of multiple markers on a set of cancers. There are some issues in their use such as the representative nature of small cores of heterogeneous tumours requiring more than one sample per tumour to be analysed.

When the sub-group analysis has been undertake the sample size of the groups becomes too small to generate statistical significance. To account for this the initial sample group would have had to been doubled in size and covered 2 years worth of cancers presenting to this unit or cancer samples from a second unit could have been recruited.

With all of the issues raise above the constraints of funding and time were of a limiting factor.

4 The Cell Model Experiment.

The results of the clinical study undertaken into the use of transcription factors as prognostic markers in breast cancer suggested that out of the three factors studied STAT3 was potentially the most significant. The clinical study showed that STAT3 over expression was significantly associated with disease recurrence at 5 years this was more significant in the node negative i.e. “good” prognosis cancers. It was shown that STAT3 expression was associated with ER negative cancers and EGFR positive cancers. As a direct result of these finding it was thought to be important to go on and study the effect of estrogen stimulation and EGF stimulation on the activation of STAT3. To do this it was decided to use 3 cell line models.

The aim of this section of the study was to ascertain the effect of estrogen and EGF stimulation on cells of differing receptor expression. The hypothesis based upon what is known about STAT3 stimulation is that EFG will stimulate STAT3 phosphorylation but estrogen will have no effect.

4.1. Methodology of cell model experiment

The cell line study was conducted in the department of Surgery and Reproductive Sciences at the University of Newcastle upon Tyne. The experiments were performed in the flow cytometry facility under the supervision of Dr Brian Shenton.

4.1.1 Selected Cell lines

The following 3 cell lines were selected for the cell models. The selections were made due to the receptors expressed and the documented response to oestrogen and EGF stimulation. The cell lines were provided by the Flow Cytometry Group, under the direction of Dr B.K. Shenton, Department of Surgery and Reproductive Sciences, University of Newcastle upon Tyne, where these cell lines were routinely cultured and used for experimental purposes.

4.1.1.1 MCF7 Breast Cancer cell line.

This cell line was derived from a malignant pleural effusion in a Caucasian patient with ductal carcinoma of the breast in 1970 (Soule, 1973). This cell line was of epithelial type cultured as an adherent monolayer. It has been documented to express ER, PR, and very low levels of EGFR (Hall et al., 1990, Imai et al., 1982a) and be sensitive to tamoxifen. MCF 7 is HER2 negative (Bacus et al., 1990). The doubling time of this cell line is 29 hours in ideal conditions. This cell line is routinely used in this lab and was initially purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, Wiltshire, UK.)

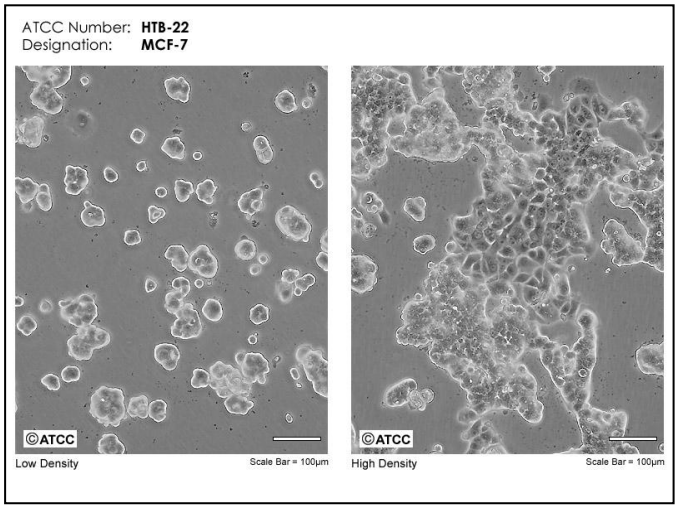

Fig. 4.1: MCF7 cells in culture (x400) (courtesy of ATCC)

4.1.1.2 SKBr -3 Breast Cancer cell line.

SKBr-3: again this cell line was derived from a 43 year old female patient's malignant pleural effusion in 1975 (Trempe, 1976). It was of an adherent epithelia monolayer type. It has been show to be ER, PR negative, HER-2 and EGFR positive (Karlin et al., 2005) and tamoxifen resistant SKBr3 has a doubling time of between 19 and 29 hours depending on density rates of seeding. It was used in this lab and a sample was originally sourced from the American Type Culture Collection (ATCC, Manassas, Virginia, USA.)

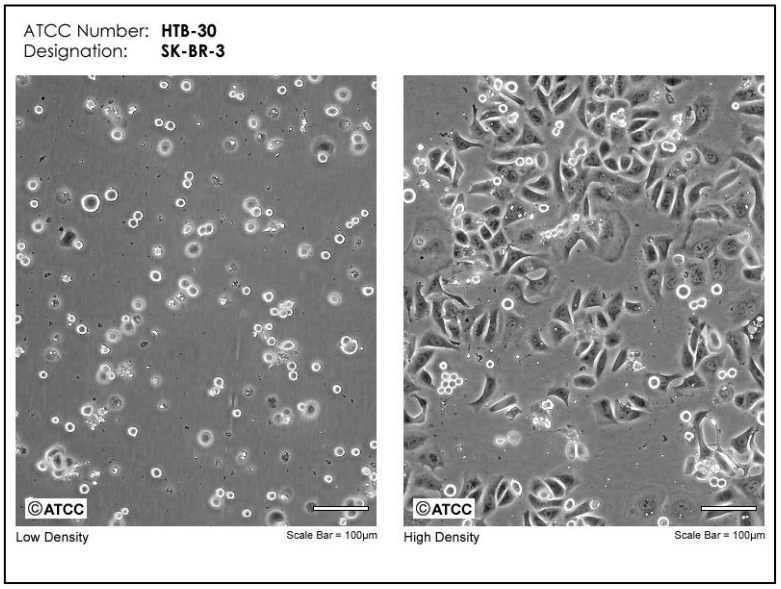

Fig. 4.2: SK-BR-3 cells in culture (x400) (courtesy of ATCC)

4.1.1.3 MDA-MB-231.

MDA-MB-231: established in 1973 from a pleural effusion. The cells grew as an epithelial type monolayer. Under the microscope they had a spindle shape morphology (Cailleau et al., 1978). They are documented to be ER-ve and PR-ve (Cailleau et al., 1978), EGFR+ve (Hall et al., 1990), HER-2 negative (Lewis et al., 1993) and tamoxifen resistant. The doubling time of MDA-MB-231 is 28.1hr ± 1.2 (Watanabe et al., 2001) This cell line routinely used in this lab was originally sourced from the European Collection of Animal Cell Cultures (ECACC, Salisbury, Wiltshire, UK.)

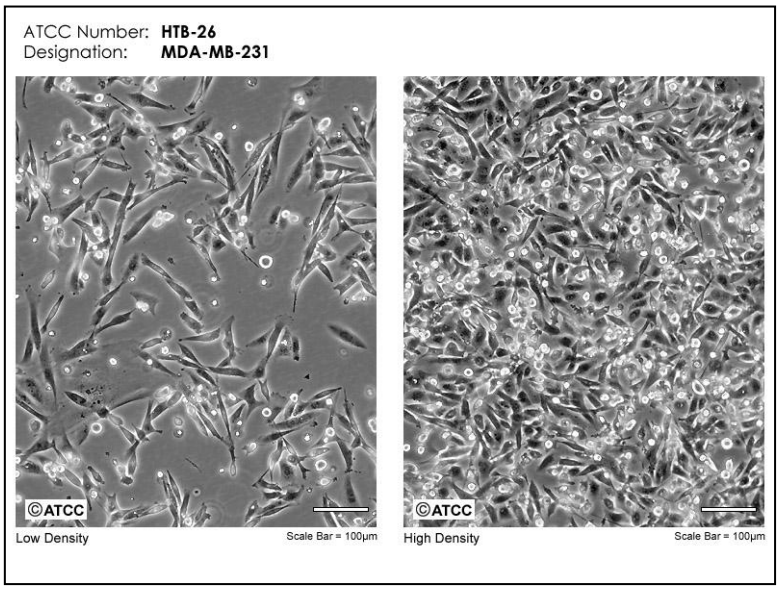

Fig. 4.3: MDA-MB-231 cells in culture (x400) (courtesy of ATCC)

4.1.2 Cell Culture

4.1.2.1 Cell lines and basic cell culture.

In the Department of Surgery and Reproductive Sciences, University of Newcastle upon Tyne, breast cancer cell lines are routinely cultured for the purpose of experimentation. Cell lines, when originally sourced, were tested for mycoplasma then frozen down in aliquots to provide a bank of original cells that could be returned to as a fresh stock for each individual experiment. The aim of this was to prevent phenotypic drift due to excessive passaging of the cells. For this cell model adherent cell lines were cultured in Complete Dulbecco's Medium in 150cm^3 cell culture flasks (Corning Ltd, High Wycombe, UK). The flasks were incubated at 37^oC with 5% CO_2 in a humidified environment. The medium was changed over the cells ever 72 hours. When the cells reached the target of 80-90% confluence the flasks were stripped, the resulting cells were then re-suspended, frozen for storage or used for experimentation.

4.1.2.2 Culture medium.

Dulbecco's Modified Eagle Medium (DMEM) containing 1000mg/l glucose, without pyruvate and phenol red (GIBCO Life Technologies, Paisley, UK) was used as the base medium for the MCF7 and MDA cell lines. McCoy's 5a medium (Sigma, Pool, UK) was used as the base medium for the SKBr3 cell line. These media were supplemented with 10% heat in-activated FBS (Sigma) and the essential amino acid L-Glutamine 2mM. To this 50U/ml penicillin and 50µ/ml streptomycin (Sigma) was added. As the planned cell model required the assessment of the affects of growth factors a stripped medium devoid of these factors was required. To the base media

(DMEM or McCoy's) 10% heat inactivated charcoal/dextran treated FCS (Hyclone, Perbio science Ltd Cheshire, UK) replaced the FBS usually added.

4.1.2.3 Stripping of Cells.

Once the cells had reached target confluence the culture medium was decanted and 21ml of warm (37^{o}C) Trypsin/EDTA (Sigma) was added to the flask. The flask was then returned to the incubator for 3-5 minutes until the cells had detached from the flask wall. The flask was then agitated to aid detachment, the resulting suspension decanted into a 30ml universal centrifuge tube (Bibby Sterilin Ltd, Staffs, UK), and spun at 1500 rpm (1230G) for 5 minutes. The pellet was then washed in 10ml DMEM for a further 5 minutes at 1500 rpm (1230G). The resulting pellet was the re-suspended as required.

4.1.2.4 Storage of cell lines.

When not needed for active culture the cell lines were stored in cryo-stasis in liquid nitrogen. The cells were suspended in a freezing medium consisting of 80% complete DMEM (GIBCO), 10% heat inactivated FBS (Sigma) and 10% Dimethyl Sulphoxide (DMSO; Sigma). Following vortex mixing the suspension was transferred to 2ml cryogenic vials (Corning) in aliquots of $5x10^{6}$ cells in 1ml. The vials were initially placed in a -20^{0}C freezer for 2 hours then into a -80^{0}C freezer for a minimum of 4 hours prior to transfer to liquid nitrogen storage. As required the cells could be returned to culture. The cells were defrosted in a water bath at 37^{0}C. The suspension was then transferred to a 30ml universal centrifuge tube (Bibby,) and washed at 1500 rpm (1230G) in 10 ml pre-warmed FBS. Once the DMSO had been removed by washing the cells were transferred to a culture flask along with 35ml of complete DMEM.

4.1.2.5 Cells required for slide culture.

For the confirmation of cell receptor status with IHC cells were cultured on glass cover slips. Glass cover slips were sterilised in 70% ethanol (Sigma) for 5 minutes. The ethanol was poured off and the cover slips when dry were placed individually in 6 well plates (Corning). 2ml of cell suspension (approx $2x10^5$ cells) in complete DMEM (GIBCO) was placed in each well and the plates returned to the incubator. The media was changed every 72hrs until an 80-90% cell confluence was reached.

To fix the cells the media was decanted from the wells and 2ml of 1% paraformaldehyde (Sigma) added to each well. The plates were left to fix in the dark for 15 min, the formalin removed and the cover slips washed x2 in full media then 4ml sterile PBS (Sigma). The cover slips were then transferred to a sterile petri dish then covered with PBS and stored in the dark at 4^0C.

4.1.2.6 Harvesting of Lymphocytes.

To provide a control for the titration of flow cytometry antibodies lymphocytes from a healthy volunteer (Dr A Davison) were harvested. 5 ml of blood was taken into a EDTA tube. The blood was then transferred to a 30ml universal centrifuge tube (Bibby) and mixed gently with 5ml of PBS. 10ml of Lymph-prep (Cedarlane, Ontario, Canada) was then pippetted as a layer on to of the blood/PBS mixture. The tube was then spun at 800G for 30 minutes in the centrifuge. The resulting cloudy layer of lymphocytes was carefully aspirated into to a fresh 30ml universal tube and then washed with an excess of PBS at 2500rpm. The resulting pellet was then re-suspended in 2ml PBS for use.

4.1.2.7 Calculating cell numbers.

Many of the experiment conducted require cells to be seeded at known concentrations and know volumes. To do this a method of cell counting has to be used. In this study cells were counted by using a haemocytometer. The haemocytometer is a counting chamber with grids of a known area and a chamber of a known depth. To calculate the number of cells in a solution the following method was used;

- The cells were harvested washed and suspended in a known volume (usually 1ml).
- The suspension is then introduced into the counting chamber which is then placed onto the microscope.
- The number of cells in 5 of the small grids is then counted.
- The volume of the small grids = $0.04mm^2$ and 0.1mm deep = $0.004mm^3$
- Total number of cells per ml = x cells counted in $0.02mm^3$ (5x $0.004mm^3$)
- x cells / 0.02 = y cells per mm^3, there are 1000 mm^3 in 1ml.
- Total number of cells per ml = y cells x 1000.

For example;

- 187 cells counted in 5 small grids.
- Total number of cells per ml = (187/0.02 = 9350) x 1000 = 9,350,000 cells per ml.

When the concentration of cells per ml is known then accurate numbers of cells can be used to see well plates for experimental purposes.

4.1.3 Flow Cytometry.

The analysis of protein phosphorylation can be done by a number of techniques including western blot, ELISA or more recently flow-cytometry. It has been suggested that flow cytometry may have a number of advantages over western blot in these type of experiment. As will be explained flow cytometry allows the rapid analysis of single cells rather than a combined lysate of a population of cell in western. The flow cytometer is unique in analyzing single cells, rivalled only by microscopy techniques (which are limited in the number of the cells that can be analyzed). Westerns and ELISAs measure epitopes from whole populations of cells. Because flow cytometry acquires data for individual cells, the technique is inherently rich in statistical properties such as population means, medians, standard deviations, and coefficients of variation (Krutzik et al., 2004). When the protocol is refined flow cytometry allows the rapid assessment of multiple samples at multiple time points which would be difficult when using a western blot or ELISA technique and this specific advantage is important when conducting a time course experiment in triplicate. The laboratory that is under the supervision of Dr Shenton provided an important flow cytometry service to the University of Newcastle therefore I had access to both the facilities and expertise it offered.

Western Blot	Flow Cytometry
Population analysis Obtain average value of multiple cells	*Single Cell analysis* Collects data for each individual cell
Homogeneous sample Limited to cultured or purified cells	*Heterogeneous cell types* Complex primary samples
One parameter Obtain data sets individually	*Multi-parameter* Correlate multiple markers simultaneously
Large number of cells Requires in vitro derived cultures of rare cells	*Small number – rare subsets* Direct analysis of rare cell types (i.e. DC)
Time consuming for large sample sets Not amenable to large screening efforts	*Rapid and Scalable* Performed in 96-well plates in parallel
Protein size and Ab specificity Ab selectivity for target is clearly visible	*Ab must be validated* Ab must have high affinity and selectivity

Table 4.0: A comparison of Western Blot and Flow cytometry (Krutzik et al., 2004)

4.1.3.1 Basics of flow cytometry.

Flow cytometry is a sensitive experimental method which allows the simultaneous assessment of multiple properties of individual cells or particles. The flow cytometer is an instrument consisting of a fluid system, an optical system and a computer interface. The fluid system takes a suspension of cells and passes them under pressure rapidly through a flow chamber as single cells in a laminar flow stream. Within this flow chamber the stream of single cells is then "interrogated" by the beam from a laser. The optical systems consists of the laser and a series of dichroic mirrors and filters which separate, filter and direct the resulting light into a series of photomultiplier tubes (PMT). The role of the PMTs is to record the resulting light and convert this energy into an analogue or digital signal. The computer interface then takes these signals and translates them into graphical results in the form of dot plots or histograms that can be subsequently analysed and interpreted.

In this study 2 different flow cytometers were used, for the cell cycle and DNA analysis the FACscan was used (Becton and Dickinson, Oxford, UK) and for the STAT3 assessment a LSR II (BD) was employed. The FACscan contains 1 laser (Argon blue 488nm), 5 channel detection and uses analogue storage of 1024 channels of data which then interfaces with an Apple Macintosh computer using Cell Quest Pro (BD) software. The LSR II contains 4 lasers (HeCd 325nm UV, Solid State 405nm violet, Solid State 488nm blue and HeNe 633 red), 12 channel detection and uses digital storage of >250,000 channel levels, this then interfaces with a PC using FACSDIVA software (BD).

4.1.3.2 Light scatter.

When a cell or particle intercepts a laser a number of things may happen. The beam may be stopped or it may be reflected of refracted at various angles (scattered). The cells or particles may auto-fluoresce or molecular dyes attached to the cells may fluoresce following excitation by the laser. The resulting scatter and fluorescence contain useful information which is then gathered and interpreted by the flow cytometer.

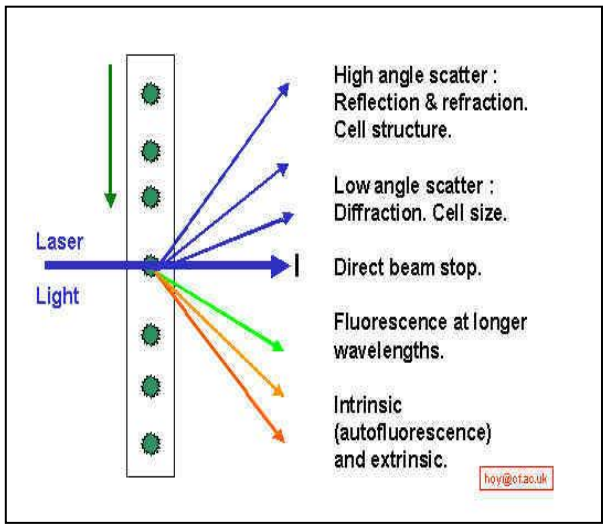

Fig 4.4 Diagrammatic representation of the flow cell.

Forward scatter (FSC) is the term ascribed to the diffracted light close to the angle of the interrogating laser, represented by the low angle scatter (fig 4.4). This FSC can be used to infer approximate information about the cell size as a property of the cells refractive index.

Side scatter (SSC) is the term ascribed to light which is reflected at angles closer to 90^0 from the incidental light source, high angle scatter (fig 4.4). This light is a measure of the cell's reflective index as compared to its refractive index. The reflective index of a cell is dependent on a number of cellular properties such as the cells granularity, its shape and properties of the cell surface. Cell granularity is affected by its internal contents, cells with a complex of internal structures will have a high SCC.

4.1.3.3 Fluorescence.

As a florescent molecule is excited by energy from a laser its molecules and electrons move to a higher orbit (higher energy state). As they return to their rest state this energy absorbed is emitted at a particular wave length. Experimentally cells are deliberately "labelled" with fluorescent molecules. These molecules may be attached to the cell membrane, cytoplasm or nucleus; antibodies to specific cellular molecules can be conjugated to fluorochromic dyes.

As a labelled cell passes though the flow chamber the resulting fluorescence is separated from the refracted and reflected light by an array of dichroic mirrors and band pass filters and focused onto specific PMTs. This light can then be analysed to ascertain the fluorescent properties of that cell.

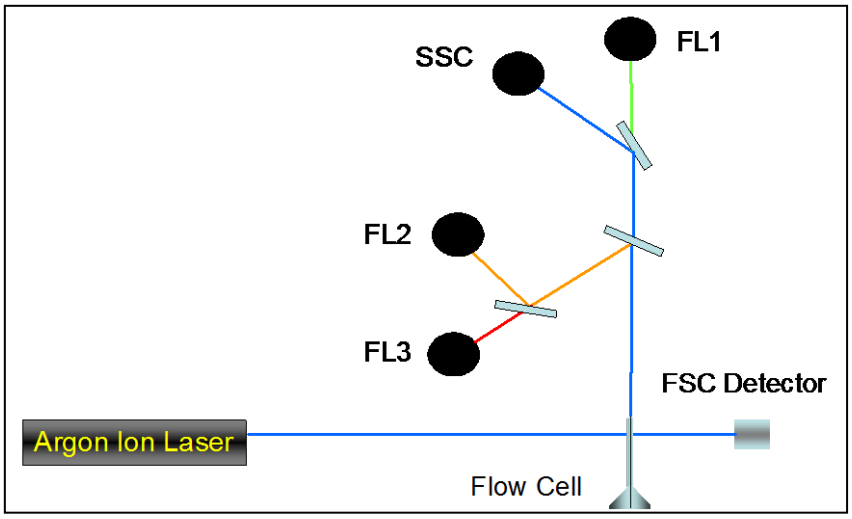

Fig. 4.5: The arrangement of the laser and the detectors of the FACScan (BD) (Courtesy of Dr A. Davison, Department of Surgery, University of Newcastle upon Tyne.)

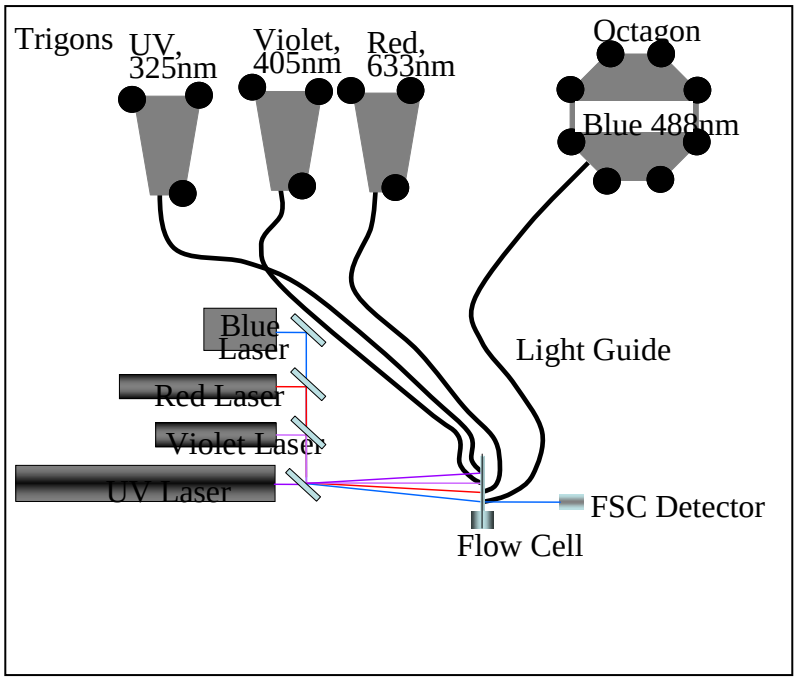

Fig. 4.6: The arrangement of the 4 lasers and multiple PMTs of the LSR II (BD). (Courtesy of Dr A. Davison, Department of Surgery, University of Newcastle upon Tyne.)

4.1.3.4 Fluorescence Compensation.

Flow cytometers are particularly useful as they allow the simultaneous measurement of multiple factors on the same cell. This is performed sometimes by labelling the cell with more than one fluorescent dye. This unfortunately raises the problem of cross over between the chosen dyes emission spectra. Two commonly used fluorochromic dyes are FITC and PE which have the similar absorbance of 495nm and emission spectra of 520nm and 576nm respectively, but as shown in fig 4.7 there is an overlap of their emission spectra.

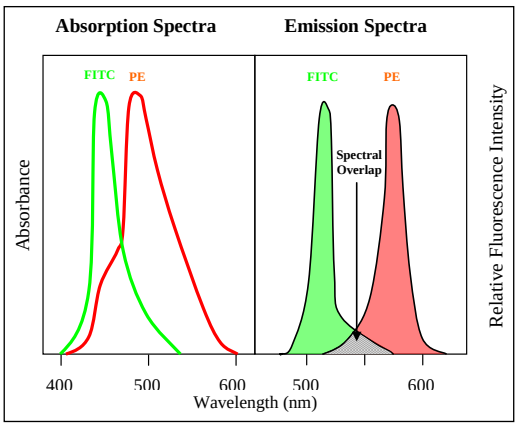

Fig.4.7: The absorption and emission spectra of FITC and PE.

Due to the overlap of these two emission spectra it is logical to understand that a proportion of the FITC emission will be detected as PE emission and vice versa. If the two dyes are used to measure independent factors then this cross over will skew the results. The flow cytometer thus has to compensate for the proportion of each emission spectra incorrectly assigned to the wrong detector. This compensation is done by using the machines software and running compensation samples labelled with individual dyes.

Along with compensation there has to be standardisation of measurement by the flow-cytometers to account for inter-machine and interval variations. This was done using fluorescent micro-bead standards (Spherotech Inc, Libertyville, Illinois, USA) at regular intervals as part of the flow cytometry department protocol.

4.1.4 Assessment of Cell Cycle.

As cells grow, the DNA content in the nucleus passes through stages of replication. Flow cytometry can be used to determine the position of a cell along the path of replication from the G0/G1 phase via S phase to the G2/M phase. When this is applied to a population of cells we can determine the percentage of that population at each point on the cell replication pathway.

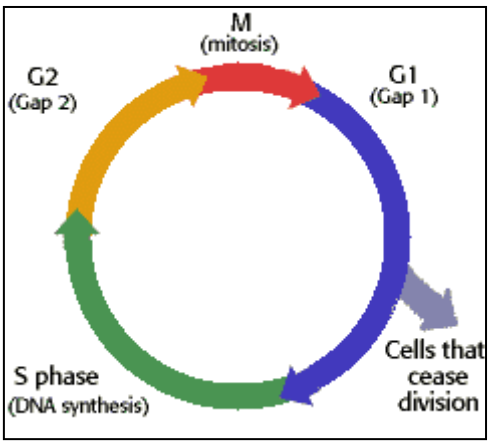

Figure 4.8: The basic cell cycle. (Courtesy of The Biology Project, University of Arizona, USA)

4.1.4.1 Synchronisation of cell lines.

Prior to the assessment of cell cycle the cell lines under study have to be synchronised. This synchronisation of the cells aims to produce the majority of the cells in the G0/G1 phase. The process also removes the majority of the exogenous and secreted stimulating steroids. Synchronisation allows accurate observation of the effects of stimulation on the cell population.

Cells from standard cultures were stripped, washed and re-suspended in stripped media at a concentration of $2x10^5$ cells per ml. 6 well plates (Corning) were then seeded with 2ml of the suspension and placed in the incubator.

Following 24 hours incubation to allow cell adhesion the media was aspirated and fresh stripped media applied, this process was repeated every 24 hours for 72 hours. Following 72 hours of culture in stripped media the cells was considered to be synchronised. The cells were then tested by cell cycle analysis.

4.1.4.2 DNA staining.

Propidium Iodide (PI; Sigma) is a fluorescent dye which can be excited by a 488nm Argon laser and emits on a range of wave lengths which can be detected by the Fl2 or FL3 PMTs. It irreversibly inter-calculates between the bases in DNA and RNA, therefore it is useful when assessing the DNA content of a cell. PI is not able to cross an intact cell membrane therefore to allow DNA staining the cell membrane has to be disrupted by the use of a detergent, in this case 5% Triton X-100 (BDH). To remove the RNA which PI also binds to RNAse A (Sigma) was added to the cell preparation.

Cells, once synchronised in a 6 well plate, were stripped washed and re-suspended in 350µml of PBS in 3ml FACS tubes (BD). To this 100µml of PI (0.25mg/ml) in 5% triton X-100 and 50µml of 1mg/ml RNAse A was added. Following mixing by vortex the cells are ready for analysis on the FACScan using pre-defined settings.

4.1.4.3 DNA analysis.

Using Multi-cycle (Phoenix Flow Systems, San Diego, USA,) an initial dot plot was generated of FSC against FL2 area. This dot plot allowed the determination of intact cells and these intact cells were gated and plotted in a second dot plot of FL2 area against FL2 width. This plot was used to determine if a cell on the plot was truly a single cell in G2 or a doublet (2 single cells adhered to each other). A doublet will have the same fluorescence as a G2 cell but due to its increase overall size will have a prolonged time in flight across the flow chamber, FL2 width is a measure of time in flight across the chamber (fig 6.9). A gate was then drawn around the population of singlet cells and these cells were then plotted as a histogram of cell number against FL2 height (fig 6.9). From this histogram the Multi-cycle AV (Phoenix Flow Systems, San Diego, USA) software then fitted a cell cycle model to the plot and determines the percentage of the cell population in G1, S and G2 phases. Multi-cycle does this by calculating the area below each curve on the histogram, with each curve representing a stage of the cell cycle see fig 4.9. From the figure below it can be seen that the fluorescent channel with the highest number of events, the median channel fluorescence for the G2 population (shown in green) was double the fluorescence of the G1 population shown in red; this was representative of the doubling of the DNA content of the G2 population compared to the G1 population.

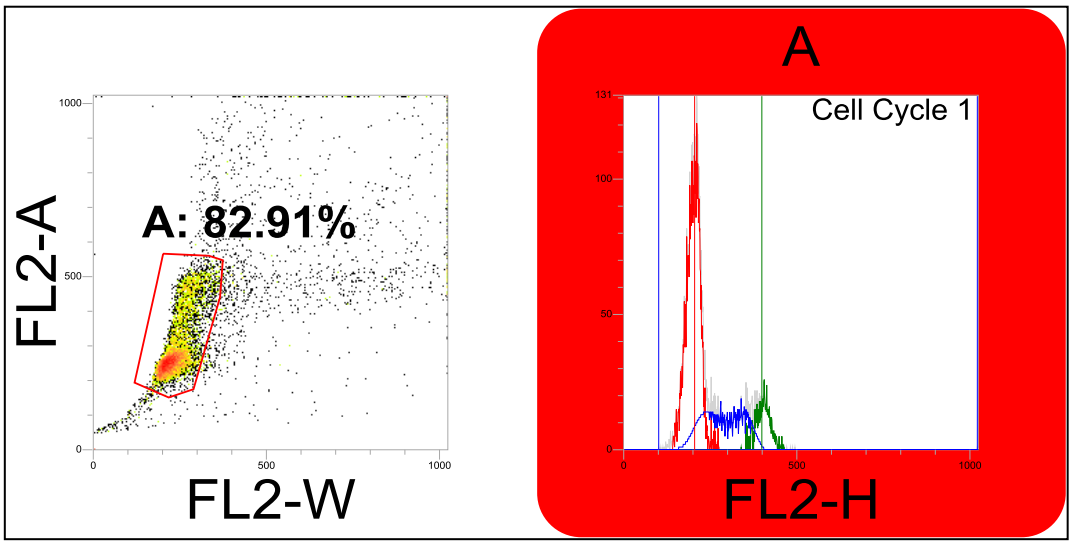

Fig. 4.9: SKBr3 cells stimulated with EGF ($1x10^{-8}$). Left; dot plot of FL2-area over FL2 width showing the gated population (A) as the intact single cells. Right; histogram of FL2 height showing the G1 population (red curve) the S population (blue curve) and the G2 population (green curve).

4.1.5 Assessment of Cell numbers.

To determine the affect of treatments on cell lines it was necessary to be able to determine the numbers of live, dead and apoptotic cells within a population. This has been traditionally done using a haemocytometer and MTT assay. An MTT assay relies on the action of mitochondria in living cell reducing the Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) to purple formazan This water insoluble crystal is then liberates from the cells into a solution using acidified isopropanol. The absorption of this purple solution cen then be measured using a spectrometer. As this reduction reaction only takes place in living cells then the degree of absorption is proportional to the number of live cells. This technique doesn't take account of the variability within a heterogeneous cell population or provided information on the apoptotic population. To overcome these issues flow cytometry uses differences within a cells' membrane during healthy growth, apoptosis and cell death.

4.1.5.1 Detection of apoptosis and dead cells.

Apoptosis is defined as the process of programmed cell death. As opposed to necrosis, which is cell death due to an external insult, apoptosis is a normal physiological process to maintain homeostasis in a multi-cellular organism. This process is a pathway of events which include loss of plasma membrane asymmetry and attachments, condensation of the cytoplasm and nucleus and DNA cleavage. Loss of the cellular membrane integrity is an early step in this process, it is characterised by the translocation of some of the phospholipids from the internal leaf of the membrane

to the external leaf, this event exposes those phospholipids to the extra-cellular environment. The phospholipid Phosphatidylserine (PS) is one of these molecules.

Annexins are a large group of intra and extra cellular proteins. Annexin V is a 36-kDa protein which binds specifically to the PS phospholipid via a calcium dependent mechanism. Annexin V can be conjugated to various fluorochromic dyes such as FITC and Phycoerythrin (PE). As the externalisation of PS is an early event in apoptosis, these conjugated dyes to Annexin provide an accurate identification of cells in early apoptosis using flow cytometry.

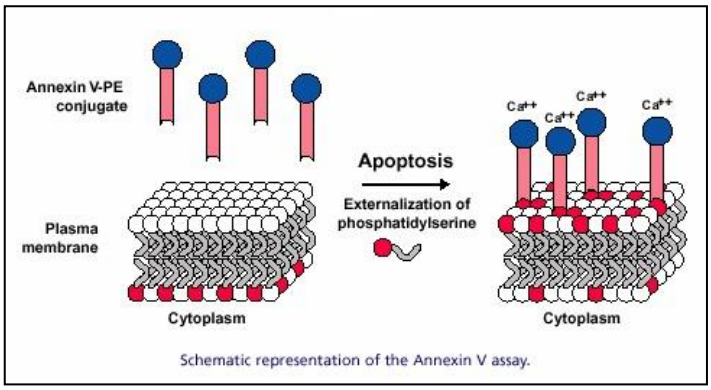

Fig. 4.10: A diagrammatic representation of the externalisation of PS in early apoptosis and the specific binding of PE labelled Annexin V.(Courtesy of BD Biosciences, Ca, USA)

The addition of a vital dye to this assay allows the detection of dead cells. 7-Amino-actinomycin (7-AAD) is a vital dye that intercalates within double stranded DNA binding strongly to the GC rich areas. It is a fluorescent dye which can be excited using a 488nm argon laser. 7-AAD is unable to penetrate an intact cell membrane which makes it useful in the identification of dead cells and cells in late apoptosis. The combination of both these stains can identify three populations of cells.

Cell Fluorescence	Cell Status
Annexin V-PE negative & 7-AAD negative	Healthy cell
Annexin V-PE positive & 7-AAD negative	Early apoptosis
Annexin V-PE positive & 7-AAD positive	Late apoptosis / Dead cell

4.1.5.2 Calculating cell numbers.

Flow count beads are used for the calculation of cell numbers. The beads are 10μm polystyrene particles which fluoresce in a range of 525nm to 700nm when excited by a 488nm argon laser. The beads are provided in an aqueous solution at 1006/μl concentration (Flow-count Fluorospheres; Beckman Coulter). These beads are easily identified on a FSC-height vs. FL2 dot plot due to their low FSC and high FL2 values. By adding a know volume of a set concentration of beads to the sample, cell concentrations can by calculated thus;

$$\frac{\text{Total No. Beads in Assay}}{\text{No. Bead Events}} \times \text{No. Cell Events} = \text{No. Cells/ml}$$

4.1.5.3 Protocol for Live / Apoptotic / Dead staining.

The Proliferation studies were conducted using the Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen). Cells stripped from the 6 well plates with 2ml of cool trypsin were placed in 3ml FACS tubes (BD) and washed twice. The pellet was then re-suspended in 100μml of full media and incubated at 37^0C for 30 minutes. This allowed the repair of any cell membrane damage cause in the stripping and washing process. The cells were then pelleted and re-suspended in 100μml of Annexin V binding Buffer (BD). To this suspension 5μml of Annexin V-PE and 5μml of 7-AAD was added and the sample mixed by vortex. The tubes were the placed in the dark at room temperature for 15 minutes. Once the staining was complete 400μml of the Annexin V binding buffer and 10μml of Flow-count beads (Beckman Coulter) were added to the tube and the sample analysed within 1 hour.

4.1.5.4 Analysis of proliferation studies.

The proliferation samples were run on the FACScan (BD) using pre-set parameters. The tubes were thoroughly mixed by vortexing prior to analysis to evenly distribute the cells and beads throughout the solution. Initially a Dot plot of FSC vs. SCC was generated and a parameter is drawn around the intact cells and the bead population (fig 4.11).

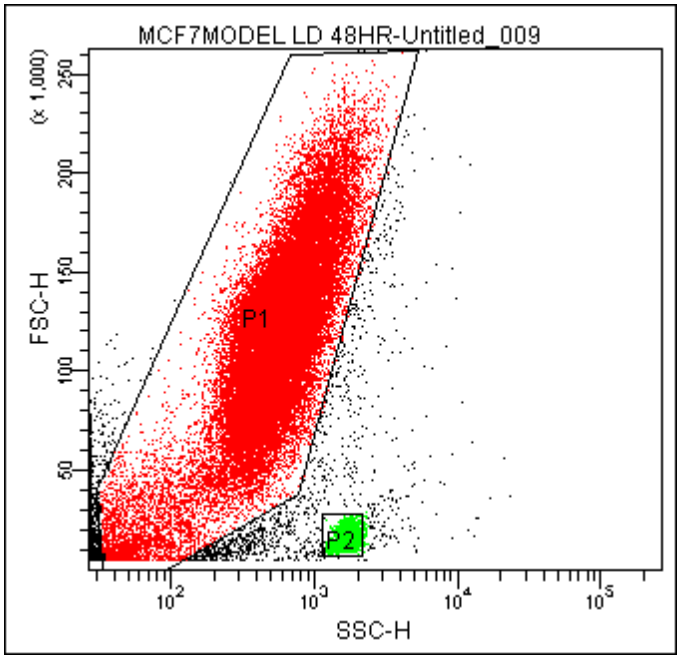

Fig. 4.11: FSC vs SSC plot of MCF7 cells, P1 gated around intact cells, P2 gated around Bead population.

A second dot plot of Annexin-PE vs. 7-AAD was gated to P1 and from this the 3 population of cells, live cells, apoptotic calls and dead cells (fig 4.12). The software then provided a results table with the numbers of each of the cell populations and the total number of beads (fig 4.13). By applying the formula described above, accurate cell numbers and percentage in each stage of proliferation was calculated.

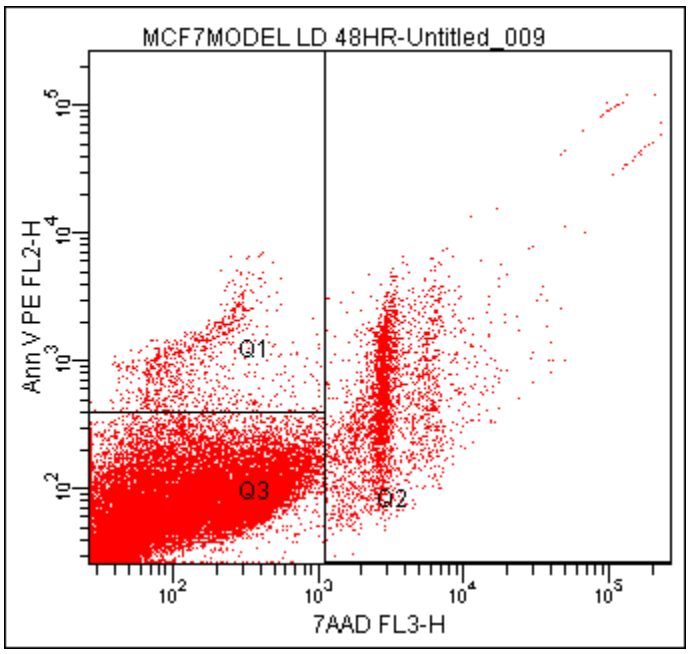

Fig. 4.12: Annexin V PE vs 7AAD plot showing the 3 populations of cells, Q1 population Annexin +ve 7AAD –ve apoptotic cells, Q2 population Annexin +ve 7AAD +ve necrotic/dead cells (note the 2 vertical population of G1 and G2 cells) and Q3 Annexin and 7AAD negative cells which are alive.

Tube: Untitled_009

Population	#Events	%Parent	%Total
All Events	50,000		100.0
P1	37,331	74.7	74.7
Q1	846	2.3	1.7
Q2	3,135	8.4	6.3
Q3	33,350	89.3	66.7
Q4	0	0.0	0.0
P2	3,563	7.1	7.1

Fig.4.13: Figure showing the numbers and % of the 3 cell populations (Q1, Q2,Q3) and the total numbers of counter beads (P2).

4.1.6 Titration of Oestrogen concentration.

Previously in this lab many stimulation experiments have been run analysing the affects of oestrogen on cell lines. The optimum concentration of oestrogen for stimulation of ER positive MCF7 cells is 10^{-9}M. These growth rate experiments were carried out by Dr A Davison on MCF7 cell lines at various E2 concentrations as part of his work towards his PHD thesis. Many other studies have considered 10^{-9}M to be the physiological concentration of estrogen required to stimulate ER positive cells (Alyea and Watson, 2009, Natoli et al., 1983, Stoica et al.).

4.1.7 Titration of EGF concentration.

To ascertain the optimum concentration of EGF required for maximum stimulation of the cell lines under investigation the following titration experiment was performed. Recombinant EGF (Sigma) was reconstituted with 0.01M HCl to produce a stock concentration of $1x10^{-4}$M, this stock was then used to produce the test solutions. 1 ml of 1 x 10^5/ml of MDA-231 breast cancer cell line were seeded into 6 well plates. These were synchronised in stripped media SDMEM for 72 hours (see 4.1.4.1).The following 6 well plates were then set up in triplicate:

Plate 1	Control DMEM
Plate 2	Control DMEM and 0.01M HCl
Plate 3	EFG 10^{-7}
Plate 4	EGF 10^{-8}
Plate5	EGF 10^{-9}
Plate 6	EGF 10^{-10}

The plates were then incubated at 37^0C for 96 hours with the test media changed every 48 hours. At the following time points each triplicate was stripped and assessed for proliferation and cell cycle (see 4.1.4 & 4.1.5). Time points 0hr, 24hr, 48hr, 72hr, 96hr.

4.1.7.1 Results of EGF titration.

The results for each test at each time point were plotted.

Cell proliferation results:

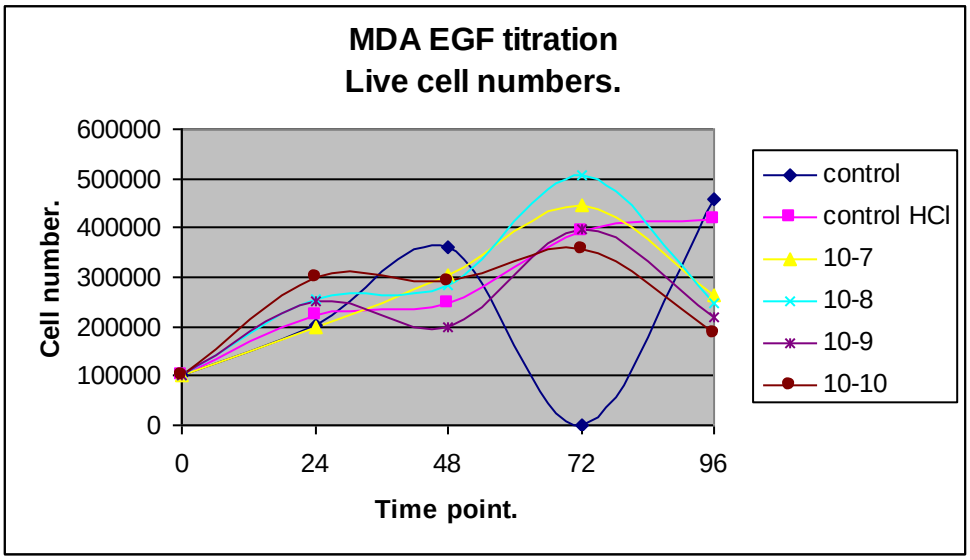

Fig. 4.14: Graph showing live cell numbers for each treatment over time course in hours.

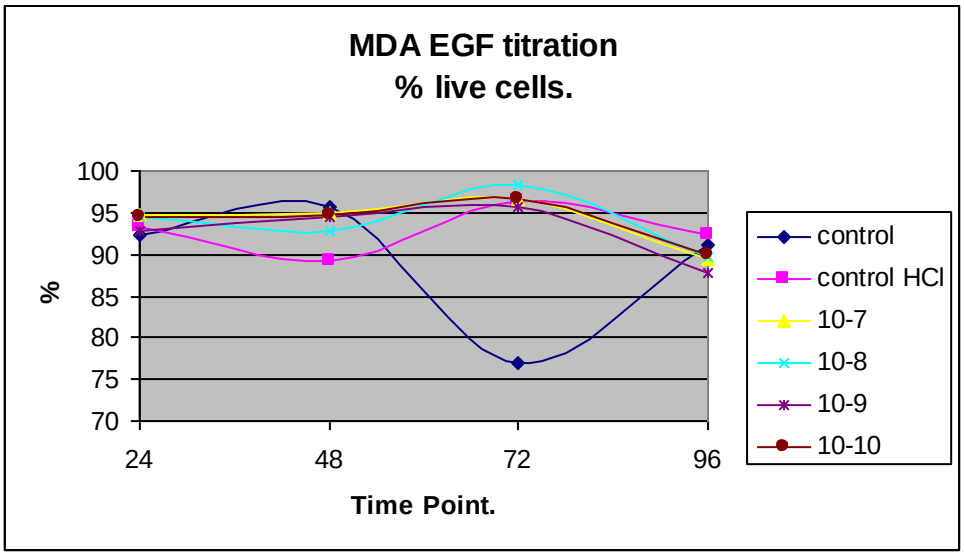

Fig 4.15: Graph showing % population of live cells for each treatment over time course in hours.

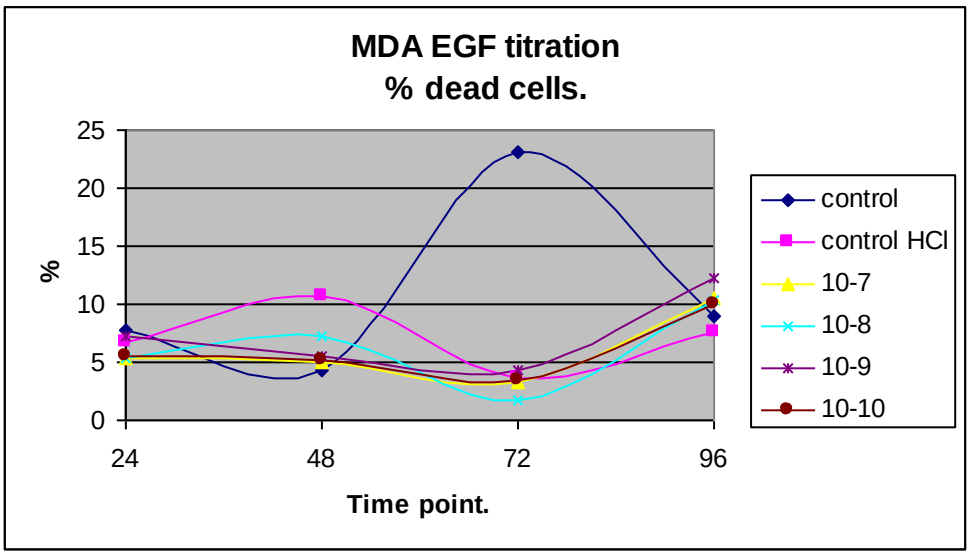

Fig.4.16: Graph showing % dead cells for each treatment over time course in hours.

Results of Cell Cycle analysis;

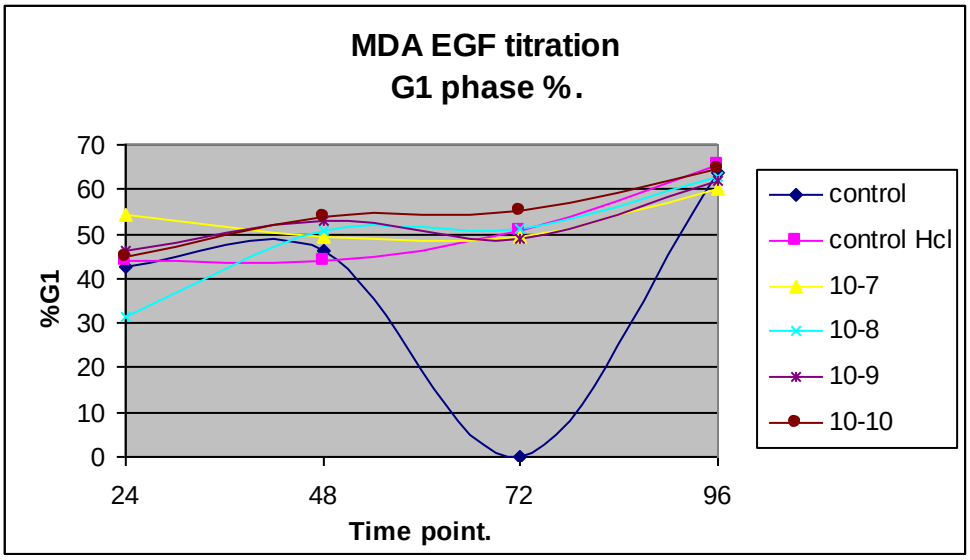

Fig. 4.17: Graph showing % cell population in G1 phase for each treatment over time course in hours.

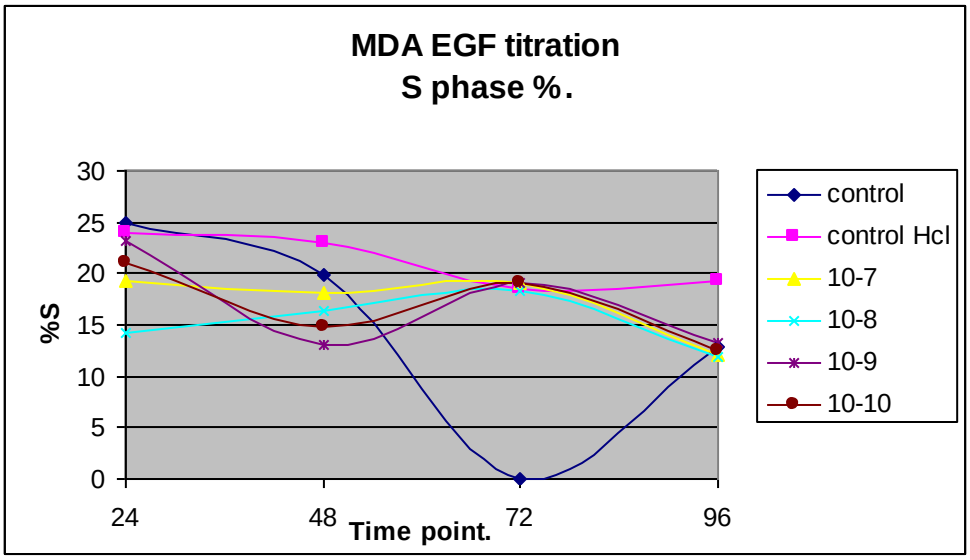

Fig. 4.18: Graph showing % cell population in S phase for each treatment over time course in hours.

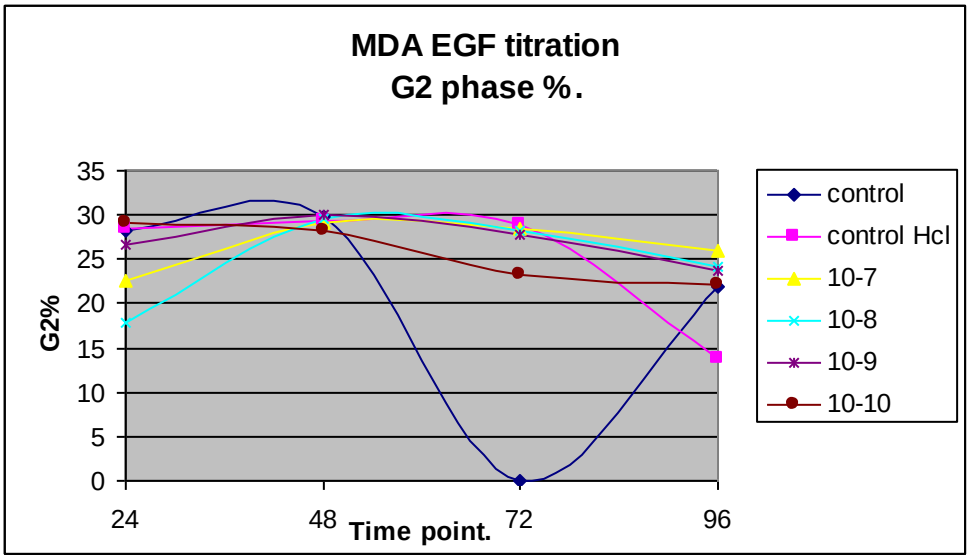

Fig. 4.19: Graph showing % of cell population in G2 phase for each treatment over time course in hours.

4.1.7.2 Analysis of EGF titration.

The results of this titration demonstrated that although the percentage of the population in each phase at each time point did not significantly differ, an EGF concentration of 10^{-8}M induced the highest proliferation. There was an obvious problem with the 72 hour control time point which was probably due to a technical error. The percentage live cells and overall live cell numbers fell off after 96 hours which may have been due to overgrowth within the wells or lack of growth factors after 72 hours culture in stripped medium supplemented only with EGF.

Following this time course experiment it was decided to use a test EGF concentration of $1x10^{-8}$ M. The $1x10^{-8}$ M concentration is equivalent to 123ng/ml, previous experimental papers on EGF stimulation of STAT3 have used concentration from 10ng/ml (Grant et al., 2002, Li and Shaw, 2002, Selander et al., 2004b) to 100ng/m (Imai et al., 1982a, Garcia et al., 2001, Lo et al., 2005b). We therefore considered a $1x10^{-8}$ solution to be adequate to maximally stimulate the cell lines and STAT3 phosphorylation.

4.1.8 Titration of pY705 Stat3 Antibody.

Stat3 is activated via tyrosine phosphorylation at Tyr705. To assess the effects of estrogen and EGF stimulation on Stat3 the assay used had to recognise the phosphorylated form of Stat3. BD Biosciences Pharmingen (San Jose, USA.) produce a range of Phos-flow antibodies specifically for detecting phosphorylated signalling proteins using flow cytometry. Following failure in the testing of various other anti-bodies and protocols the PE conjugated Anti-Stat3 pY705 antibody was used and the appropriate stain buffer and isotype control (IgG2a, κ) (see data sheet in appendix).

Due to the transient and temporary nature of signalling protein phosphorylation it was a concern that any delay in the assessment of the pY705 Stat3 during the time course experiment may induce error in the experiment. This delay may occur due to the fact that at each time point during the experiment every triplicate cell sample had to be run for proliferation and cell cycle status as well as protein expression. It was decided that during the time course experiment the protein assessment samples would be snap frozen to preserve the phosphorylation status of the Stat3 to allow assessment at a later date. Using IHC we had demonstrated Stat3 expression in MCF7 cells.

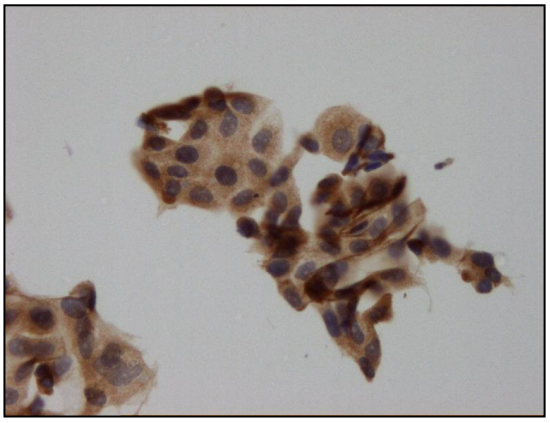

Fig. 4.20: MCF7 breast cancer cells stained for STAT3 using IHC. Cell show a strong cytoplasmic stain with some nuclear staining.(x400)

4.1.8.1 Protocol for pY705 Stat3 titration.

Un-stimulated MCF7 cells were routinely cultured in 6 well plates. Lymphocytes were harvested as a control cell. The Stat3 antibody was titrated at volumes to cover the recommended 20µl test volume. Isotype control volumes were matched to antibody volumes.

Tube Number	Cell type	Antibody volume
1	MCF7	Control (no antibody)
2	MCF7	Antibody 5µl
3	MCF7	Antibody 10µl
4	MCF7	Antibody 15µl
5	MCF7	Antibody 20µl
6	MCF7	Antibody 30µl
7	MCF7	Isotype 5µl
8	MCF7	Isotype 15µl
9	MCF7	Isotype 30µl
10	Lymphocytes	Control (no antibody)
11	Lymphocytes	Antibody 5µl
12	Lymphocytes	Antibody 10µl
13	Lymphocytes	Antibody 15µl
14	Lymphocytes	Antibody 20µl
15	Lymphocytes	Antibody 30µl
16	Lymphocytes	Isotype 5µl
17	Lymphocytes	Isotype 15µl
18	Lymphocytes	Isotype 30µl

The staining and analysis protocol for the pSTAT3 experiment was based on the protocol provided on the technical data sheet (contained in appendix) for this specific antibody. Each tube was repeated in triplicate.

Cells seeded in 6 well plates were stripped with 2ml cool trypsin. They were then washed twice with PBD at 2500rpm (2050G) for 5 minutes and then fixed with 2% paraformaldehyde/PBS for 30 minutes at 37^0C. Following fixing the cells were washed again in PBS then frozen at -80^0C.

The cell samples were then defrosted at 37^0C, washed again with PBS and then divided into three 3ml FACS tubes (BD). To allow the antibody to permeate the cells the cells were permeablised by the addition of 1ml cold 90% methanol for 30 minutes on ice. The samples were then washed twice at 2500 rpm (2050G) for 5 minutes with BD Pharmingen Stain Buffer. The appropriate volume antibody, isotype control or control PBS was then added to the tube and incubated for 30 minutes in the dark at room temperature. Following staining the sample was washed in stain buffer to remove unbound antibody then re-suspended in 500µl of stain buffer for analysis on the LSR II flow cytometer.

Following analysis a dot plot of SSC-height vs. FITC-area identified the cell population. This gated population was then plotted as a FITC-height vs. FITC-area to distinguish single cells from doublets. The singlet population was then plotted as PE 575/26 area vs. SSC- height to determine the specific cell fluorescence. The cell fluorescence was then plotted as a histogram on a log scale and the median channel fluorescence calculated (fig 4.21 & 4.22). In the cell model experiment, to ascertain the fluorescence shift specific to the binding of the STAT3 antibody we subtract the median channel florescence in the isotype control tube from the median channel fluorescence of the antibody tube to give the specific median channel shift.

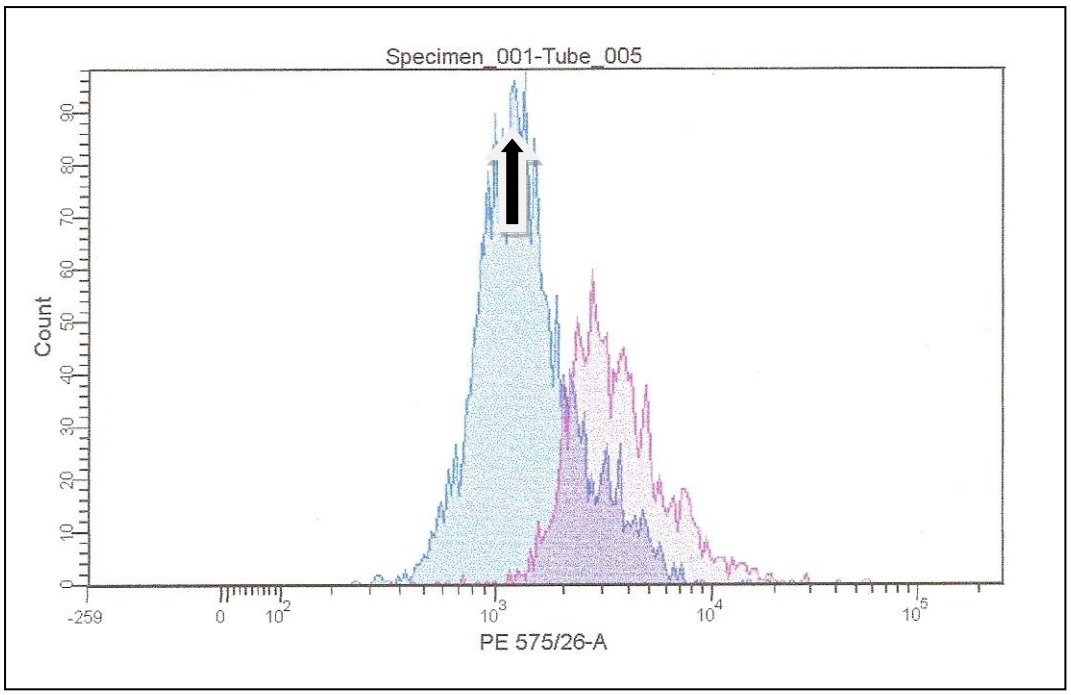

Fig. 4.21: Un-stimulated MCF7 breast cancer cells stained with a stat3 isotype control with the median channel for the singlet population(black arrow) of 1259.

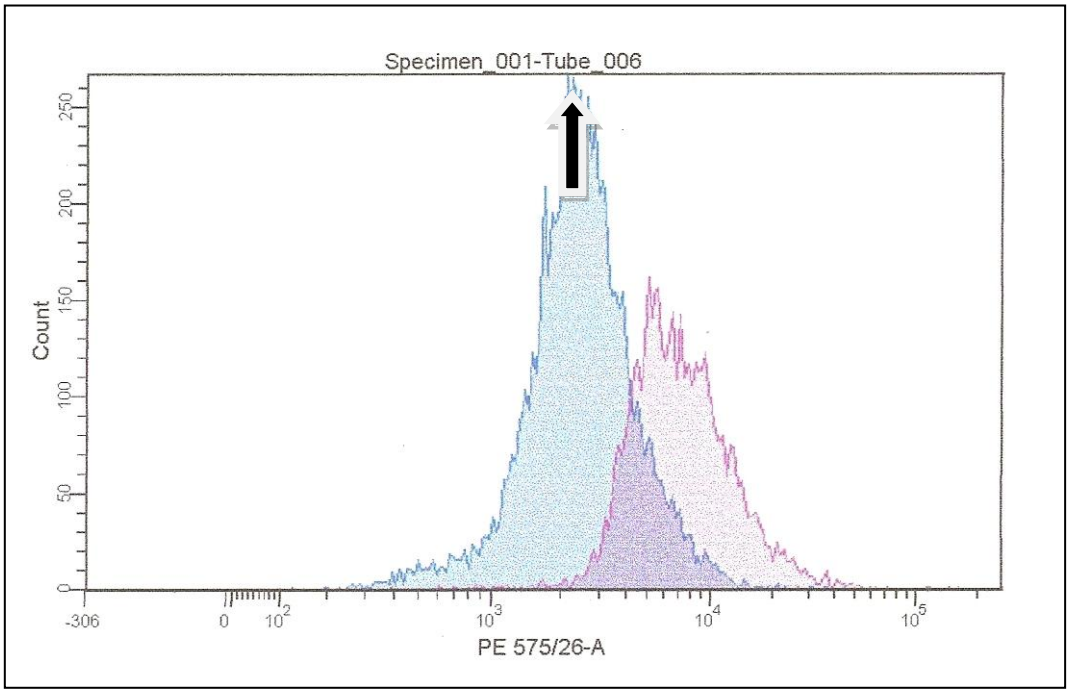

Fig. 4.22:Un-stimulated MCF7 breast cancer cells stained with a stat3 antibody with the median channel for the singlet population of 2364 (black arrow).

Figure 4.21 and 4.22 show the histograms generated for the 2 tubes of MCF7 cells analysed on the flow cytometer. The first tube (fig 4.21) contains MCF7 cells stained with the isotype control which showed the non specific binding and fluorescence of the PE 575/26A dye. The second tube (fig 4.22) contained MCF7 cells stained with the STAT3 specific antibody. The difference in the median channel between these 2 tubes was the median channel shift specific which is a measure of the pY705 phospho-STAT3 expression in un-stimulated MCF7 cells. For example using these 2 tubes the median channel shift would be 1095 channels (2364 – 1269)

4.1.8.2 Results of pY705 Stat3 Titration.

In this titration experiment the median shift was the difference between the antibody or isotype control and the control tube. The average median channel shift for the triplicate of each titration tube was then calculated then plotted a graph.

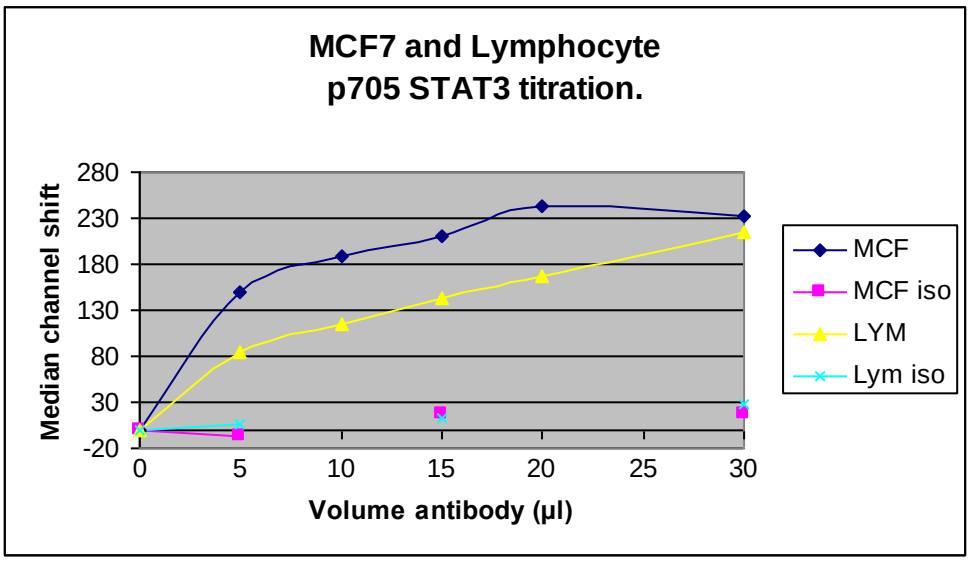

Fig. 4.23: Graph showing the median channel shift see for each volume of antibody and isotype control in both MCF7 breast cancer cells and human lymphocytes.

4.1.8.3 Analysis of pY705 STAT3 titration.

The results demonstrate that there was no specific binding of the isotype control in either of the tested cells. It appeared that the lymphocytes do demonstrate pY705 Stat3 expression and this pStat3 was not saturated in this experiment. When the MCF7 curve was analysed it demonstrated the classical titration curve for pStat3 antibody with a saturation point of 20µl and the curve reaching a plateau at this point (fig 4.23).

This titration experiment confirmed that using our protocol for staining we were using a specific stain which saturated the sample at a volume identical to the manufacturer's suggested test volume of 20µl.

4.1.9 The cell model time course experiment.

The Stat3 analysis time course experiments were conducted with each of the 3 cell lines consecutively. Each cell line model followed the same protocol. There were 7 treatments for each cell line;

Treatment 1. Control: Stripped DMEM only.

Treatment 2. Control: ETOH (20µl 100% ETOH in 19.98ml DMEM.)

Treatment 3. Control: HCl (20µl 0.01M HCl in 19.98ml DMEM.)

Treatment 4. Control: ETOH & HCl (20µl 100% ETOH & 20µl 0.01M HCl in 19.96 ml DMEM)

Treatment 5. Test E_2: 10^{-9}M E_2 (20µl E_2 10^{-6} stock in 19.98ml DMEM.)

Treatment 6. Test EGF: 10^{-8} EGF (20µl 10^{-5} EGF stock in 19.98ml DMEM.)

Treatment 7. Test E_2 & EGF: 10^{-9} E_2 & 10^{-8} EGF (20µl E_2 10^{-6} stock and 20µl 10^{-5} EGF stock in 19.96ml DMEM)

6 well plates of the selected cell lines were set up in parallel for both the Stat3 expression and for the cell cycle / live dead and apoptotic study. Each test point was conducted in triplicate. The cells were synchronised for 72 hours as described earlier.

4.1.9.1 Stat3 expression assessment.

87 wells of 200,000 cells were seeded for each experiment in 2ml of media. These represented the 7 treatments to be tested at 0hrs, 12hrs, 24hrs, 48hrs, and 72hrs. The test media was changed at 0hrs, 24hrs and 48hrs.

The selection of time points for the analysis of STAT3 was based upon the fact that the phosphorylation of STAT3 is a relatively rapid event but the effect of pSTAT3 induction of cell proliferation has a longer time course. Some studies have shown that in the inflammatory setting phosphorylation of STAT3 occurs maximally at 3 hours when cardiac cell are stimulated with IFN-γ (Wang et al., 2002). In breast cancer cell lines MCF7 cells show a peak in STAT3 phosphorylation at a 6h then 24h time point when stimulated by hypoxia (Lee et al., 2006). Other studies have shown that depending on cell line type stimulation with IL-6 causes a peak of phosphorylation at 6-12hr or 24-48hrs. There is little published work on the time course of estrogen of EGF stimulated phosphorylation of STAT3 in breast cancer cell lines. It is hope that the selected time points in this study will detect these peaks in phosphorylation.

The 6 well plates were then stripped at the appropriate time point and frozen for analysis as per the protocol above. Stat3 analysis was then conducted as per the protocol described above with an antibody test volume of 20µl.

4.1.9.2 Cell cycle, Live / Dead and apoptotic time course.

66 wells of 100,000 cells were seeded for each experiment in 2 ml of media. These represented the 7 treatments to be tested at 0 hrs, 24hrs, 48 hrs and 72hrs, with the media changed every 24 hours,

The wells were then stripped at the given time point and divided into 2 x 3ml FACS tubes. 1 tube was analysed for cell cycle using the protocol described above and the second tube was used to calculate the live/dead and apoptotic populations as per protocol described above.

4.1.9.3 Statistical analysis of cell model results.

Each treatment at each time point was run in triplicate simultaneously. Cell numbers, cell cycle proportions and pSTAT3 median channel shifts were all calculated as a mean of the triplicates for each treatment and time point. The treatments were compared to the specific controls for that treatment i.e. estrogen to ethanol, EGF to HCl.

The comparison of these means were made using a paired t-test, a result was considered significant with a p value of 0.05 or less. Statistical analysis was carried out using SPSS Statistical Software, 15.0 version. (SPSS, Inc, Chicago, IL, USA.)

4.2 Results of Cell Model.

The results for the cell model experiments are presented for each cell line individually. For each cell line a conclusion table is shown for the cell number results, the cell cycle results and the STAT3 induction of expression results. Selected graphs are shown to illustrate significant trends or results. The raw data and statistical analysis for each experiment is contained in the appendix. All significant results are bases on a two sided t-test with significance level of 0.05 or less. The significant results are expressed when there is a significant difference between the treatments and the specific controls for the treatment at that time. To show these differences results are plotted on the bar charts for the treatments with the specific control

4.2.1 MCF7 cell model results.

4.2.1.1 Cell number results.

The mean cell number of the triplicate experiments was compared to that of the treatment specific control using a using a paired t-test and significant values (p=<0.05) are shown in the tables. Where no significant difference no result is shown.

Treatment \Time point	24 hours	48 hours	72 hours
E2	↑ Total cells ↑Live cells	↑ Total cells ↑Live cells ↑Apoptotic cells	↑ Total cells ↑Live cells
EGF	■	■	■
E2 & EGF	■	↑ Total cells ↑Live cells ↑Apoptotic cells	↑ Total cells ↑Live cells

Table 4.1: Table showing the significant results for the MCF7 cell number experiment. Significant increase. Significant decrease. *(p=0.05)*

The following graphs illustrate these significant results.

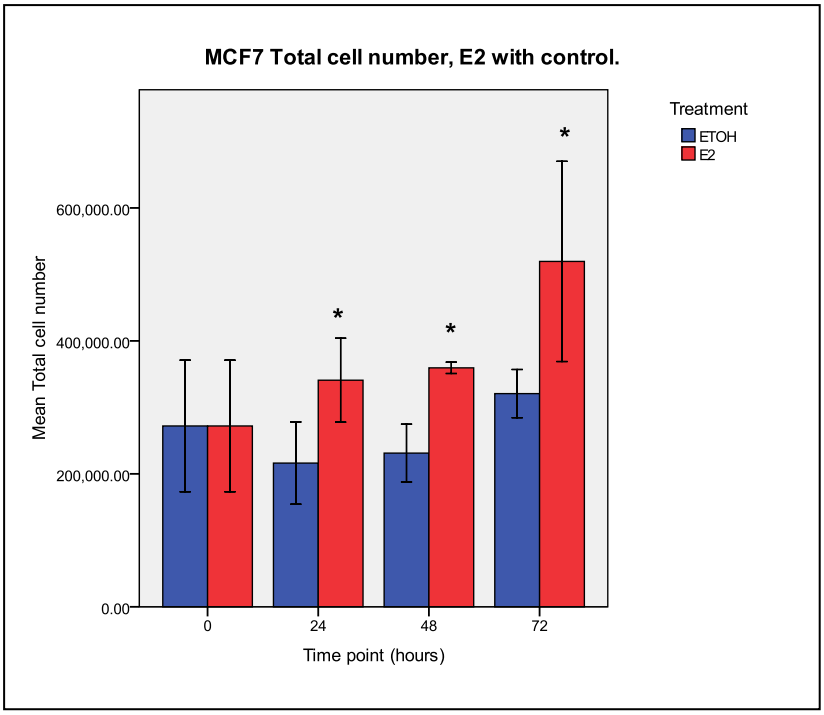

*Fig 4.24: Graph showing the mean total cell numbers of E2 stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

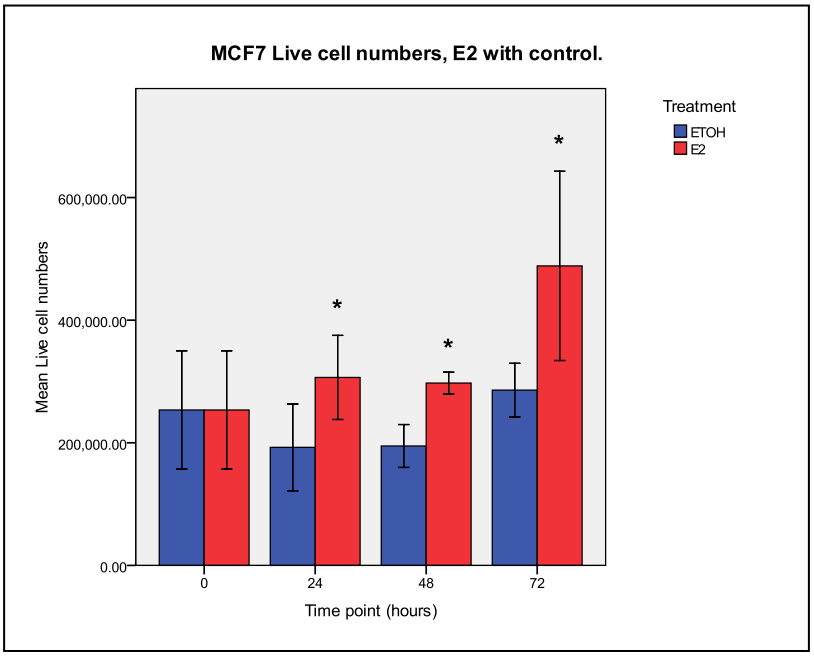

*Fig 4.25: Graph showing the mean live cell numbers of E2 stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

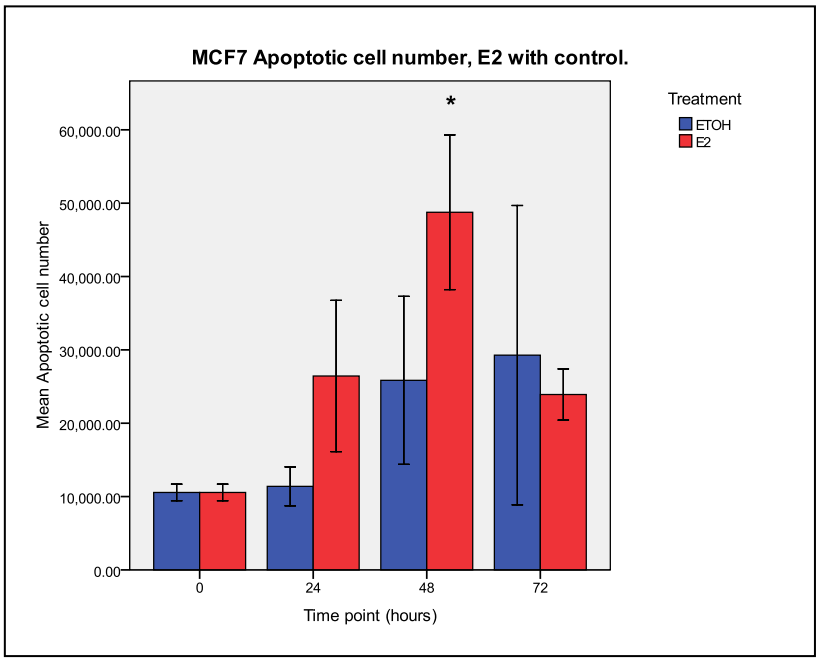

*Fig 4.26: Graph showing the mean apoptotic cell numbers of E2 stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

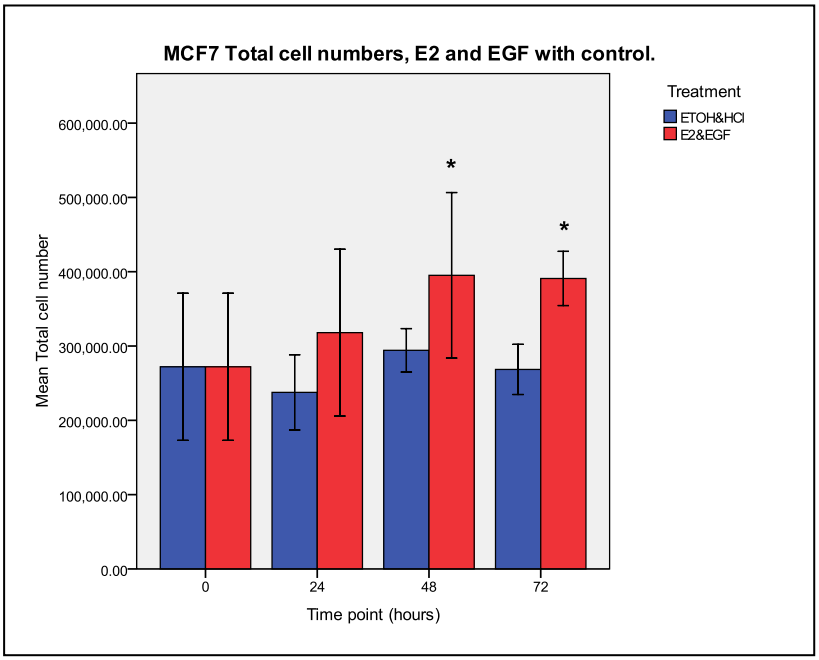

*Fig 4.27: Graph showing the mean total cell numbers of E2and EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

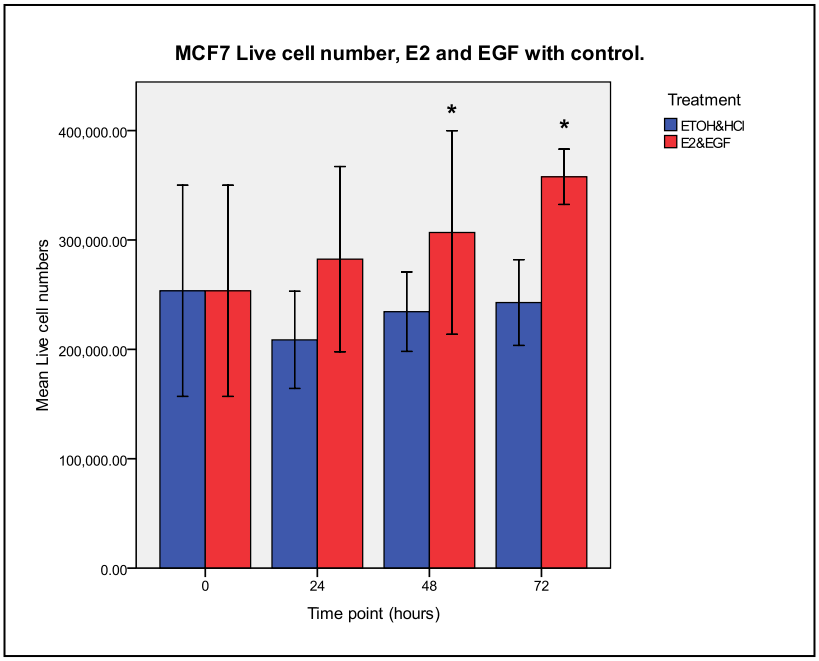

*Fig 4.28: Graph showing the mean live cell numbers of E2 and EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

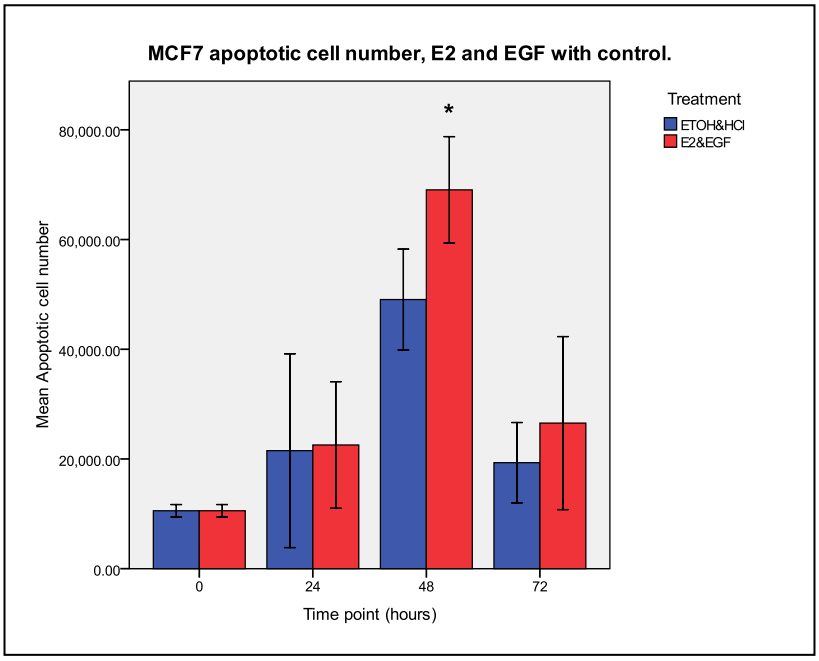

*Fig 4.29 Graph showing the mean live cell numbers of E2 and EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

4.2.1.2 Cell cycle results.

The mean of the triplicate % populations between the treatments and controls were compared. This table shows the significant increases (in red) and decreases (in blue) of the % population in the G1, S and G2 phases for the MCF7 cell model.

Treatment \Time point	24 hours	48 hours	72 hours
E2	↓*G1*, ↑*S*↑G2	↓*G1*	↓*G1*, ↑*S*↑G2
EGF	↓*G1*, ↑*S*	↑*S*	↑*S*
E2 & EGF	↓*G1*, ↑*S*↑G2	↓*G1*, ↑*S*	↑S↑G2

Table 4.2: Table showing the results of the MCF7 cell cycle experiment. . Significant increase. Significant decrease. *(p=0.05)*

The following graphs illustrate these significant results.

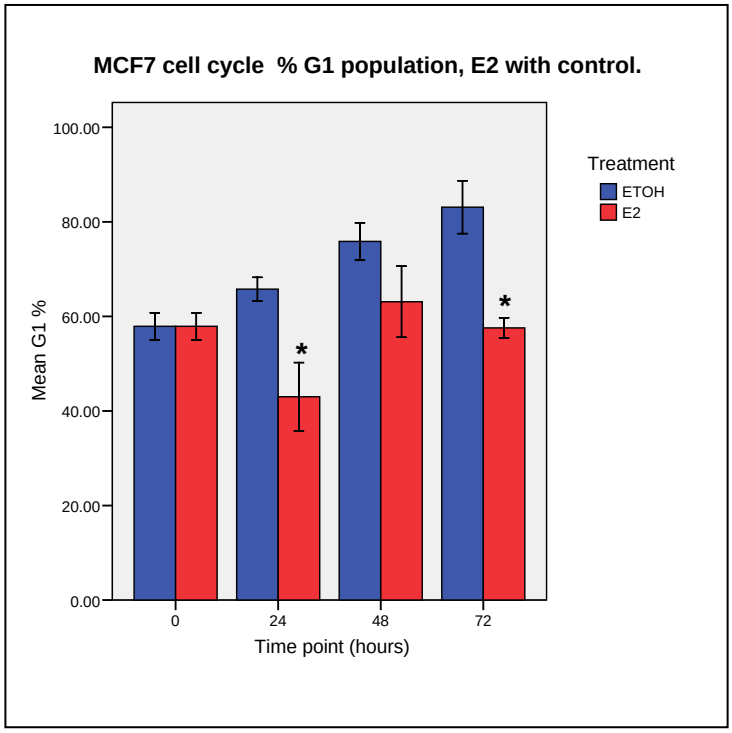

*Fig 4.30: Graph showing the mean G1 % of E2 stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

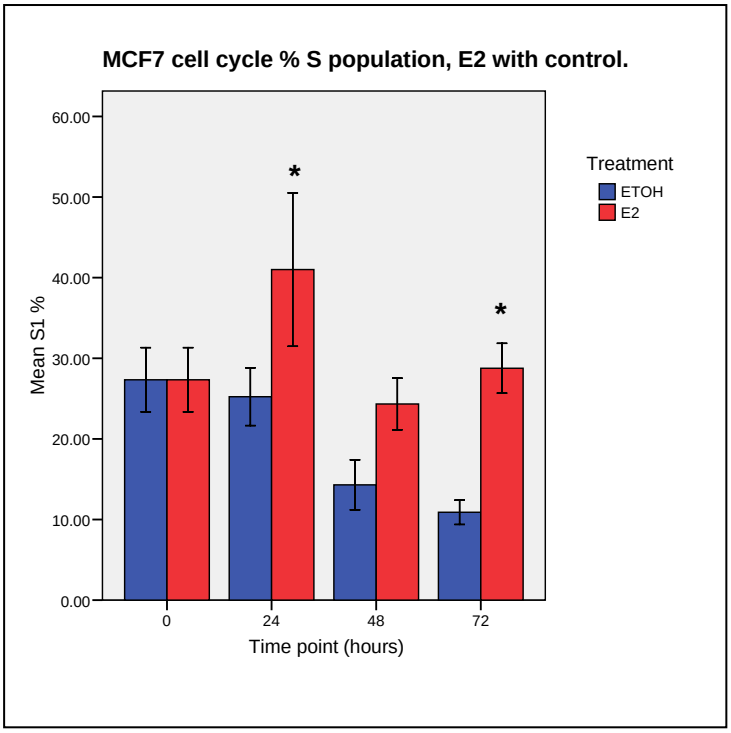

*Fig 4.31 Graph showing the mean S % of E2 stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

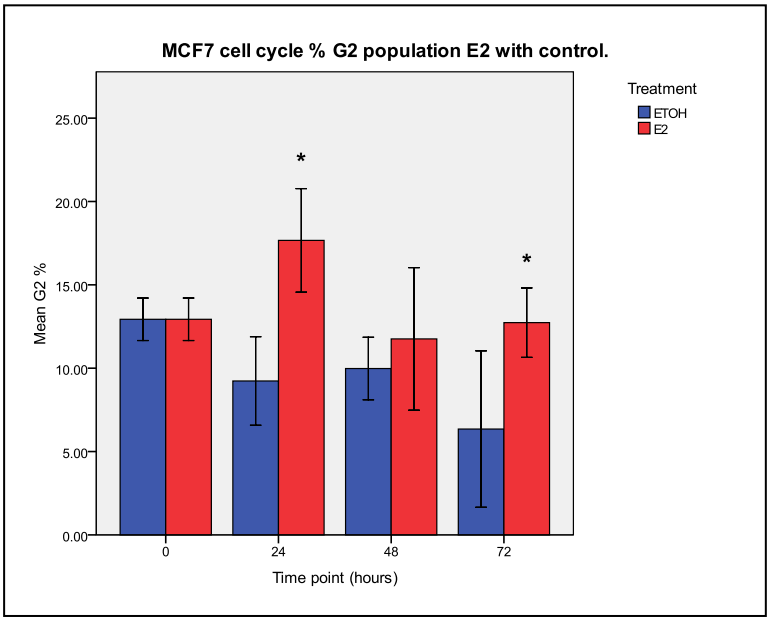

*Fig 4.32 Graph showing the mean G2 % of E2 stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

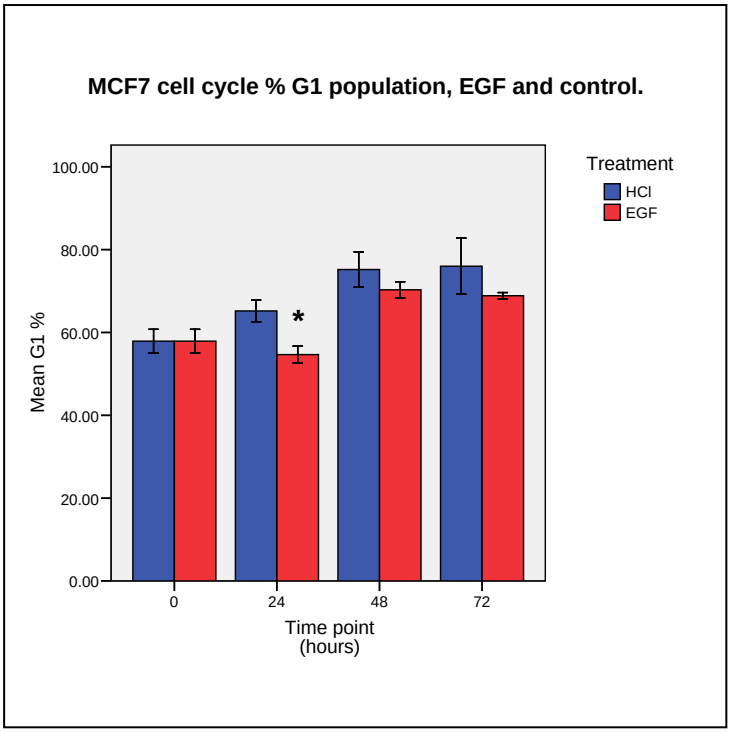

*Fig 4.33: Graph showing the mean G1 % of EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

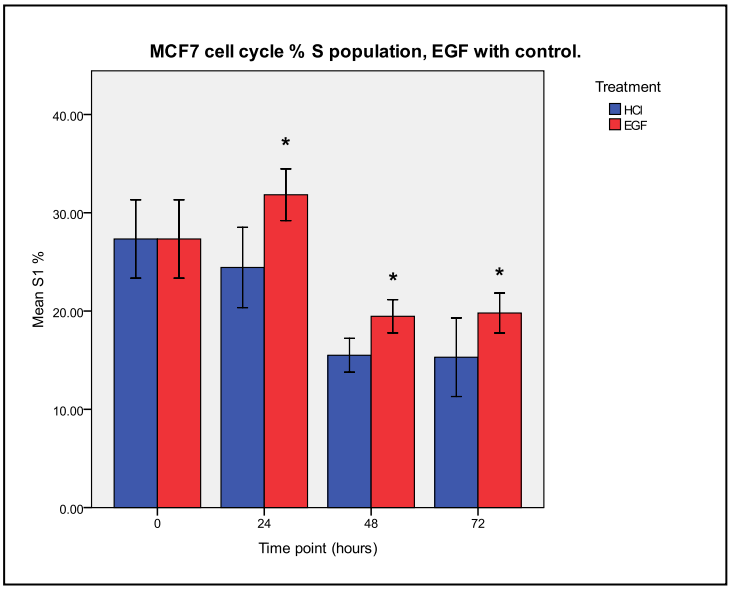

*Fig 4.34: Graph showing the mean S % of EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

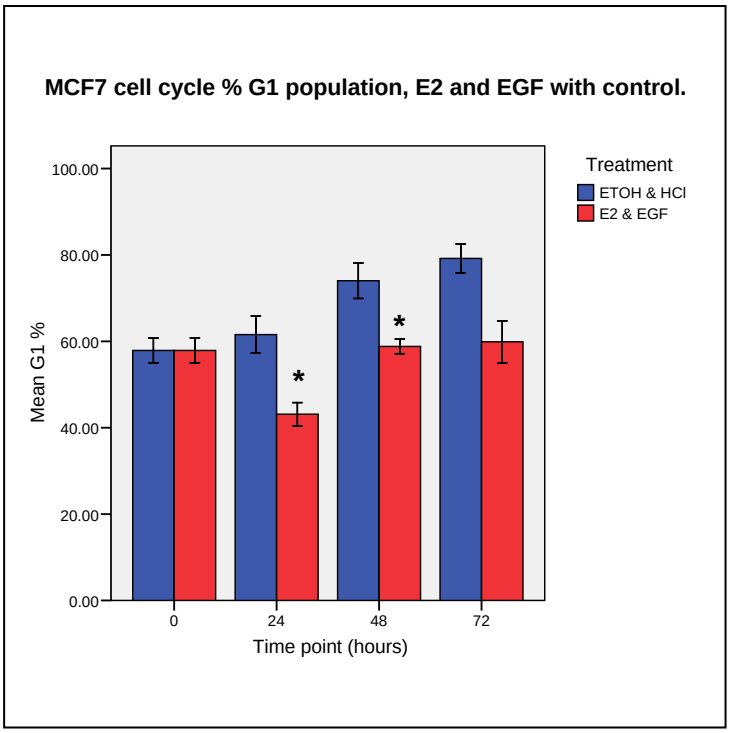

*Fig 4.35: Graph showing the mean G1 % of combined E2 and EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

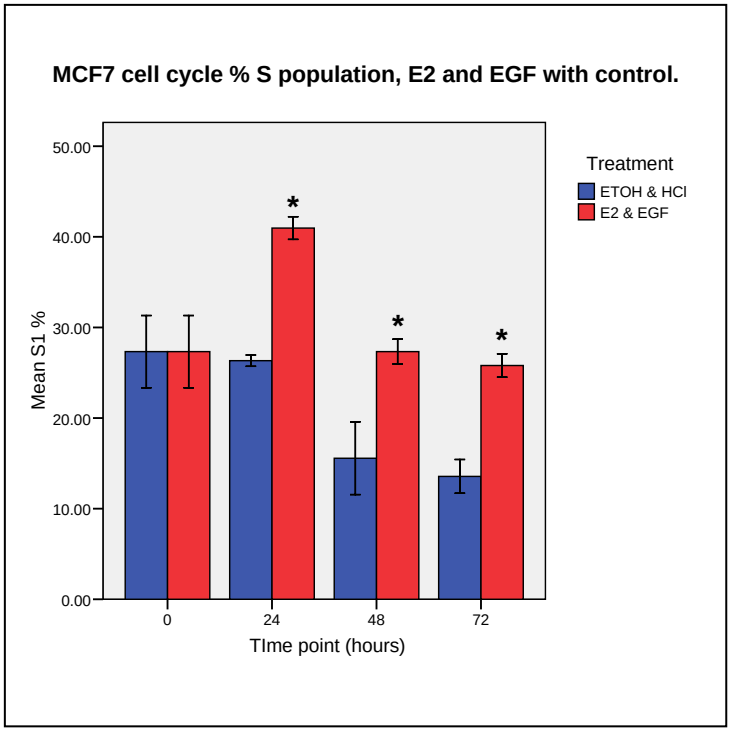

*Fig 4.36: Graph showing the mean S % of combined E2 and EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

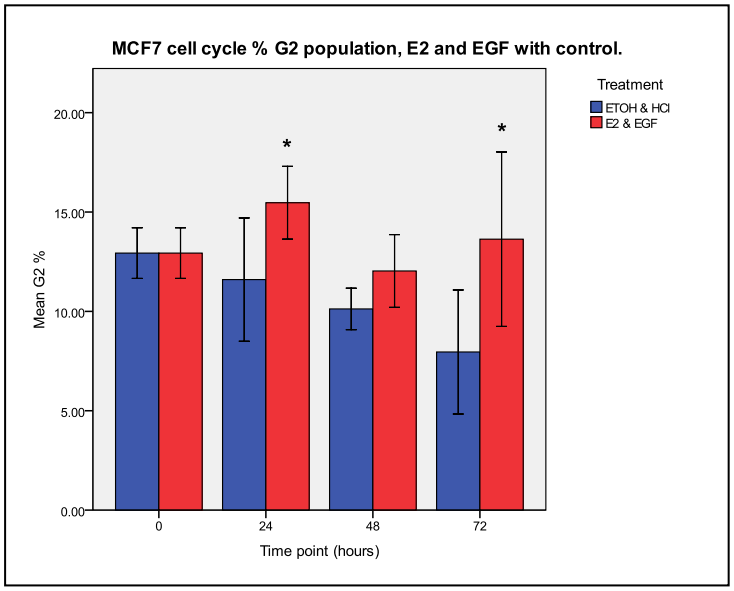

*Fig 4.37: Graph showing the mean G2 % of combined E2 and EGF stimulated MCF7 cells. * = significant difference compared to control. 95% CI bars shown.*

4.2.1.3 STAT3 expression results.

This table shows the change in pSTAT3 expression when measured as a significant change in median channel shift for each treatment at each time point when compared to its specific control. Where there is no significant difference no result is shown.

Treatment \ Time point	12 Hr	24 Hr	48 Hr	72 Hr
E2	■	■	↑	■
EGF	↑	■	↑	■
E2 and EGF	■	■	■	■

Table 4.3: Table showing the significant results for the MCF7 STAT3 induction experiment. Significant increase. Significant decrease. *(p=0.05)*

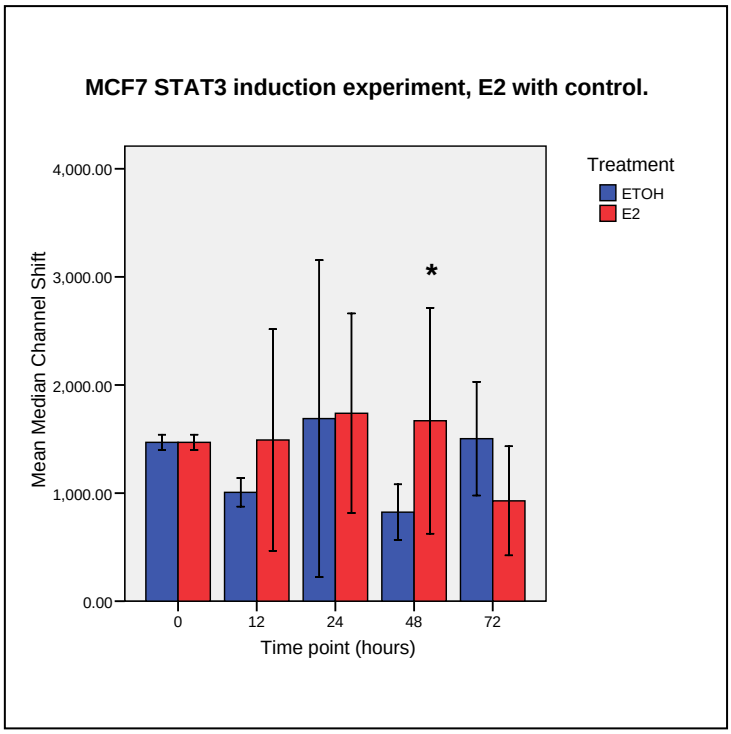

Fig 4.38: Graph showing the mean median channel shift of pSTAT3 in MCF7 cells with E2 stimulation.

** = significant difference compared to control. 95% CI bars shown.*

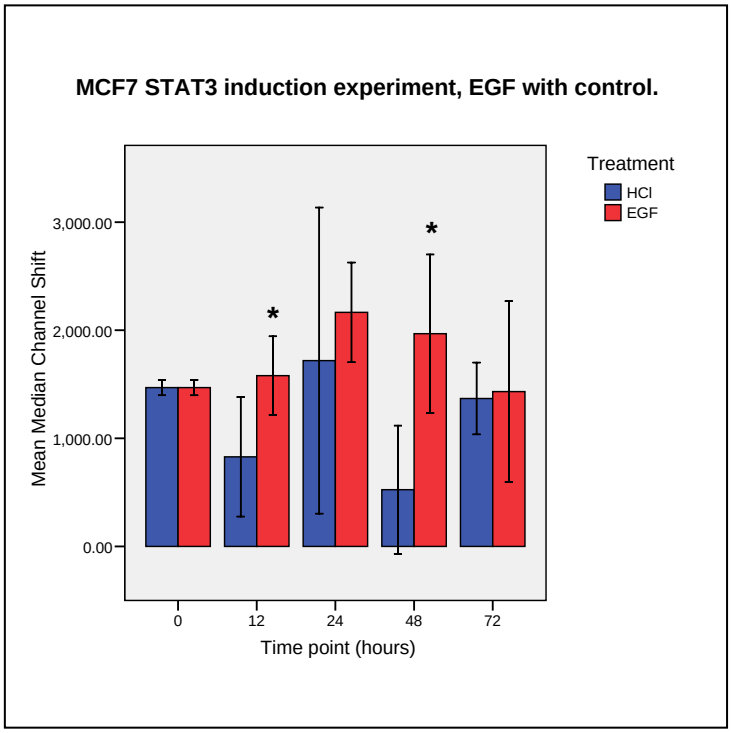

*Fig 4.39:Graph showing the mean median channel shift of pSTAT3 in MCF7 cells with EGF stimulation. * = significant difference compared to control. 95% CI bars shown.*

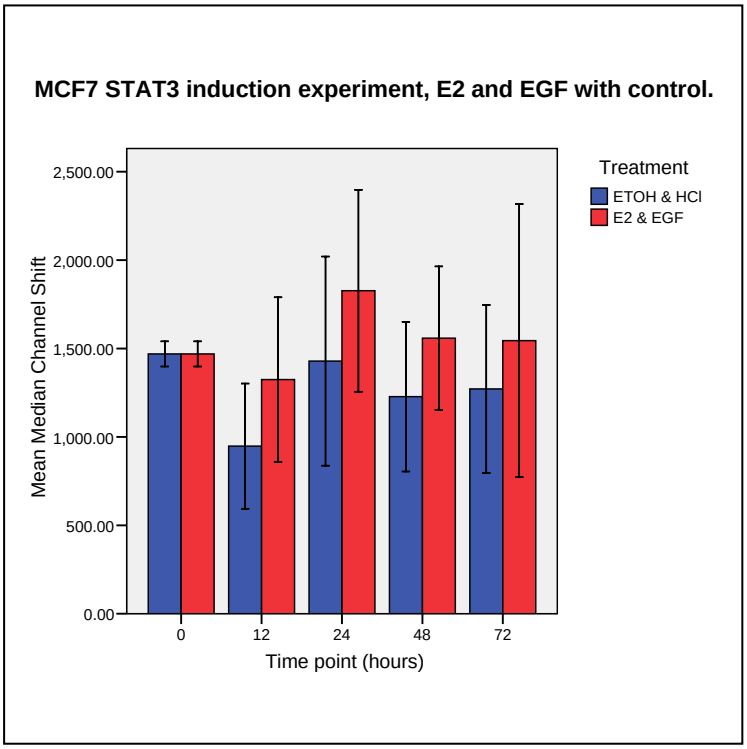

Fig 4.40:Graph showing the mean median channel shift of pSTAT in MCF7 cells with E2 and EGF stimulation. No significant difference compared to control. 95% CI bars shown.

To illustrate the effect of the increased stimulation of STAT3 phosphorylation by EGF alone compared to estrogen and EGF two of the histograms plotted are shown below.

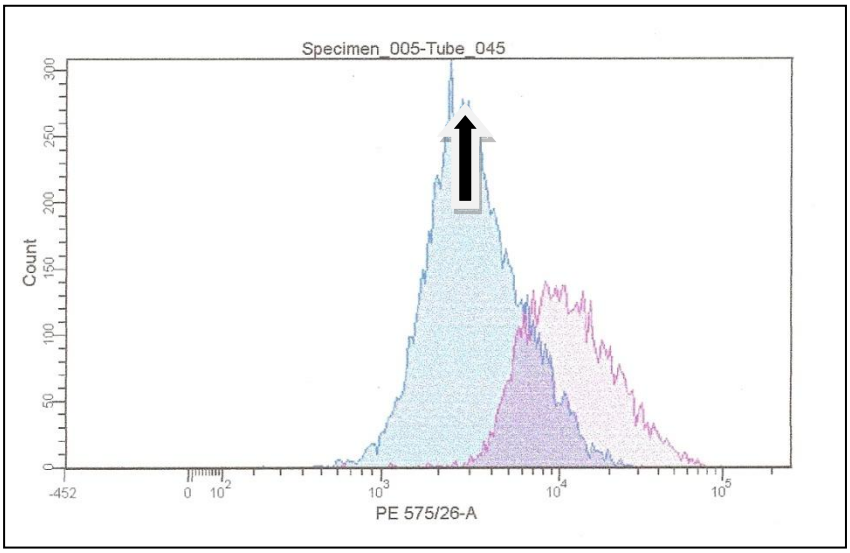

Fig 4.41Histogram of pSTAT3 expression in MCF7 cells stimulated with EGF after 48hrs. The black arrow (single cells) shows a median channel fluorescence of 2920.

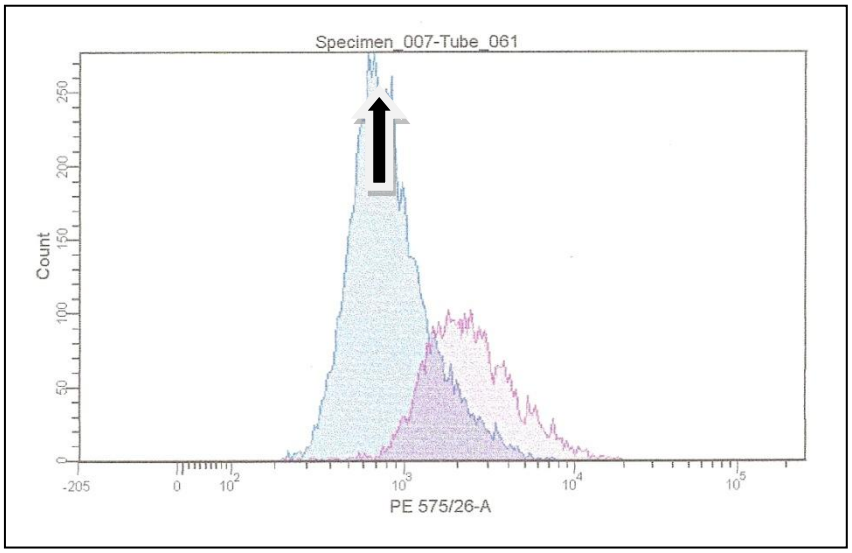

Fig 4.42 Histogram of pSTAT3 expression in of MCF7 cells stimulated with estrogen and EGF after 48hrs. The black arrow (single cells) shows a median channel fluorescence of 756.

The 2 histograms above clearly show the inhibiting effect on pSTAT3 expression of the addition of estrogen to EGF stimulation in MCF7 breast cancer cells.

To demonstrate the effect of the treatment on the induction of pSTAT3 a graph showing the ratio change of the pSTAT3 induction for the treatment compared to its specific control at each time point.

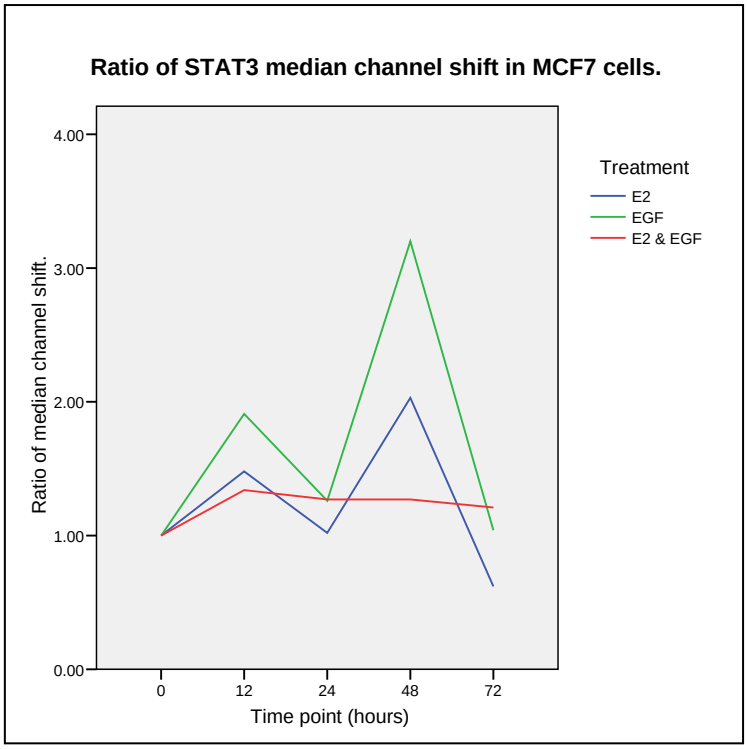

Fig 4.43 Graph showing ratio of median channel shift for pSTAT3 induction in MCF7 cells.

4.2.1.4 Summary of results for MCF7 cell model experiment.

The results for the cell numbers and cell cycle have shown that MCF7 cell were stimulated to grow and divide by estrogen and to a lesser extent EGF. The pSTAT3 results have shown that EGF stimulation causes a biphasic increase in the expression of phosphorylated STAT3 at 12 and 48 hours. Estrogen does appear also to have a small affect at 48 hours. The addition of E2 to EGF stimulation appears to inhibit EGF ability to stimulate pSTAT3 expression both at 12 and 48 hours.

4.2.2 SKBr3 cell model results.

4.2.2.1 Cell number results.

This table shows the significant changes in the numbers of total, live, apoptotic and dead cells over time for each of the treatments when compares to their control at that time point. Where there is no significant difference no result is shown.

Treatment \Time point	24 hours	48 hours	72 hours
E2	■	■	■
EGF	■	■	↑ Total cells ↑Live cells
E2 & EGF	■	■	↑ Total cells ↑Live cells

Table 4.4: Table showing the significant results for the SKBr3 cell number experiment. Significant increase. Significant decrease. *(p=0.05)*

The following graphs represent these significant results.

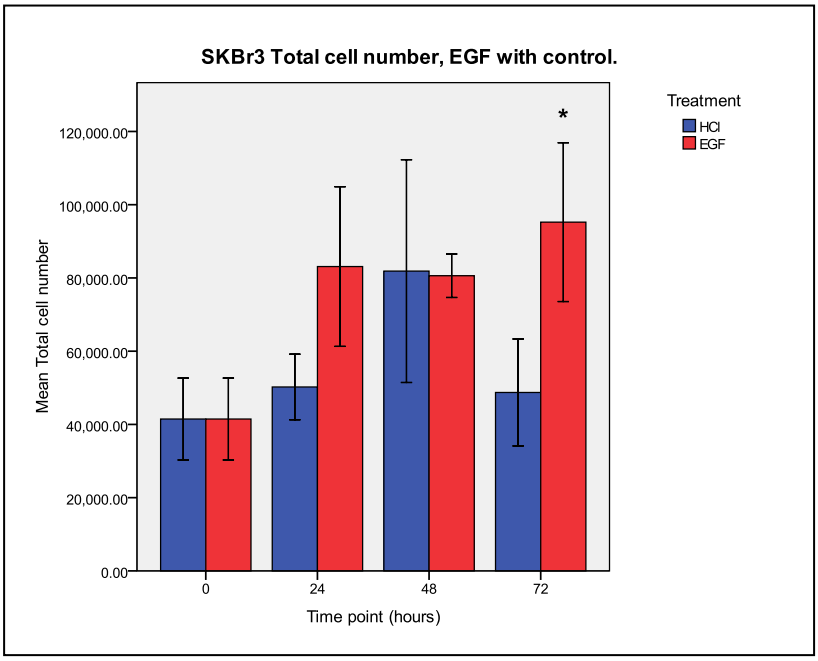

*Fig 4.44: Graph showing the mean total cell numbers of EGF stimulated SKBr3 cells. * = significant difference compared to control. 95% CI bars shown.*

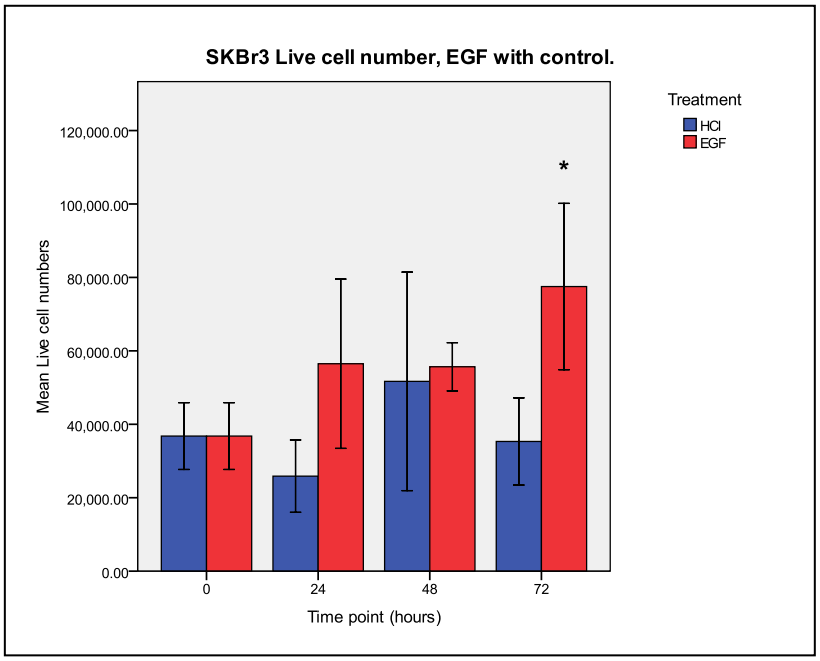

*Fig 4.45: Graph showing the mean live cell numbers of EGF stimulated SKBr3 cells. * = significant difference compared to control. 95% CI bars shown.*

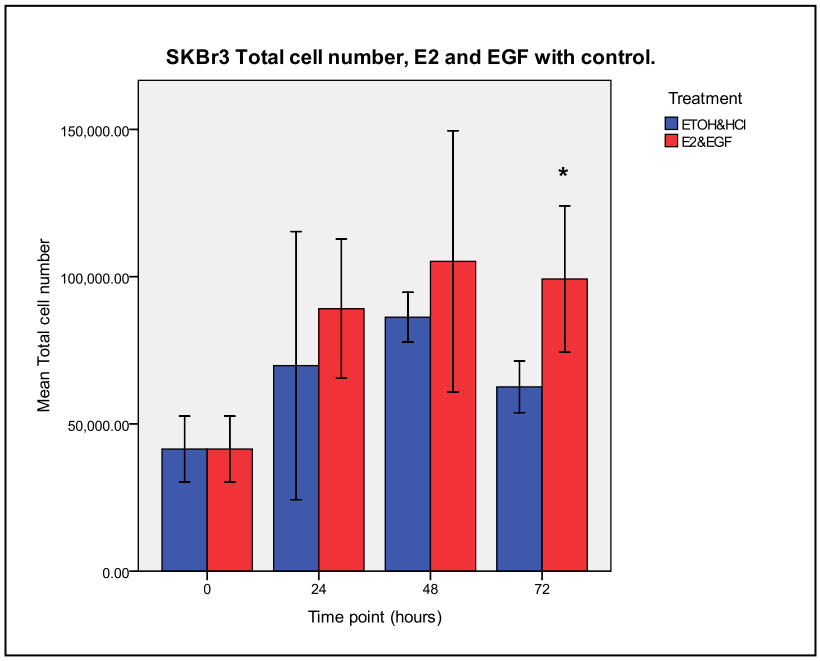

*Fig 4.46: Graph showing the mean total cell numbers of E2 and EGF stimulated SKBr3 cells. * = significant difference compared to control. 95% CI bars shown.*

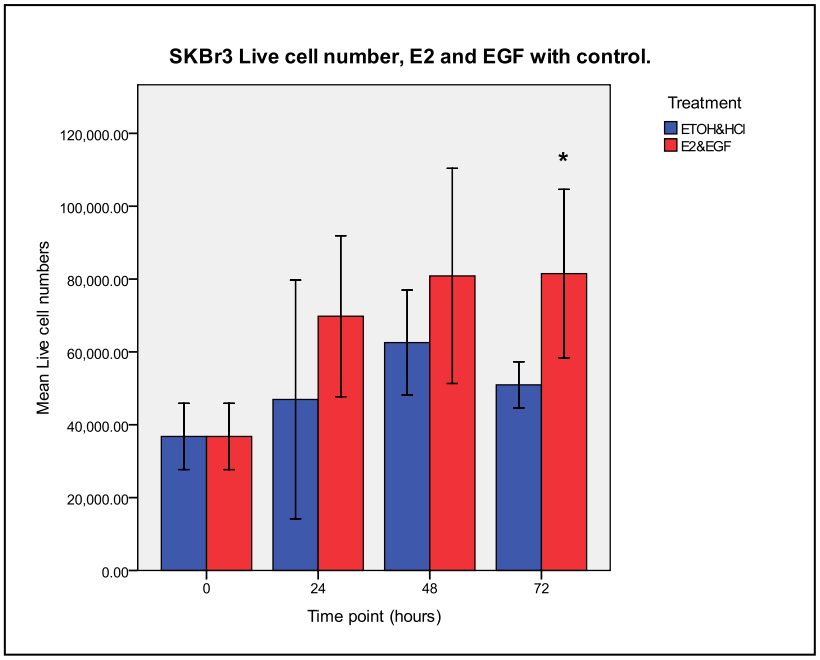

*Fig 4.47: Graph showing the mean live cell numbers of E2 and EGF stimulated SKBr3 cells. * = significant difference compared to control. 95% CI bars shown.*

4.2.2.2 Cell cycle results.

This table shows the significant increases (in red) and decreases (in blue) of the mean % population in the G1, S and G2 phases for the SKBr3 cell model experiment. Where there is no significant difference no result is shown.

Treatment \Time point	24 hours	48 hours	72 hours
E2	■	■	■
EGF	■	↑S ↓G1	■
E2 & EGF	■	■	■

Table 4.5: Table showing the results of the SKBr3 cell cycle experiment. . Significant increase. Significant decrease. *(p=0.05)*

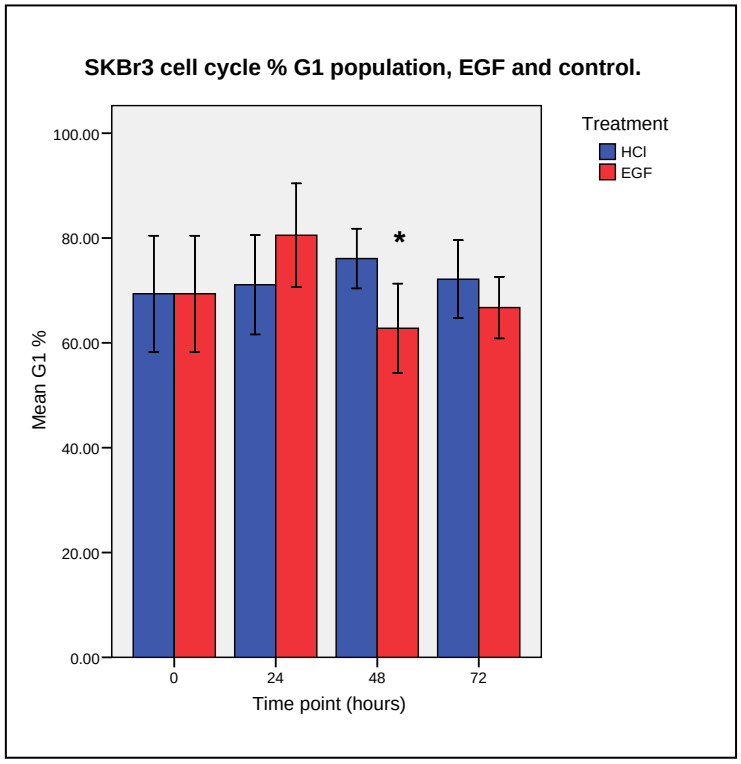

*Fig 4.48: Graph showing the mean G1 % of EGF stimulated SKBr3 cells. * = significant difference compared to control. 95% CI bars shown.*

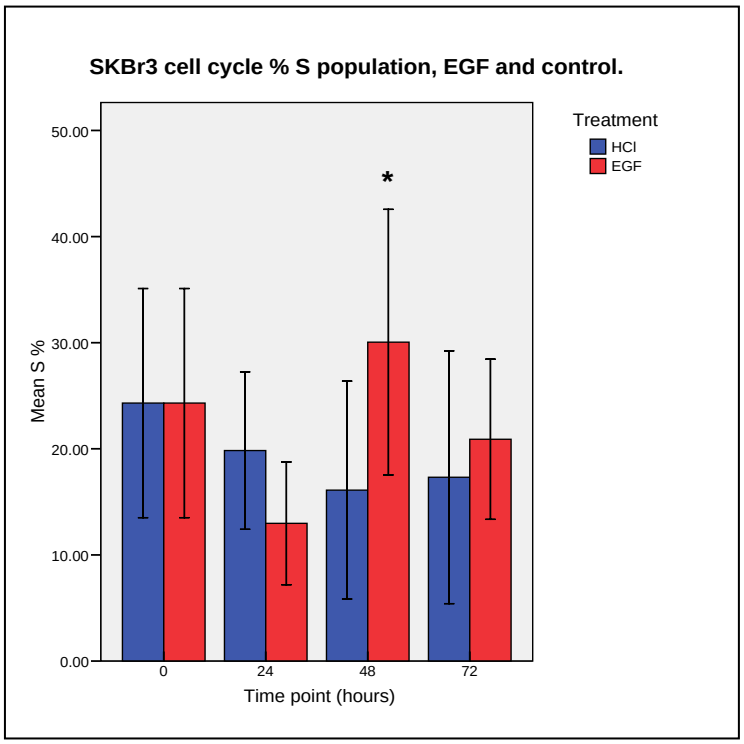

*Fig 4.49: Graph showing the mean S % of EGF stimulated SKBr3 cells. * = significant difference compared to control. 95% CI bars shown.*

4.2.2.3 STAT3 expression results.

This table shows the change in pSTAT3 expression when measured as a significant change in median channel shift for each treatment at each time point when compared to its specific control. Where there is no significant difference no results is shown.

Treatment \ Time point	12 Hr	24 Hr	48 Hr	72 Hr
E2	■	■	■	■
EGF	■	↑	■	↑
E2 and EGF	■	↑	■	↑

Table 4.6: Table showing the significant results for the SKBr3 STAT3 induction experiment. Significant increase. Significant decrease. *(p=0.05)*

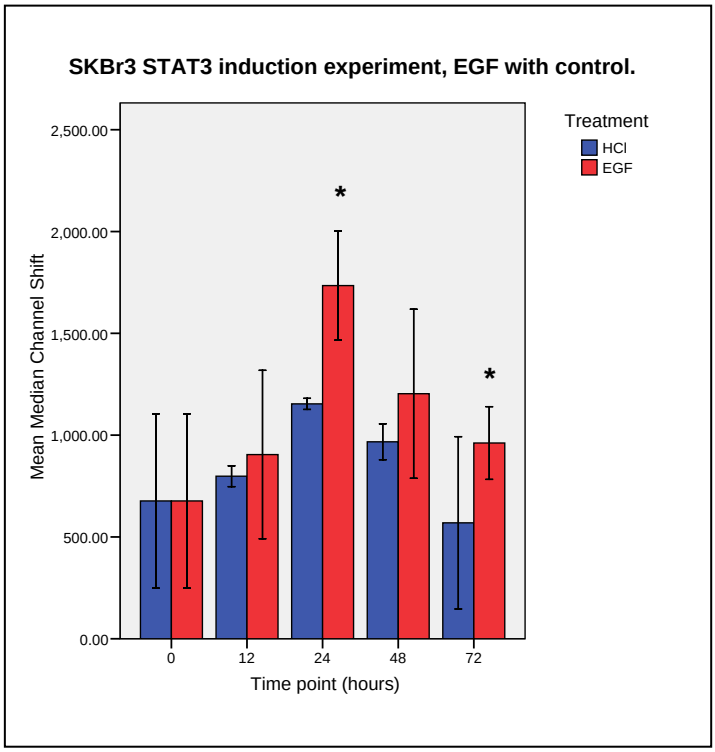

*Fig 4.50: Graph showing the mean median channel shift of pSTAT3 in SKBr3 cells with EGF stimulation. * = significant difference compared to control. 95% CI bars shown.*

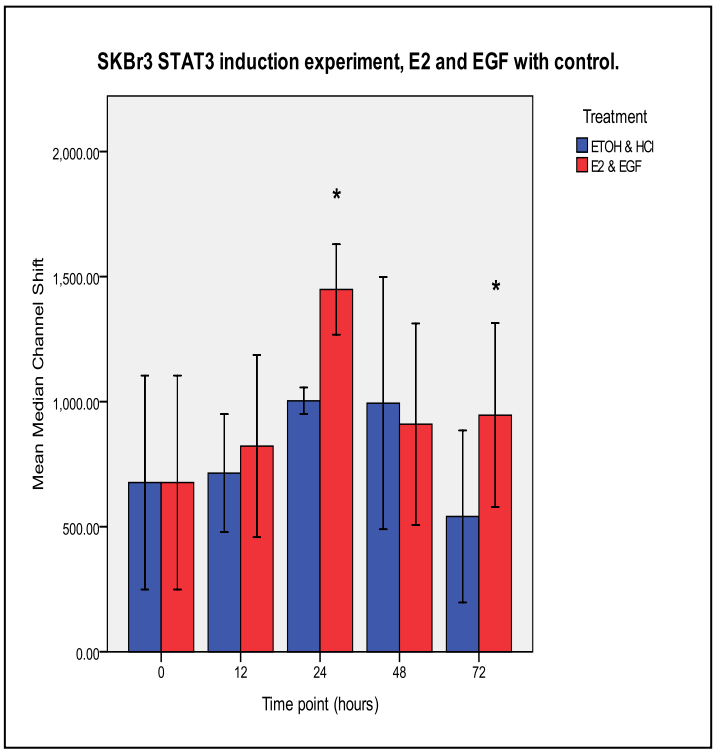

*Fig 4.51: Graph showing the mean median channel shift of pSTAT3 in SKBr3 cells with E2 and EGF stimulation. * = significant difference compared to control. 95% CI bars shown.*

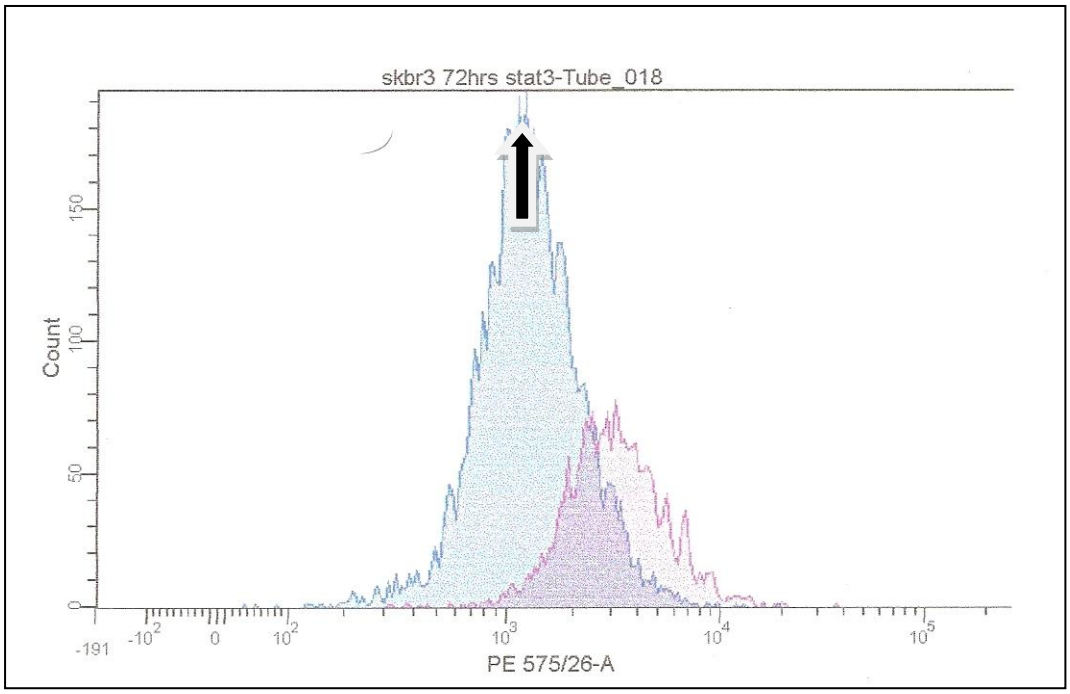

Fig 4.52: Histogram of pSTAT3 expression in SKBr3 cells stimulated with EGF after 72hrs. The black arrow (single cells) shows a median channel fluorescence of 1212.

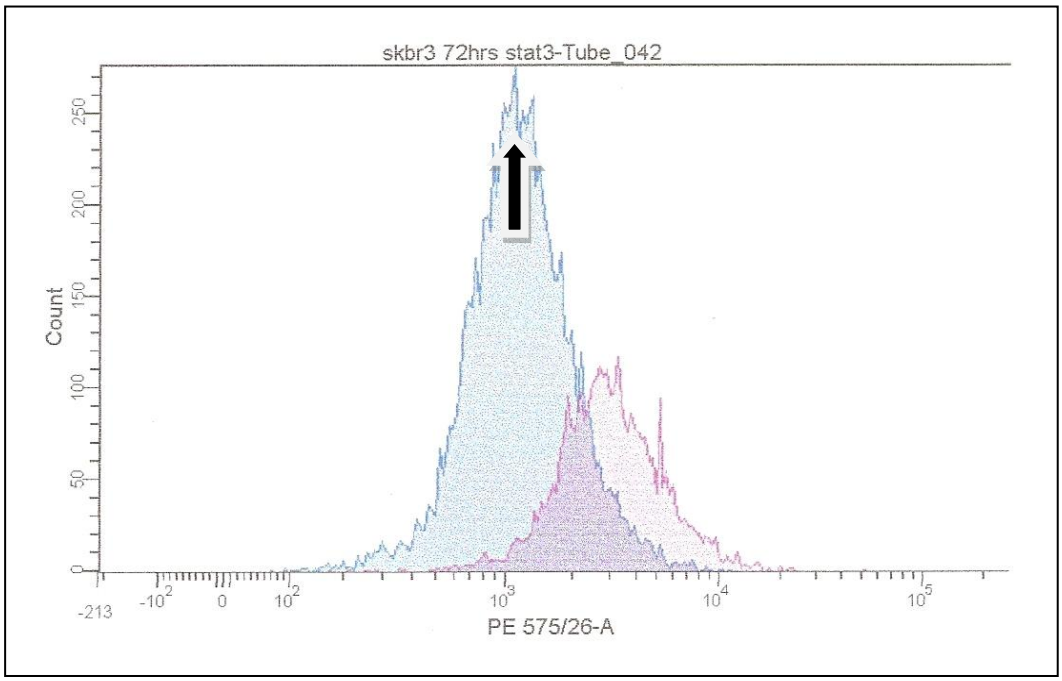

Fig 4.53: Histogram of pSTAT3 expression in SKBr3 cells stimulated with E2 and EGF after 72hrs. The black arrow (single cells) shows a median channel fluorescence of 1110.

The 2 histograms above shows that, unlike MCF7 cells, the addition of E2 to EGF stimulation does not inhibit STAT3 phosphorylation in SKBr3 cells. Again to show this a ratio of the median channel shift of treatment vs. control was plotted

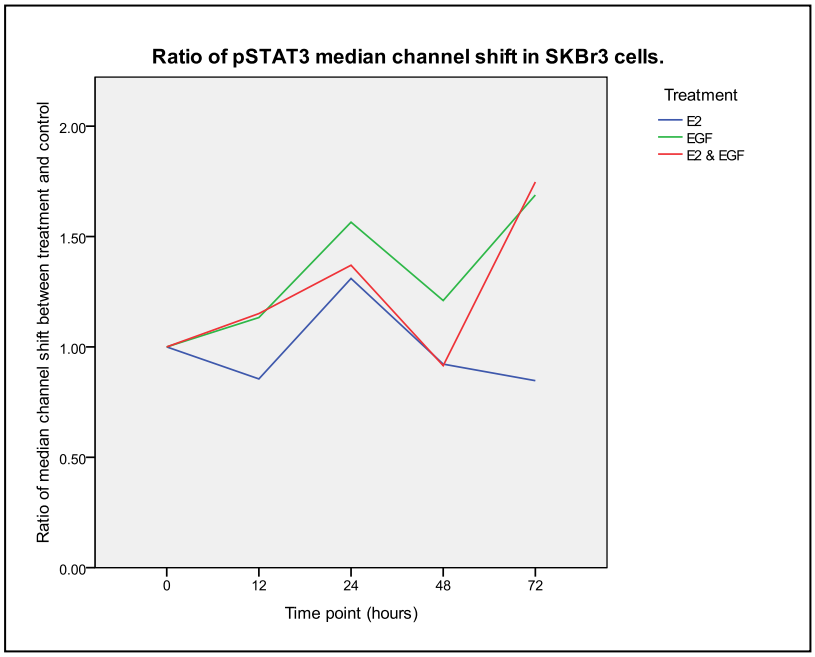

Fig 4.54: Graph showing ratio of median channel shift for pSTAT3 induction in SKBr3 cells.

4.2.2.4 Summary of results for SKBr3 cell model experiment.

The results for the cell cycle have shown that SKBr3 cells were stimulated to grow and divide by EGF with a decrease in the G1 population and an increase in S phase population at 48 hours. The STAT3 results have shown that EGF stimulation in SKBr3 cells again causes a biphasic increase in the expression of phosphorylated STAT3 this time at the 24 and 48 hour time point. Estrogen has no affect on STAT3 expression in this cell line. The addition of E2 to EGF stimulation in this cell line appears not to inhibit EGF ability to stimulate STAT3 expression especially at the 72 hour point unlike theMCF7 cell line.

4.2.3 MDA 231 cell model results.

After the analysis of the cell model results, and despite running the same cell model twice, we have been unable to ascertain and significant differences in cell number, cell cycle or STAT3 expression. The reasons for this are postulated in the discussion of results.

4.2.4 Summary of the cell line experiment.

The table below is shown to summarise the overall effect on STAT3 phosphorylation of stimulation with estrogen, EGF and combined stimulation on the 2 cell lines MCF7 and SKBr3.

	Cell Line Receptor Status		Stimulation.		
Cell Line	ER	EGFR	E2	EGF	E2&EGF
MCF7	Positive	Weak	↑	↑	0
SKBr3	Negative	Positive	0	↑	↑

Table 4.7: Table showing the stimulation of STAT3 phosphorylation in MCF7 and SKBr3 cell lines following treatment with E2, EGF or combined E2 and EGF.

The overall results showed that in the ER positive EGFR weak MCF7 cells estrogen and EGF alone resulted in an increase in STAT3 phosphorylation whereas combined stimulation did not increase STAT3 phosphorylation. These results were different from the ER negative EGFR positive SKBr3 cells where estrogen had no effect on STAT3 phosphorylation and EGF and combined estrogen and EGF increased STAT3 phosphorylation.

4.3. Discussion of cell model results.

The aim of the cell model study was to delineate the role of E2 and EGF stimulation on levels of STAT3 phosphorylation. To do this we selected the ER positive EGFR negative MCF7 cell line and compared its response to the ER negative EGFR positive cell lines MDA-231 and SKBr3. Overall stimulation of growth of the cell line was assessed using analysis of cell numbers and changes to the cell cycle populations. Changes in STAT3 phosphorylation was assessed by looking at significant increases in the median channel shift in florescence caused by E2 or EGF stimulation and combined stimulation when compared to the controls.

4.3.1 Cell line stimulation.

When considering the MCF7 cell line, we expected that upon stimulation with E2 and with the combination of E2 and EGF that the cell line would grow and proliferate. This stimulation would be represented by an increase in overall cell numbers and an increase in the live cell population. We would also see the cells progressing though a change in the DNA with a decrease in the G1 population compared with an increase in the S phase population and eventually a rise in the G2 population as the cell prepare to divide.

Table 4.1 shows the results for the MCF cell number experiment. In this estrogen sensitive breast cancer cell line, stimulation with estrogen caused a significant increase in total cell number and in the live cell number at all time points when compared to the ETOH control (fig 4.25 & 4.25). At the 48 hour time point with the estrogen stimulation there was also a significant increase in the apoptotic cell population (fig 4.26,) this was an indication of increased cell turnover.

The combined stimulation with estrogen and EGF also lead to an increase in total and live cell numbers at the 48 and 72hr time point (fig 4.27 & 4.28) and the apoptotic cells at 48hrs (fig 4.29). This would be predominantly a result of the estrogen stimulation due to the very low level if EGFR present in MCF7 cells.

The results for the cell cycle experiment are shown in table 4.2. These results show that stimulation with estrogen did result in a significant decrease in the G1 % population at the 24, 48 and 72 hour time point, the decrease in comparison to the ETOH control is shown in fig 4.30. This decrease in G1 % population was matched with the associated increase in the S phase proportion at the same time points, at 48 hours this increase did not quite reach statistical significance though the trend can be seen in fig. 4.31. The results also showed a significant increase in the G2 % population at the 24 72 hour time point (fig 4.32).

Stimulation with EGF did appear to decrease the initial G1 % population and increase the S phase % population at 24, 48 and 72 hours. Though only small (fig 4.33 & 4.34) the changes did appear to be significant. MCF7 is classical thought to be EGFR deficient but studies have shown low levels of functional EGFR below the lower limits for IHC detection (Imai et al., 1982b). This apparent stimulation in cell cycle was not reflected in a significant change in the cell numbers (table 4.1)

The combined stimulation with E2 and EGF showed similar results to the E2 alone stimulation with a decrease in G1 % population and an increase in S phase % population at all time points (fig 4.35 & 4.36). The G1 % drop at 72 hours though not statistically significant due to the slightly larger 95% CI became more obvious when

plotted on the graph (fig 4.35). Once again we also saw a significant increase in G2 % at the 24 and 72 hours time points (fig 4.37).

These results demonstrated that the MCF7 cell line was stimulated by E2 alone and E2 combined with EGF. Work in MCF7 cells has shown that in optimum stimulated conditions 85% of the cells had a mean cell cycle length of 21.3 hrs with cells passing through G2M phase in 3 hours (Taylor et al., 1983). These results have shown that a G2 % population increase within occurred the first 24 hours.

The cell number results, for the SKBr3 cell line (table 4.4), has shown that E2 stimulation had little effect on the cell line. Stimulation with EGF and combined EGF and estrogen caused an increase in total and live cell numbers at most of the time points (fig 4.44 – 4.47) but these increases did not become statistically significant until the 72 hr time point.

The cell cycle results have shown that E2 and the combined E2 and EGF stimulation had no significant effect on the % population in each phase (table 4.5). There was a significant decrease in G1 % and increase in S phase % at the 48 hour time point with EGF stimulation, indicating that the cell were moving though the cell cycle towards division (fig 4.48 & 4.49).

Though these results were not as impressive as hoped with regard to the increase in cell numbers and the progression through to G2 phase of the cell cycle they did indicate that SKBr3 was undergoing stimulation by EGF. Though previous work has shown that 72 hours is a sufficient length of time to show this progression through the

cell cycle it appears that the cells used in this model had a slower than expected growth rate. With prolonged culture beyond 72 hours this progression may well have been evident. Other studies have documented a longer proliferation time with the drop in G1 population proportion not evident until 4-6 days and similar difficulties in showing an increase in the G2 population proportion (Ingrid et al., 1997).

4.3.2 STAT3 induction.

The results of the STAT3 induction are presented as absolute differences of mean median channel shift between the treatment and its control and as a ratio of this difference.

When considering the MCF7 cell model we have shown that there is a significant increase in pSTAT3 when the cell line is stimulated with EFG (table 4.3). This appeared to be a biphasic response with a peak at 12 hours and a further peak at 48 hours (fig 4.39 and fig 4.43). Though as discussed above MCF7 are classically thought to be EGFR negative these results show that pSTAT3 induction via an EGF mediated route requires only a very low level of EGFR receptor expression. This STAT3 induction despite low levels of EGFR has been reported before in MCF7 cell lines (Li and Shaw, 2002) and again in MDA-231 cell lines deficient in detectable EGFR (Selander et al., 2004a).

The results of the EGFR positive SKBr3 cell model again, as demonstrated this bi-phasic response to EGF stimulation in the activation of pSTAT3 (see table 4.6). In the SKBr3 cell model this response occurs slightly later at the 48 and 72 hour time point (fig 4.50 & 4.54).

It has been suggested that STAT3 activation via EGF can occur via a rapid JAK mediated signal pathway with some studies demonstrating increases in nuclear STAT3 complexes after 30 min of treatment with EGF (Lo et al., 2005a). This rapid response has been termed “acute STAT3 activation” (Li and Shaw, 2002). A slower STAT3 response has been shown when starved cell lives have been re-suspended in

full serum and in this case it was suggested that this slower response was mediated via an IL6 pathway. There has been little published documentation of this bi-phasic response following continued EGF stimulation. The kinetics of this response may suggest that the first 12 hour peak is due to the phosphorylation of STAT3 which is constitutionally present in the cytoplasm whereas the second peak is due to an up regulation of the STAT3 gene itself. It has been shown that the STAT3 gene expression is up regulated by the sub-unit of the IL6 receptor gp130 (Ichiba et al., 1998). Gp130 activation and up regulation occurs in response to EGF stimulation in breast cancer cells (Grant et al., 2002). It has also been shown that inhibition of gp130 in breast cancer cell decreased STAT3 activation (Selander et al., 2004a). This provides a mechanism of up regulation of STAT3 gene expression via EGF stimulation which would account for this second peak seen in fig 4.50 and 4.54. It appears that EGF induction of STAT3 can be blocked by inhibition of the JAK with the pan-Jak inhibitor P6 but the joint EGFR/HER2 inhibitor used in clinical practice (Gefitinib) does not decrease EGF mediated STAT3 activation (Berishaj et al., 2007).

ER activation is not classical considered as one on the predominate pathways of STAT3 activation. Our results in the MCF7 cell line have shown that though the initial acute phase of STAT3 activation is not present there is a delayed peak of pSTAT3 at the 48 hour time point. This ER activation of STAT3 has been demonstrated in other studies (Bjornstrom and Sjoberg, 2002) and it has been shown that the addition of Tamoxifen can inhibit this activation (Yeh et al., 2006b). This stimulation is likely not to be a direct activation of STAT3 by ER, seen with the acute EGF response, but activation via non-genomic cross talk as discussed in section 1.2.2.

This cross talk may be via G-protein coupling with EGFR receptors or the up regulation of Scr and MAPK pathways (Wong et al., 2002b).

The results of the SKBr3 model showed that when the cell line is deficient in ER there is no STAT3 activation upon stimulation with estrogen (table 4.6). This result, though expected, is important as we have shown that EGFR expression below detectable levels in the MCF7 cell line is adequate for STAT3 stimulation, the converse in the SKBr3 cell line with ER was not true.

An interesting and novel finding in our results is the inhibition of EGF pSTAT3 induction when the cells were stimulated with EGF in combination with estrogen (see table 4.3). Though many experiments have been directed at the induction of STAT3 with EGF or E2 little work has been published on the affect of dual stimulation. This inhibition of EGF pSTAT3 stimulation by estrogen was seen only in the ER positive MCF7 cell line. In the ER negative SKBr3 cell line the dual stimulation still results in the bi-phasic increase in STAT3 activation (table 4.6 & fig 4.50 & 4.54). The 2 histograms plotted (fig 4.52 & 4.53) showed that there was no difference between the pSTAT3 median channel fluorescence at 72hrs in SKBR3 cells stimulated with EGF alone or EGF and estrogen combined. This is in contrast to the histograms (fig 4.41 & 4.42) which showed the drop in pSTAT3 median channel fluorescence at 48hrs when MCF cells are stimulated with EGF and estrogen compared with EGF alone.

There is no clear mechanism to account for this inhibition of EGF action on pSTAT when combined with E2 stimulation. There is some evidence that though ER can stimulate STAT3 activation it has also been shown that when MCF7 cell lines were

stimulated with IL6 the resultant STAT3 activation was inhibited by the addition of E2. It was also demonstrated that this inhibition could be reversed by the addition of Tamoxifen (Yamamoto et al., 2000a). It was suggested in this study that this inhibition was due to a direct interaction between ERα and STAT3. STAT3 action in Multiple Myeloma has been shown to be inhibited by PIAS3 (protein inhibitor of activated STAT3). Cell line experiments in Multiple Mylomas have shown that E2 treatment increases the synthesis of PIAS3 (Wang et al., 2001). Both of these studies may supply a mechanism to explain the results seen in this work but further work is obviously necessary to clarify this interaction of dual stimulation.

4.3.3 The MDA-231 cell model.

Unfortunately many difficulties were encountered with the MDA-231 cell line during culture. The cells were ascertained form in house stock and during the EGF titration experiment appeared to behave as expected with EGF stimulation. In the titration experiment the live cell numbers increased with stimulation and the cell moved through the G1, S and G2 cycle over the 96 hours. When the cell model experiment was conducted the same stock of MDA-231 was used but it became apparent on the analysis of the cell number and cell cycle data that they did not respond to any of the treatments with no difference between the controls and the treatments. We are currently at a loss as to the reasons for this but it is likely to be a technical problem with the culture of that stock of cells. It is unfortunate that these results are lacking as it would have been interesting to have seen the difference in STAT3 activation with this cell line and if they would respond in the same way to the SKBr3 cell line which had a similar receptor expression profile.

4.3.4 Critique of the methodology and suggestions for improvements to the cell line models.

Though this experiment has yielded some interesting results there were a number of metholodical issues that arose both in the running of the cell model and the analysis of the results. In this section I will identify some of those issues and offer suggestion on how this experiment could be developed and refined in the future.

The basis of the cell model is sound, to ascertain the effects of the phosphorylation of STAT3 by estrogen and EGF it is sensible to have cell lines which have a different

expression of the 2 receptors for the hormones of interest i.e. one to act as a control for another. The MCF 7 line provided an ER positive line and the SKBr3 a strong EGFR positive ER negative line to allow this comparison. The fact that the MCF7 line was also a low expresser of EGFR did lead to the interesting results seen with the co-stimulation with estrogen and EFG discussed above. To explore this further a truly ER positive EGFR negative line could be added in. A second option to provide a more thorough negative control would be the use of a receptor inhibition arm to the experiment. This would be provided by the use of tamoxifen as the estrogen control or Cetuximab (a monoclonal antibody against EGFR) as the EGF control.

The second issue with the model was the time points of analysis. As explained in section 4.1.9.1 there is a variation in the phosphorylation pattern of STAT3 depending on the stimulation used and the cell line under stimulation. It is of concern that as this is a relatively fluid and transient processes the peak of phosphorylation may be missed with the time points chosen. To address this further optimisation experiments could be performed now it is know that EGF does stimulate STAT3 phosphorylation in SKBr3 cells. This experiment could be designed along the lines of a brief period of stimulation followed by the analysis of the cell population at rapid multiple time points i.e. 15 min 30 min 1hr 2hr 6hr etc. This would ensure that an earlier peak of phosphorylation was not missed in this cell model.

There is a question of validity of the results seen using flow cytometry alone to assess STAT3 phosphorylation. Now flow cytometry is widely recognised for this purpose but at the time of the experiments there was less published experience in its use in this field. To further validate these results other methods could have been used alongside

the flow cytometry. These could have included western blot to ascertain a semi-quantitative result for STAT3 phosphorylation or possibly the use of confocal fluorescence microscope which would allow the assessment of nuclear localisation of pSTAT3 in stimulated cells.

Another point of discussion is the analysis of the results with regards to what values to use as the control points. There were two possible methods to do this one would be to compare the level of pSTAT3 with the time point zero result in the same stimulated arm of the experiment or to compare the level of pSTAT3 with the control un-stimulated arm at each time point. It was decided that as STAT3 phosphorylation is a variable process then it was more valid to compare its level to the control arm at each time point, this was to take into account the possible natural fluctuation if pSTAT3 levels across the time span of the experiment. I do however appreciate the arguments in favour of the other approach of going back to time point zero as the control value. This again could be addressed by the use of western blotting films at each time point to add more validity to this method.

5 Conclusion of the thesis.

The two aims of this study were firstly to ascertain if the over expression of transcription factors STAT3 SP1 and NFKB in invasive breast cancer are related to the disease outcome and secondly to assess the role of estrogen and EGF in the induction of pSTAT3 in ER and EGFR positive and negative cell lines.

Immunohistochemistry was used to determine the expression of the 3 transcription factors in 204 breast cancer samples in the study group. The study group selected was shown to be representative for the range of patient age and tumour types with regards to histology, grade and stage. The transcription factor "status" in the tumour was then compared to important pathological prognostic features and to overall patient outcome.

The transcription factor STAT3 was over expressed in 27% of the cancers analysed. It was shown that nuclear expression of STAT3 was significantly associated with the poor prognostic markers of high grade, larger tumour size, vessel space invasion, node metastasis and a high NPI group. STAT3 expression was also significantly associated with ER and PR negative status but not to HER2 expression. STAT3 nuclear expression was related to EGFR positive cancers.

With regards to patient outcome we have shown that STAT3 over expression was significantly associated with disease recurrence but not with overall survival at 5 years. Although STAT3 over expression was not shown to be an independent prognostic marker it was shown that it was a significant marker of poor survival in

node negative breast cancers. This sub-group of node negative patients may be being under treated with regards to adjuvant therapy with current management protocols.

Sub-group analysis dependent on the HER2 status appeared to show that the dual positive cancers (HER2 +ve and STAT3 +ve) had a much poorer outcome than the HER2 +ve STAT3 -ve cancers. The significance of this was limited due to the size of these sub groups. These findings could have significant clinical importance if it is confirmed that the negative survival effect of HER2 positivity is only significant in the STAT3 positive patients. Anti HER2 (Herceptin) therapy may be more effectively targeted to these dual positive patients. This would target treatment to those with most to benefit; reducing the overall numbers of patient treated unnecessarily, which is important when considering the implications of Herceptin therapy. A further study into STAT3 expression in HER2 receiving Herceptin could well identify that the population who benefit from Herceptin is the population which express STAT3.

Nuclear or transcriptionally active SP1 was seen in 33% of the breast cancers tested. There were no significant correlations with any of the pathological features recorded but there was an association between SP1 and ER positive cancers. Though SP1 is an important transcription factor in the expression of ER it is not necessary for a tumour to have nuclear SP1 staining to express ER. Where SP1 does seem to be important is in the identification of patients who will suffer from a late disease recurrence. When survival after 2 years was plotted dependent on SP1 status, the only recurrences fell in the SP1 negative group of patients. This effect was independent of initial ER status. These results may allow the identification of patients with the risk of late recurrence to be targeted with further adjuvant treatments.

When testing our patient samples it was shown that nuclear localisation of NFKB was present in 15% of the group. This nuclear localisation was significantly associated with larger and higher grade cancers but not with node status. Due to the correlation with these larger more aggressive tumours there was also a relationship between nuclear NFKB and high NPI score. As in other studies a link between NFKB and ER negative tumours was shown. Despite these initial finding no link between NFKB expression and disease outcome was demonstrated. The sub group analysis failed to show any survival impact of NFKB expression, as of yet a there appears not to be a role for the IHC determined expression of NFKB in breast cancers.

This IHC based clinical study has yielded some new and significant findings which may clinical and management implications. Further studies looking at STAT3 in HER2 positive cancers are needed determine if the initial trends seen in this study become significant with increased numbers of patients tested. Extended follow up in both the STAT3 and SP1 groups may yield significant late survival effects for each of these transcription factors and a larger cohort would allow more significant sub-group analysis.

The cell model was designed to ascertain the effects of cell stimulation with estrogen, EGF or combined stimulation on the levels of phosphorylated STAT3. These results have confirmed that EGF stimulation in the EGFR rich cell line, SKBr3, results in an increase in STAT3 phosphorylation, it has also been shown that this is a bi-phasic response to EGF stimulation. These results have shown that, despite very low levels of expressed EGFR, the MCF7 cell line again exhibits a bi-phasic phosphorylation of STAT3 with EGF stimulation.

In the ER rich MCF7 cells it was shown that estrogen alone can increase STAT3 phosphorylation and in the discussion we have proposed a mechanism to explain this. In this experiment the results suggested the novel finding that the addition of estrogen to EGF stimulation in the ER positive MCF7 cells there is an inhibition of STAT3 phosphorylation. This inhibition did not occur in the ER negative SKBr3 cell line. It may be considered that ER stimulation of this predominantly ER driven cell line causes a preferential cell growth via an ER pathway rather than an EFGR/STAT3 pathway i.e. the ER pathway overrides the EGFR/STAT3 phosphorylation. This could be important in a clinical setting when we consider an ER positive cancer which also has the potential for EGFR/STAT3 driven cell growth. If this cancer, currently driven via an estrogen stimulated ER pathway is then deprived of estrogen by an adjuvant therapy, such as aromatase inhibitor therapy, then they may be switch to an EGFR/STAT3 pathway of cell proliferation. This could cause and unexpected proliferation in tumour growth.

These results generate many interesting hypothesis which deserve further investigation. The cell model experiment could be extended to include ER inhibition/blocking and EGFR inhibition. This may confirm firstly estrogen does inhibit EGF phosphorylation of STAT3 and whether or not this is via an ER mediated pathway.

This hypothesis could also be tested in the clinical setting. Patients who do not have surgery for their tumour, undergoing primary endocrine therapy instead, provided a clinical setting to test this hypothesis. Samples of ER positive breast cancers which were treated with primary endocrine therapy (i.e. Letrazole) could be examined for

the levels of pSTAT3 before treatment and then at time points during the treatment. Some of these tumours will progress or recur after an initial response to primary endocrine therapy and if these tumours displayed an increase in pSTAT3 levels then this would add weight to the hypothesis that intervention with estrogen deprivation causes a shift to pSTAT3 mediated proliferation.

This study has added to what is known about the role of STAT3, SP1 and NFKB expression in breast cancer patients and in so it has generated some important questions which should be the subject of further investigation. The cell model has raised the suggestion that estrogen deprivation may increase STAT3 mediated cell proliferation and the impact of this has been discussed.

Breast cancer was one of the first tumours where biological markers were used to dictated treatment. With the increasing understanding of the molecular biology of breast cancer we have had a glimpse of the future of breast cancer management. That future is that upon presentation a patient's tumour will be subjected to an entire panel of molecular markers which will identify the proliferative pathways in use by that cancer and future pathways available to that tumour. This will allow a bespoke package of adjuvant therapies to be tailored to the patient which will ensure the best possible outcome. Until this point we will always be faced with the patient who should have done well dying from their disease.

References.

(1982) The World Health Organization, Histological Typing of Breast Tumors second edition. *American Journal of Clinical Pathology,* 78**,** 806-16.

(1996) Collaborative Group on Hormonal Factors in Breast Cancer, Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Collaborative Group on Hormonal Factors in Breast Cancer. *Lancet,* 22**,** 1713-27.

(1997a) Collaborative Group on Hormonal Factors in Breast Cancer, Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. *Lancet,* 11**,** 1047-59.

(1997b) Office for National Statistics, Twentieth Century Mortality - 95 years of mortality data in England and Wales by age, sex, year and underlying cause. IN STATISTICS, O. F. N. (Ed.). London, Office for National Statistics.

(2002) Collaborative Group on Hormonal Factors in Breast Cancer, Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease. *Lancet,* 20**,** 187-95.

(2003) The Million Women Study, Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet,* 362**,** 419-427.

(2005a) Information and Statistics Division, Cancer registrations 2002. IN SCOTLAND, N. (Ed.) *ISD online.* Edinburgh, NHS Scotland.

(2005b) Office for National Statistics, Cancer Statistics registrations: Registrations of cancer diagnosed in 2002, England. *MB1 no 33.* London., National Statistics.

(2005c) Welsh Cancer Intelligence and Surveillance Unit, Cancer registrations 2002.

AALTOMAA, S., LIPPONEN, P., ESKELINEN, M., KOSMA, V. M., MARIN, S., ALHAVA, E. & SYRJÄNEN, K. (1991) Hormone receptors as prognostic factors in female breast cancer. *Annals of Medicine,* 23**,** 643-8.

AGGARWAL, B. B. (2004) Nuclear factor-ºB: The enemy within. *Cancer Cell,* 6**,** 203-208.

AGGARWAL, B. B., KUNNUMAKKARA, A. B., HARIKUMAR, K. B., GUPTA, S. R., THARAKAN, S. T., KOCA, C., DEY, S. & SUNG, B. (2009) Signal Transducer and Activator of Transcription-3, Inflammation, and Cancer. *Annals of the New York Academy of Sciences,* 1171**,** 59-76.

AGGARWAL, B. B., SCHWARZ, L., HOGAN, M. E. & RANDO, R. F. (1996) Triple Helix-forming Oligodeoxyribonucleotides Targeted to the Human Tumor Necrosis Factor (TNF) Gene Inhibit TNF Production and Block the TNF-dependent Growth of Human Glioblastoma Tumor Cells. *Cancer Research,* 56**,** 5156-5164.

ALLRED, D. C., HARVEY, J. M., BERARDO, M. & CLARK, G. M. (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol,* 11**,** 155-168.

ALYEA, R. & WATSON, C. (2009) Nongenomic mechanisms of physiological estrogen-mediated dopamine efflux. *BMC Neuroscience,* 10**,** 59.

ARTEAGA, C. B. O., A. MOULDER, S.L. PINNS, M. SPARANO, J.A. SLEDGE, G.W. DAVIDSON, N.E. (2008) A phase I-II study of combined blockade of the ErbB receptor network with trastuzumab and gefitinib in patients with HER2 (ErbB-2) overexpressing metastatic breast cancer. *Clinical Cancer Research,* 14**,** 6277-6283.

ATAC, THE, ATAC, TRIALIST & GROUP. (2002) Anastrazole alone or in combination with Tamoxifen versus Tamoxifen alone for the treatment of post menopausal women with early breast cancer. *Lancet,* 359**,** 2131-2139.

BABU, M. M., LUSCOMBE, N. M., ARAVIND, L., GERSTEIN, M. & TEICHMANN, S. A. (2004) Structure and evolution of transcriptional regulatory networks. *Current Opinion in Structural Biology,* 14**,** 283-291.

BACUS, S. S., KIGUCHI, K., CHIN, D., KING, C. R. & HUBERMAN, E. (1990) Differentiation of cultured human breast cancer cells (AU-565 and MCF-7) associated with loss of cell surface HER-2/neu antigen. *Molecular Carcinogenesis,* 3**,** 350-362.

BALSLEV, I., AXELSSON, C. K., ZEDELER, K., RASMUSSEN, B. B., CARSTENSEN, B. & MOURIDSEN, H. T. (1994) The Nottingham Prognostic Index applied to 9,149 patients from the studies of the Danish Breast Cancer Cooperative Group (DBCG). *Breast Cancer Research and Treatment,* 32**,** 281-290.

BARDOU, V. A., G. ELLEDGE, R.M. OSBORNE, C.K. CLARK, G.M. (2003) Progesterone receptor status significantly improves outcome prediction over estrogen receptor alone for adjuvant endocrine therapy in two large breast cancer databases. *Journal of Clinical Oncology,* 21**,** 1973-1979.

BEATSON, G. T. (1896) On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet,* ii**,** 104-107, .

BECKER N. (1998) Cancer mortality and prevention in the European Union. *European Journal of Surgical Oncology.,* 24**,** 370 -374.

BERCLAZ, G., ALTERMATT, H. J., ROHRBACH, V., SIRAGUSA, A., DREHER, E. & SMITH, P. D. (2001) EGFR dependent expression of STAT3 (but not STAT1) in breast cancer. *Int J Oncol,* 19**,** 1155-60.

BERGSTROM, A., PISANI, P., TENET, V., WOLK, A. & ADAMI, H. O. (2001) Overweight as an avoidable cause of cancer in Europe. *Int J Cancer,* 91**,** 421-30.

BERISHAJ, M., GAO, S. P., AHMED, S., LESLIE, K., AL-AHMANDIE, H., GERALD, W. L., BORNMANN, W. & BROMBERG, J. F. (2007) Stat3 is tyrosine-phosphorylated through the interleukin-6/glycoprotein 130/janus kinase pathway in breast cancer. *Breast Cancer Research,* 9.

BISWAS, D. K., CRUZ, A. P., GANSBERGER, E. & PARDEE, A. B. (2000) Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. *Proc Natl Acad Sci U S A,* 97**,** 8542-7.

BISWAS, D. K., MARTIN, K. J., MCALISTER, C., CRUZ, A. P., GRANER, E., DAI, S. C. & PARDEE, A. B. (2003) Apoptosis caused by chemotherapeutic inhibition of nuclear factor-kappaB activation. *Cancer Res,* 63**,** 290-5.

BISWAS, D. K., SHI, Q., BAILY, S., STRICKLAND, I., GHOSH, S., PARDEE, A. B. & IGLEHART, J. D. (2004) NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc Natl Acad Sci U S A,* 101**,** 10137-42.

BJORNSTROM, L. & SJOBERG, M. (2002) Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. *Mol Endocrinol,* 16**,** 2202-14.

BJORNSTROM, L. & SJOBERG, M. (2004) Estrogen receptor-dependent activation of AP-1 via non-genomic signalling. *Nucl Recept,* 2**,** 3.

BLAMEY, R. W., ELLIS, I. O., PINDER, S. E., LEE, A. H. S., MACMILLAN, R. D., MORGAN, D. A. L., ROBERTSON, J. F. R., MITCHELL, M. J., BALL, G. R., HAYBITTLE, J. L. & ELSTON, C. W. (2007) Survival of invasive breast cancer according to the Nottingham Prognostic Index in cases diagnosed in 1990-1999. *European Journal of Cancer,* 43**,** 1548-1555.

BREASTED, J. H. (1980) *The Edwin Smith surgical papyrus,* Chicargo, The university of Chicargo Press.

BROMBERG, J. & DARNELL, J. E. (2000) The role of STATs in transcriptional control and their impact on cellular function. *Oncogene,* 19**,** 2468-2473.

BROMBERG, J. F., WRZESZCZYNSKA, M. H. & DEVGAN, G. (1999) STAT3 as an oncogene. *Cell,* 98**,** 295-303.

BUCHHOLZ, T. A., GARG, A. K., CHAKRAVARTI, N., AGGARWAL, B. B., ESTEVA, F. J., KUERER, H. M., SINGLETARY, E., HORTOBAGYI, G., PUSZTAI, L., CRISTOFANILLI, M. & SAHIN, A. (2005) The nuclear transcrition factor êß / bcl-2 pathway correlates with pathological complete response to doxorubicin based neoadjuvant chemotherapy in human breast cancer. *Clinical Cancer Research,* 11**,** 8398-8402.

BUENO-DE-MESQUITA, J. M., NUYTEN, D. S. A., WESSELING, J., VAN TINTEREN, H., LINN, S. C. & VAN DE VIJVER, M. J. (2009) The impact of inter-observer variation in pathological assessment of node-negative breast cancer on clinical risk assessment and patient selection for adjuvant systemic treatment. *Annals of Oncology,* 21**,** 40-47.

BURKE, W. M., JIN, X., LIN, H. J., HUANG, M., LIU, R., REYNOLDS, R. K. & LIN, J. (2001) Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cells. *Oncogene,* 20**,** 7925-34.

BURKE, W. M. J., X. LIN, H. HUANG, M. LIU, R. REYNOLDS, R. LIN, J. (2001) Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cell lines. *Oncogene,* 20**,** 7925-7934.

CAILLEAU, R., OLIVE, M. & CRUEIGER, Q. V. J. (1978) Long term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro,* 14**,** 911-915.

CALDERON-MARGALIT, R. & PALTIEL, O. (2004) Prevention of breast cancer in women who carry BRCA1 or BRCA2 mutations: A critical review of the literature. *International Journal of Cancer,* 112**,** 357-364.

CALDON, L. J. M., WALTERS, S. J., REED, J. A., MURPHY, A., WORLEY, A. & REED, M. W. R. (2005) Case-mix fails to explain variation in mastectomy rates: management of screen-detected breast cancer in a UK region 1997–2003. *Br J Cancer,* 92**,** 55-59.

CALO, V., MIGLIAVACCA, M., BAZAN, V., MACALUSO, M., BUSCEMI, M., GEBBIA, N. & RUSSO, A. (2003) STAT proteins: from normal control of cellular processes to tumourogenisis. *Journal of Cellular Physiology.,* 197**,** 157-168.

CARMICHAEL, A., SAMI, A. S. & DIXON, J. M. (2003) Breast cancer risk among the survivors of atomic bomb and patients exposed to therapeutic ionising radiation. *Eur J Surg Oncol,* 29**,** 475-9.

CARTER C.L., A. C., HENSON D.E. (1989) Relation of tumor size lymph node status and survival in 24,740 breast cancer cases. *Cancer,* 63**,** 181-7.

CASTRO-RIVERA, E., SAMUDIO, I. & SAFE, S. (2001) Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. *J Biol Chem,* 276**,** 30853-61.

CENTRE, T. I. (2008) Breast Screening Program England 2006 - 2007. London, Office for National Statistics.

CHAMBLISS, K. L., YUHANNA, I. S., MINEO, C., LIU, P., GERMAN, Z., SHERMAN, T. S., MENDELSOHN, M. E., ANDERSON, R. G. & SHAUL, P. W. (2000) Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signalling module in caveolae. *Circ Res,* 87**,** E44-52.

CHAPMAN R, LOURENCO P, TONNER E, FLINT D, SELBERT S, TAKEDA K, AKIRA S, CLARKE A & C, W. (1999) Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3

Genes Dev, 13**,** 2604-2616.

CIOCCA, D., GAGO, F. E., FANELLI, M. A. & CALDERWOOD, S. K. (2006) Co-expression of steroid receptors (estrogen receptor alpha and/or progesterone receptors and Her-2/neu: Clinical implications. *Journal of Steroid Biochemistry and Molecular Biology,* 102**,** 32-40.

CIOCCA, D. G., F.E. FANELLI, M.A. CALDERWOOD, S.K. (2006) Co-expression of steroid receptors (estrogen receptor alpha and/or progesterone receptors and Her-2/neu: Clinical implications. *Journal of Steroid Biochemistry and Molecular Biology,* 102**,** 32-40.

CIOCCA, D. R., FUJIMURA, F. K., TANDON, A. K., CLARK, G. M., LEE-CHEN, G. J., POUNDS, G. W., VENDELEY, M. A., OWENS, M. A., PANDIAN, M. R. & MCGUIRE, W. L. (1992) Correlation of HER-2/neu amplification with expression and with other prognostic factors in 1103 breast cancers. *Journal of the National Cancer Institute,* 84**,** 1279-1282.

CIOCCA, D. R. F., F.K. TANDON, A.K. CLARK, G.M. LEE-CHEN, G.J. POUNDS, G.W. VENDELEY, M.A. OWENS, M.A. PANDIAN, M.R. MCGUIRE, W.L. (1992) Correlation of HER-2/neu amplification with expression and with other prognostic factors in 1103 breast cancers. *Journal of the National Cancer Institute,* 84**,** 1279-1282.

CLARKE, R., LIU, M. C., BOUKER, K. B., GU, Z., LEE, R. Y., ZHU, Y., SKAAR, T. C., GOMEZ, B., O'BRIEN, K., WANG, Y. & HILAKIVI-CLARKE, L. A. (2003) Antiestrogen resistance in breast cancer and the role of estrogen receptor signalling. *Oncogene,* 22**,** 7316-39.

CLAVEL-CHAPELON, F. (2002) Differential effects of reproductive factors on the risk of pre- and postmenopausal breast cancer. Results from a large cohort of French women. *Br J Cancer,* 86**,** 723-7.

CLEVENGER, C. V. (2004) Roles and regulation of stat family transcription factors in human breast cancer. *Am J Pathol,* 165**,** 1449-60.

COGSWELL, P. C., GUTTRIDGE, D. C., FUNKHOUSER, W. K. & BALDWIN, A. S., JR. (2000) Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. *Oncogene,* 19**,** 1123-31.

COHEN, S. (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new born animal. *Journal of Biological Chemistry.,* 237**,** 1555-1562.

COHEN, S. & CARPENTER, G. (1975) Human growth factor isolation and chemical and biological properties. *Proc. Natl. Acad. Sci. USA,* 72**,** 1317-1321.

COHEN, S., CARPENTER, G. & KING, L. (1980) Epidermal growth factor receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *Journal of Biological Chemistry.,* 255**,** 4834-4842.

COLE, M. P., JONES, M. P. & TODD, I. D. (1971) A new anti-estrogenic agent in late breast cancer. An early appraisal. *British Journal of Cancer.,* 25**,** 270-275.

COLEMAN, M. P. (2004) Trends and socioeconomic inequalities in cancer survival in England and Wales up to 2001. *British Journal of Cancer,* 90**,** 1367 - 1373.

COLLABORATORS, M. W. S. (2003) Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet,* 362**,** 419-427.

CONNELY, O. M., MULAC-JERICEVIC, B., LYDON, J. P. & DE MAYO, F. J. (2001) Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. *Molecular Cellular Endocrinology,* 179.

COOMBES, R. C., HALL, E. & L.J., G. (2004) A randomised control trial of exemestane after two to three years of tamoxifen in post-menopausal women with primary breast cancer. *N Engl J Med,* 350**,** 1081-1092.

COOMBES, R. C., KILBURN, L. S., SNOWDON, C. F., PARIDAENS, R., COLEMAN, R. E., JONES, S. E., JASSEM, J., VAN DE VELDE, C. J. H., DELOZIER, T., ALVAREZ, I., DEL MASTRO, L., ORTMANN, O., DIEDRICH, K., COATES, A. S., BAJETTA, E., HOLMBERG, S. B., DODWELL, D., MICKIEWICZ, E., ANDERSEN, J., LØNNING, P. E., COCCONI, G., FORBES, J., CASTIGLIONE, M., STUART, N., STEWART, A., FALLOWFIELD, L. J., BERTELLI, G., HALL, E., BOGLE, R. G., CARPENTIERI, M., COLAJORI, E., SUBAR, M., IRELAND, E. & BLISS, J. M. (2007) Survival and safety of exemestane versus tamoxifen after 2-3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. *The Lancet,* 369**,** 559-570.

COWIE, A. T. (1974) Overview of the mammary gland. *J Invest Dermatol1.1,* 63**,** 2-9.

DEGRAFFENRIED, L. A., CHANDRASEKAR, B., FRIEDRICHS, W. E., DONZIS, E., SILVA, J., HIDALGO, M., FREEMAN, J. W. & WEISS, G. R. (2004) NF-kappa B inhibition markedly enhances sensitivity of resistant breast cancer tumor cells to tamoxifen. *Ann Oncol,* 15**,** 885-90.

DEGRAFFENRIED, L. A., HILSENBECK, S. G. & FUQUA, S. A. (2002) Sp1 is essential for estrogen receptor alpha gene transcription. *J Steroid Biochem Mol Biol,* 82**,** 7-18.

DEO, S. V. S., BHUTANI, M., SHUKLA, N. K., RAINA, V., RATH, G. K. & PURKAYASTH, J. (2003) Randomized trial comparing neo-adjuvant versus adjuvant chemotherapy in operable locally advanced breast cancer (T4b N0-2 M0). *Journal of Surgical Oncology,* 84**,** 192-197.

DETRE, S., SACCANI-JOTTI, G. & DOWSETT, M. (1995) A "quick score" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast cancer. *Journal of Clinical Pathology,* 48**,** 876-878.

DIAZ, L. K., SAHIN, A. & SNEIGE, N. (2004) Interobserver agreement for estrogen receptor immunohistochemical analysis in breast cancer: a comparison of manual and computer-assisted scoring methods. *Annals of Diagnostic Pathology,* 8**,** 23-27.

DIAZ, N., MINTON, S., COX, C., BOWMAN, T., GRITSKO, T., GARCIA, R., EWEIS, I., WLOCH, M., LIVINGSTON, S., SEIJO, E., CANTOR, A., LEE, J. H., BEAM, C. A., SULLIVAN, D., JOVE, R. & MURO-CACHO, C. A. (2006a) Activation of stat3 in primary tumors from high-risk breast cancer patients is associated with elevated levels of activated SRC and survivin expression. *Clinical Cancer Research,* 12**,** 20-28.

DIAZ, N., MINTON, S., COX, C., BOWMAN, T., GRITSKO, T., GARCIA, R., EWEIS, I., WLOCH, M., LIVINGSTON, S., SEIJO, E., CANTOR, A., LEE, J. H., BEAM, C. A., SULLIVAN, D., JOVE, R. & MURO-CACHO, C. A. (2006b) Activation of stat3 in primary tumours from high-risk breast cancer patients is associated with elevated levels of activated SRC and survivin expression. *Clinical Cancer Research,* 12**,** 20-28.

DIEN, J., AMIN, H. M., CHIU, N., WONG, W., FRANTZ, C., CHIU, B., MACKEY, J. R. & LAI, R. (2006) Signal transducers and activators of transcription-3 up-regulates tissue inhibitor of metalloproteinase-1 expression and decreases invasiveness of breast cancer. *American Journal of Pathology,* 169**,** 633- 642.

DIETEL, M., ELLIS, I., HÖFLER, H., KREIPE, H., MOCH, H., DANKOF, A., KÖLBLE, K. & KRISTIANSEN, G. (2007) Comparison of automated silver enhanced in situ hybridisation (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in breast carcinoma according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists. *Virchows Archiv,* 451**,** 19-25.

DOLLED-FILHART, M., CAMP, R. L., KOWALSKI, D. P., SMITH, B. L. & RIMM, D. L. (2003a) Tissue microarray analysis of signal transducers and activators of transcription 3 (Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. *Clin Cancer Res,* 9**,** 594-600.

DOLLED-FILHART, M., CAMP, R. L., KOWALSKI, D. P., SMITH, B. L. & RIMM, D. L. (2003b) Tissue microarray analysis of signal transducers and activators of transcription 3 (Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. *Cancer Research,* 9**,** 594-600.

DREW, P. J., KERIN, M. J., MAHAPATRA, T., MALONE, C., MONSON, J. R. T., TURNBULL, L. W. & FOX, J. N. (2001) Evaluation of response to neoadjuvant chemoradiotherapy for locally advanced breast cancer with dynamic contrast-enhanced MRI of the breast. *European Journal of Surgical Oncology,* 27**,** 617-620.

DUAN, R., PORTER, W. & SAFE, S. (1998) Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology,* 139**,** 1981-90.

DUAN, R., PORTER, W., SAMUDIO, I., VYHLIDAL, C., KLADDE, M. & SAFE, S. (1999) Transcriptional activation of c-fos proto-oncogene by 17beta-estradiol: mechanism of aryl hydrocarbon receptor-mediated inhibition. *Mol Endocrinol,* 13**,** 1511-21.

DYNAN, W. S. & TJIAN, R. (1983) Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell,* 32**,** 669-80.

EARLY, BREAST, CANCER, TRIALISTS, COLLABORATIVE & GROUP (1998) Tamoxifen for early breast cancer an overview of the randomised trials. *Lancet,* 351**,** 1451-1467.

EBCTCG, E. B. C. T. C. G. (1995) Effects of radiotherapy and surgery in early breast cancer. An overview of the randomised trials. N. Engl. J. Med. 1995;333:1444-1455. *N Engl J Med,* 333**,** 1444-55.

EBCTCG, E. B. C. T. C. G. (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *The Lancet,* 365**,** 1687-1717.

ELLIS I.O., G. M., BROUGHTON N., LOCKER A., BLAMEY R.W., ELSTON C.W. (1992) Pathological prognostic factors in breast cancer. II. Histological tyoe. Relationship with survival in a large study with long-term follow-up. *Histopathology,* 20**,** 479-489.

ELSTON, C. W. & ELLIS, I. O. (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in bresat cancer: experience from a large study with long-term follow-up. *Histopathology,* 19**,** 403-410.

ENCARNACION, C. A., CIOCCA, D. R., MCGUIRE, W. L., CLARK, G. M., FUQUA, S. A. & OSBORNE, C. K. (1993) Measurement of steroid hormone receptors in breast cancer patients on tamoxifen. *Breast Cancer Res Treat,* 26**,** 237-46.

ENMARK, E., PELTO-HUIKKO, M., GRANDIEN, K., ET AL (1997) Human estrogen receptor B: gene structure, chromosomal localisation and expression pattern. *Journal of Clinical Endocrinology,* 82**,** 4258-4265.

EWERTZ M, D. S., ADAMI HO, KVALE G, LUND E, MEIRIK O, MELLEMGAARD A, SOINI I, TULINIUS H. (1990) Age at first birth, parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries. *Int J Cancer,* 15**,** 597-603.

FANGER, H. & REE, H. J. (1974) Cyclical changes in human mammary gland epithelium in relation to the menstrual cycle, an ultrastructural study. *Cancer,* 34**,** 574 - 585.

FERLAY, J. (2004) *GLOBOCAN 2002, Cancer Incidence, Mortality and Prevalence Worldwide,* Lyon, IACR press.

FERNANDES, A., HAMBURGER, A. W. & GERWIN, B. I. (1999) ErbB-2 kinase is required for constitutive stat 3 activation in malignant human lung epithelial cells. *International Journal of Cancer,* 83**,** 564-570.

FISHER, B., ANDERSON, S., REDMOND, C. K., WOLMARK, N., WICKERHAM, D. L. & CRONIN, W. M. (1995) Reanalysis and Results after 12 Years of Follow-up in a Randomized Clinical Trial Comparing Total Mastectomy with Lumpectomy with or without Irradiation in the Treatment of Breast Cancer. *New England Journal of Medicine,* 333**,** 1456-1461.

FISHER, B., REDMOND, C., FISHER, E. R. & CAPLAN, R. (1988) Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node negative breast cancer patients: findings from National Surgical Adjuvant Breast and Bowel Project Protocol B-06. *Journal of Clinical Oncology,* 6**,** 1076-87.

FOEHR, E. D., LIN, X., O'MAHONY, A., GELEZIUNAS, R., BRADSHAW, R. A. & GREENE, W. C. (2000) NF-ÎºB Signaling Promotes Both Cell Survival and Neurite Process Formation in Nerve Growth Factor-Stimulated PC12 Cells. *The Journal of Neuroscience,* 20**,** 7556-7563.

FOLCA, P. J., GLASCOCK, R. F. & IRVINE, W. T. (1961) Studies with tritium-labled Hexoestrol in advanced breast cancer. *Lancet,* 2**,** 796.

FORD, D., EASTON, D. F., BISHOP, D. T., NAROD, S. A. & GOLDGAR, D. E. (1994) Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Lancet,* 343**,** 692-5.

FORD, D., EASTON, D. F., STRATTON, M., NAROD, S., GOLDGAR, D., DEVILEE, P., BISHOP, D. T., WEBER, B., LENOIR, G., CHANG-CLAUDE, J., SOBOL, H., TEARE, M. D., STRUEWING, J., ARASON, A., SCHERNECK, S., PETO, J., REBBECK, T. R., TONIN, P., NEUHAUSEN, S., BARKARDOTTIR, R., EYFJORD, J., LYNCH, H., PONDER, B. A., GAYTHER, S. A. & ZELADA-HEDMAN, M. (1998) Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *American Journal of Human Genetics,* 62**,** 676-89.

FULFORD, L., REIS-FILHO, J., RYDER, K., JONES, C., GILLETT, C., HANBY, A., EASTON, D. & LAKHANI, S. (2007) Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival. *Breast Cancer Research,* 9**,** R4.

GAGO, F. E., FANELLI, M. A. & CIOCCA, D. R. (2006) Co-expression of steroid hormone receptors (estrogen receptor α and/or progesterone receptor) and HER2/neu (c-erbB-2) in breast cancer: Clinical outcome following tamoxifen based adjuvant therapy. *Journal of Steroid Biochemistry and Molecular Biology,* 98**,** 36-40.

GAGO, F. E. F., M.A. CIOCCA D.R. (2006) Co-expression of steroid hormone receptors (estrogen receptorα and/or progesterone receptor) and HER2/neu (c-erbB-2) in breast cancer: Clinical outcome following tamoxifen based adjuvant therapy. *Journal of Steroid Biochemistry and Molecular Biology,* 98**,** 36-40.

GARCIA, R., BOWMAN, T. L., NIU, G., YU, H., MINTON, S., MURO-CACHO, C. A., COX, C. E., FALCONE, R., FAIRCLOUGH, R., PARSONS, S., LAUDANO, A., GAZIT, A., LEVITZKI, A., KRAKER, A. & JOVE, R. (2001) Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene,* 20**,** 2499-513.

GEE, J. M., ROBERTSON, J. F., ELLIS, I. O. & NICHOLSON, R. I. (2001) Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. *Int J Cancer,* 95**,** 247-54.

GEE, J. M., ROBERTSON, J. F., GUTTERIDGE, E., ELLIS, I. O., PINDER, S. E., RUBINI, M. & NICHOLSON, R. I. (2005) Epidermal growth factor receptor / HER2/ insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. *Endocrine Related Cancer.,* 12**,** S99-S111.

GHERSI, D., WILCKEN, N. & SIMES, R. (2005) A systematic review of taxane-containing regimens for metastatic breast cancer. *Br J Cancer,* 93**,** 293-301.

GHOSH, S. & KARIN, M. (2002) Missing pieces in the NF-kappaB puzzle. *Cell,* 109 supp**,** S81-S96.

GOLDHIRSCH, A., WOOD, W. C., GELBER, R. D., COATES, A. S., THÃ¼RLIMANN, B. & SENN, H. J. (2007) Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. *Annals of Oncology,* 18**,** 1133-1144.

GOSS, P. E., INGLE, J. N. & MARINO, S. (2003) A randomized control trial of letrazole in post menopausal women after five years of tamoxifen therapy for early stage breast cancer. *N Engl J Med,* 349**,** 1793-1802.

GRAHAM, J. D. & CLARKE, C. L. (1997) the physiological action of progesterone in target tissues. *Endocrine Reviews,* 18**,** 502-519.

GRANN, V. R., TROXEL, A. B., ZOJWALLA, N. J., JACOBSON, J. S., HERSHMAN, D. & NEUGUT, A. I. (2005) Hormone receptor status and survival in a population-based cohort of patients with breast carcinoma. *Cancer,* 103**,** 2241-51.

GRANT, S. L., HAMMACHER, A., DOUGLAS, A. M., GOSS, G. A., MANSFIELD, R. K., HEATH, J. K. & BEGLEY, C. G. (2002) An unexpected biochemical and functional interaction between gp130 and the EGF receptor family in breast cancer cells. *Oncogene,* 21**,** 460-474.

GREGORY, H. (1975) Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature,* 257**,** 325-327.

GREY, H. (1999) *Grey's Anatomy thirty Eighth Edition.,* London, Churchill Livingstone.

GRIGRANDE, P. H., POLLIO, G. & MCDONNELL, D. P. (1997) Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. *Journal of Biological Chemistry.,* 272**,** 32889-32900.

GRITSKO, T., WILLIAMS, A., TURKSON, J., KANEKO, S., BOWMAN, T. L., HUANG, M., NAM, S., EWEIS, I., DIAZ, N., SULLIVAN, D., YODER, S., ENKERMANN, S., ESCHRICH, S., LEE, J. H., BEAM, C. A., CHENG, J., MINTON, S., MURO-CACHO, C. A. & JOVE, R. (2006a) Persistant activation of Stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. *Clinical Cancer Research,* 12**,** 11-19.

GRITSKO, T., WILLIAMS, A., TURKSON, J., KANEKO, S., BOWMAN, T. L., HUANG, M., NAM, S., EWEIS, I., DIAZ, N., SULLIVAN, D., YODER, S., ENKERMANN, S., ESCHRICH, S., LEE, J. H., BEAM, C. A., CHENG, J., MINTON, S., MURO-CACHO, C. A. & JOVE, R. (2006b) Persistant activation on Stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. *Clinical Cancer Research,* 12**,** 11-19.

HALL, R. E., LEE, C. S., ALEXANDER, I. E., SHINE, J., CLARKE, C. L. & SUTHERLAND, R. L. (1990) Steroid hormone receptor gene expression in human breast cancer cells: inverse relationship between oestrogen and glucocorticoid receptor messenger RNA levels. *International journal of cancer,* 46**,** 1081-1087.

HALSTED, W. S. (1898) A clinical and histological study of certain adenocarcinomata of the breast. *Annals of surgery,* 28**,** 557 - 576.

HARVEY, J. M., CLARK, G. M. & OSBORNE, C. K. (1999) Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assays for predicting response to adjvant therapy in breast cancer. *Journal of Clinical Oncology,* 17**,** 1474-1485.

HAYES, D. F., THOR, A. D., DRESSLER, L. G., WEAVER, D., EDGERTON, S., COWAN, D., BROADWATER, G., GOLDSTEIN, L. J., MARTINO, S., INGLE, J. N., HENDERSON, I. C., NORTON, L., WINER, E. P., HUDIS, C. A., ELLIS, M. J. & BERRY, D. A. (2007) HER2 and Response to Paclitaxel in Node-Positive Breast Cancer. *New England Journal of Medicine,* 357**,** 1496-1506.

HELBIG, G., CHRISTOPHERSON, K. W., 2ND, BHAT-NAKSHATRI, P., KUMAR, S., KISHIMOTO, H., MILLER, K. D., BROXMEYER, H. E. & NAKSHATRI, H. (2003) NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *J Biol Chem,* 278**,** 21631-8.

HELLE, M., HELIN, M., ISOLA, J. & HELIN, H. (1989) Immunohistochemical versus biochemical estrogen-receptor and progesterone-receptor analysis: correlation with histological parameters. *Journal of Cancer Research and Clinical Oncology,* 115**,** 361-365.

HOENTJEN, F., SARTOR, R. B., OZAKI, M. & JOBIN, C. (2005) STAT3 regulates NF-ÎºB recruitment to the IL-12p40 promoter in dendritic cells. *Blood,* 105**,** 689-696.

HORWITZ, K. B. K., Y. MCGUIRE, W.L. (1978) Estrogen control of progesterone receptor in human breast cancer. *Endocrinology,* 103**,** 1742-1751.

HU, Z., FAN, C., OH, D., MARRON, J. S., HE, X., QAQISH, B., LIVASY, C., CAREY, L., REYNOLDS, E., DRESSLER, L., NOBEL, A., PARKER, J., EWEND, M., SAWYER, L., WU, J., LIU, Y., NANDA, R., TRETIAKOVA, M., ORRICO, A., DREHER, D., PALAZZO, J., PERREARD, L., NELSON, E., MONE, M., HANSEN, H., MULLINS, M., QUACKENBUSH, J., ELLIS, M., OLOPADE, O., BERNARD, P. & PEROU, C. (2006) The molecular portraits of breast tumors are conserved across microarray platforms. *BMC Genomics,* 7**,** 96.

HUANG, H. J. N. P. D., M. PARIDAENS, R. WILDIERS, H. VAN LIMBERGEN, E. BERTELOOT, P. AMANT, F. VERGOTE, I. CHRISTIAENS, M.R. (2005) Hormone receptors do not predict the HER2/neu status in all age groups of women with operable breast cancer. *Annals of Oncology,* 16**,** 1755-1761.

HYMAN, E., KAURANIEMI, P., HAUTANIEMI, S., WOLF, M., MOUSSES, S., ROZENBLUM, E., RINGNE´R, M., SAUTER, G., MONNI, O., ELKAHLOUN, A., KALLIONIEMI, O.-P. & KALLIONIEMI, A. (2002) Impact of DNA Amplification on Gene Expression Patterns in Breast Cancer. *Cancer Research,* 62**,** 6240 - 6245.

ICHIBA, M., NAKAJIMA, K., YAMANAKA, Y., KIUCHI, N. & HIRANO, T. (1998) Autoregulation of the Stat3 gene through cooperation with a cAMP-responsive element binding protein. *Journal of Biological Chemistry.,* 273**,** 6132-6138.

IMAI, Y., LEUNG, C. K., FRIESEN, H. G. & SHIU, R. P. (1982a) Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long term tissue culture. *Cancer Research,* 42**,** 4394-4398.

INGLE, J., TU, D., PATER, J., MARTINO, S., ROBERT, N., MUSS, H., PICCART, M., CASTIGLIONE, M., SHEPHERD, L., PRITCHARD, K., LIVINGSTON, R., DAVIDSON, N., NORTON, L., PEREZ, E., ABRAMS, J., CAMERON, D., PALMER, M. & GOSS, P. (2006) Duration of letrozole treatment and outcomes in the placebo-controlled NCIC CTG MA.17 extended adjuvant therapy trial. *Breast Cancer REsearch and Treatment,* 99**,** 295-300.

INGRID, A. H., BO, B., ÅKE, B. & STINA, M. O. (1997) Activated cell cycle checkpoints in epirubicin-treated breast cancer cells studied by BrdUrd-flow cytometry. *Cytometry,* 29**,** 321-327.

ISAACS, C., PESHKIN, B. & SCHWARTZ, M. (2004) Evaluation and managment of women with a strong family history. IN J. HARRIS, M. L., M. MORROW, C. OSBORNE (Ed.) *Diseases of the Breast.* 3rd ed. Philadelphia, Lippincott Williams & Wilkins.

JACKSON, S. P., MACDONALD, J. J., LEES-MILLER, S. & TJIAN, R. (1990) GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. *Cell,* 63**,** 155-65.

JAIYESIMI, I. A., BUZDAR, A. U., DECKER, D. A. & HORTOBAGYI, G. N. (1995) Use of tamoxifen for breast cancer: twenty-eight years later. *Journal of Clinical Oncology,* 13**,** 513-529.

JENSEN, E. V. & JACOBSON, H. I. (1962) basic guide to the mechanism of estrogen action. *Recent Progress in Hormone Research,* 18**,** 387-414.

JIANG, N. Y., WODA, B. A., BANNER, B. F., WHALEN, G. F., DRESSER, K. A. & LU, D. (2008) Sp1, a new biomarker that identifies a sub-set of aggressive pancreatic ductal adenocarcinomas. *Cancer Epidemiol Biomarkers and Prevention,* 17**,** 1684-1652.

JOENSUU, H., KELLEKUMPU-LEHTINEN, P. L. & BONO, P. (2006) Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med,* 354**,** 809-820.

JOERGER, M. & THÜRLIMANN, B. (2009) Update of the BIG 1-98 Trial: Where do we stand? *The Breast,* 18**,** S78-S82.

JONAT, W., MAASS, H. & STEGNER, H. E. (1986) Immunohistochemical Measurement of Estrogen Receptors in Breast Cancer Tissue Samples. *Cancer Research,* 46**,** 4296s-4298s.

JORDAN, V. C. (1998) Antiestrogenic action of raloxifene and tamoxifen: today and tomorrow. *J Natl Cancer Inst,* 90**,** 967-71.

JUMPPANEN, M., GRUVBERGER-SAAL, S., KAURANIEMI, P., TANNER, M., BENDAHL, P.-O., LUNDIN, M., KROGH, M., KATAJA, P., BORG, A., FERNO, M. & ISOLA, J. (2007) Basal-like phenotype is not associated with patient survival in estrogen-receptor-negative breast cancers. *Breast Cancer Research,* 9**,** R16.

KADONAGA, J. T., CARNER, K. R., MASIARZ, F. R. & TJIAN, R. (1987) Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell,* 51**,** 1079-90.

KANDA, N., SENO, H., KONDA, Y., MARUSAWA, H., KANAI, M., NAKAJIMA, T., KAWASHIMA, T., NANAKIN, A., SAWABU, T., UENOYAMA, Y., SEKIKAWA, A., KAWADA, M., SUZUKI, K., KAYAHARA, T., FUKUI, H., SAWADA, M. & CHIBA, T. (2004) STAT3 is constitutively activated and supports cell survival in association with survivin expression in gastric cancer cells. *Oncogene,* 23**,** 4921-4929.

KARIN, M., CAO, Y., GRETEN, F. R. & LI, Z. W. (2002) NF-kappaB in cancer: from innocent bystander to major culprit. *Nature Reviews in Cancer.,* 2**,** 301-310.

KARLIN, J. D., NGUYEN, D., YANG, S. X. & LIPKOWITZ, S. (2005) Epidermal growth factor expression in breast cancer. *Journal of Clinical Oncology,* 23**,** 8118 - 8120.

KATO, S., ENDOH, H., MASUHIRO, Y., KITAMOTO, T., UCHIYAMA, S., SASAKI, H., MASUSHIGE, S., GOTOH, Y., NISHIDA, E., KAWASHIMA, H., METZGER, D. & CHAMBON, P. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science,* 270**,** 1491-4.

KATO, T., DUFFEY, D. C., ONDREY, F. G., DONG, G., CHEN, Z., COOK, J. A., MITCHELL, J. B. & VAN WAES, C. (2000) Cisplatin and radiation sensitivity in human head and neck squamous carcinomas are independently modulated by glutathione and transcription factor NF-κB. *Head & Neck,* 22**,** 748-759.

KAWAGOE, J., OHMICHI, M., TAKAHASHI, T., OHSHIMA, C., MABUCHI, S., TAKAHASHI, K., IGARASHI, H., MORI-ABE, A., SAITOH, M., DU, B., OHTA, T., KIMURA, A., KYO, S., INOUE, M. & KURACHI, H. (2003) Raloxifene inhibits estrogen-induced up-regulation of telomerase activity in a human breast cancer cell line. *J Biol Chem,* 278**,** 43363-72.

KHAN, S., ABDELRAHIM, M., SAMUDIO, I. & SAFE, S. (2003) Estrogen receptor/Sp1 complexes are required for induction of cad gene expression by 17beta-estradiol in breast cancer cells. *Endocrinology,* 144**,** 2325-35.

KHAN, S., WU, F., LUI, S., WU, Q. & SAFE, S. (2007) Role of specificity protein transcription factors in estrogen-induced gene expression in MCF-7 breast cancer cells. *Journal of Molecular Endocrinology,* 39**,** 289-304.

KIM, H. P., LEE, J. Y., JEONG, J. K., BAE, S. W., LEE, H. K. & JO, I. (1999) Non-genomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. *Biochem Biophys Res Commun,* 263**,** 257-62.

KING, M.-C., WIEAND, S., HALE, K., LEE, M., WALSH, T., OWENS, K., TAIT, J., FORD, L., DUNN, B. K., COSTANTINO, J., WICKERHAM, L., WOLMARK, N. & FISHER, B. (2001) Tamoxifen and Breast Cancer Incidence Among Women With Inherited Mutations in BRCA1 and BRCA2. *JAMA: The Journal of the American Medical Association,* 286**,** 2251-2256.

KINSEL, L. B., SZABO, E., GREENE, G. L., KONRATH, J., LEIGHT, G. S. & MCCARTY, K. S. (1989) Immunocytochemical Analysis of Estrogen Receptors as a Predictor of Prognosis in Breast Cancer Patients: Comparison with Quantitative Biochemical Methods. *Cancer Research,* 49**,** 1052-1056.

KIUCHI N, NAKAJIMA K, ICHIBA M, FUKADA T, NARIMATSU M, MIZUNO K, HIBI M & T, H. (1999) STAT3 Is Required for the gp130-mediated Full Activation of the c-myc Gene. *J Exp Med,* 189**,** 63-73.

KLIJN, J., BERNS, P. M. & SCHMITZ, P. L. (1992) The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review of 5232 patients. *Endocrine Review,* 13**,** 3-17.

KLIJN, J., LOOK, M. P., FISGUSCH, J., W., P. & FOEKENS, J. (1994) The prognostic value of epidermal growth factor receptor (EGF-R) in primary breast cancer: Results of a 10 year follow-up study. *Breast Cancer Research and Treatment,* 29**,** 73-83.

KLIJN, J. B., P.M. SCHMITZ, P.L. (1992) The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review of 5232 patients. *Endocrine Review,* 13**,** 3-17.

KLIJN, J. L., M.P. FISGUSCH, J. PUTTEN W. FOEKENS, J. (1994) The prognositic value of epidermal growth factor receptor (EGF-R) in primary breast cancer: Results of a 10 year follow-up study. *Breast Cancer Research and Treatment,* 29**,** 73-83.

KOJO, K., PUKKALA, E. & AUVINEN, A. (2005) Breast cancer risk among Finnish cabin attendants: a nested case-control study. *Occup Environ Med,* 62**,** 488-93.

KRUTZIK, P. O., IRISH, J. M., NOLAN, G. P. & PEREZ, O. D. (2004) Analysis of protein phosphorylation and cellular signalling events by flow cytometry: techniques and clinical applications. *Clinical Immunology,* 110**,** 206-221.

KUPIER, G. J. M., CARLSSON, B., ENMARK, E., HAGGBALD, J., NISON, S., GUSTAFSSON, J. (1997) Comparison of ligand binding specificity and trasncription tissue distribution of estrogen receptors alpha and beta. *Endocrinology,* 138**,** 863-870.

KUUKASJARVI, T., KONONEN, J. & HELIN, H. (1996) Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. *Jounal of Clinical Oncology,* 14**,** 2584-2589.

LARSEN, W. J. (2001) *Human Embryology,* New York, Churchill Livingstone.

LATCHMAN, D. S. (1997) Transcription factors: An overview. *The International Journal of Biochemistry & Cell Biology,* 29**,** 1305-1312.

LAUDET, V. (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *Journal of Molecular Endocrinology.,* 19**,** 207-226.

LEAKE, R., BARNES, D., SINDER, S., ELLIS, I. O., ANDERSON, L., ANDERSON, T., ADAMSON, R., RHODES, T., MILLER, K. & WALKER, R. (2000) Immunohistochemical detection of steroid receptor in breast cancer: a working protocol. *Journal of Clinical Pathology,* 53**,** 634-635.

LEE, M. Y., JOUNG, Y. H., LIM, E. J., PARK, J.-H., YE, S.-K., PARK, T., ZHANG, Z., PARK, D. K., LEE, K. J. & YANG, Y. M. (2006) Phosphorylation and activation of STAT proteins by hypoxia in breast cancer cells. *The Breast,* 15**,** 187-195.

LEUNG, G. M. (2002) Trends in breast cancer incidence in Hong Kong between 1973 and 1999: an age-period-cohort analysis. *British Journal of Cancer,* 87**,** 982 - 988.

LEVY, D. E. & DARNELL, J. E. (2002) STATs: Transcriptional control and biological impact. *National Review of Molecular Cellular Biology,* 3**,** 651-662.

LEWIS, G. D., FIGARI, I., FENDLY, B., LEE WONG, W., CARTER, P., GORMAN, C. & SHEPARD, H. M. (1993) Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies. *Cancer Immunology, Immunotherapy,* 37**,** 255-263.

LI, L. & SHAW, P. E. (2002) Autocrine-mediated activation of STAT3 correlates with cell proliferation in breast carcinoma lines. *J Biol Chem,* 277**,** 17397-405.

LI, W.-C., YE, S.-L., SUN, R.-X., LIU, Y.-K., TANG, Z.-Y., KIM, Y., KARRAS, J. G. & ZHANG, H. (2006) Inhibition of Growth and Metastasis of Human Hepatocellular Carcinoma by Antisense Oligonucleotide Targeting Signal Transducer and Activator of Transcription 3. *Clinical Cancer Research,* 12**,** 7140-7148.

LIN, S. Y., MAKINO, K., XIA, W., MARTIN, A., WEN, Y., KWONG, K. Y., BOURGUIGNON, L. Y. & HUNG, M. C. (2001) Nuclear localisation of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol,* 3**,** 802-808.

LO, H. W., HSU, S. C., ALI-SEYED, M., GUNDUZ, M., XIA, W., WEI, Y., BARTHOLOMEUZ, G., SHIH, J. Y. & HUNG, M. C. (2005a) Nuclear interaction of EGFR and STAT3 in the acivation of the iNOS/NO pathway. *Cancer Cell,* 7**,** 575-588.

LO, H. W., HSU, S. C., ALI-SEYED, M., GUNDUZ, M., XIA, W., WEI, Y., BARTHOLOMEUZ, G., SHIH, J. Y. & HUNG, M. C. (2005b) Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell,* 7**,** 575-588.

LO, H. W., HSU, S. C. & HUNG, M. C. (2006) EGFR signalling pathway in breast cancers: from traditional signal transduction to direct nuclear translocation. *Breast Cancer Research and Treatment,* 95**,** 211-218.

MACFARLANE R, SPEERS C, MASOUDI H & S., C. (2008) Molecular changes in the primary breast cancer versus the relapsed/metastatic lesion from a large population based database and tissue microarray series. *J Clin Oncol.,* 26**,** 1000. .

MANSEL, R. E., FALLOWFIELD, L., KISSIN, M., GOYAL, A., NEWCOMBE, R. G., DIXON, J. M., YIANGOU, C., HORGAN, K., BUNDRED, N., MONYPENNY, I., ENGLAND, D., SIBBERING, M., ABDULLAH, T. I., BARR, L., CHETTY, U., SINNETT, D. H., FLEISSIG, A., CLARKE, D. & ELL, P. J. (2006) Randomized Multicenter Trial of Sentinel Node Biopsy Versus Standard Axillary Treatment in Operable Breast Cancer: The ALMANAC Trial. *J. Natl. Cancer Inst.,* 98**,** 599-609.

MASUDA, M., SUZUI, M., YASUMATU, R., NAKASHIMA, T., KURATOMI, Y., AZUMA, K., TOMITA, K., KOMIYAMA, S. & WEINSTEIN, I. B. (2002) Constitutive Activation of Signal Transducers and Activators of Transcription 3 Correlates with Cyclin D1 Overexpression and May Provide a Novel Prognostic Marker in Head and Neck Squamous Cell Carcinoma. *Cancer Research,* 62**,** 3351-3355.

MATTISSON, I., WIRFALT, E., WALLSTROM, P., GULLBERG, B., OLSSON, H. & BERGLUND, G. (2004) High fat and alcohol intakes are risk factors of postmenopausal breast cancer: a prospective study from the Malmo diet and cancer cohort. *Int J Cancer,* 110**,** 589-97.

MAURER, G., LEUPOLD, J., SCHEWE, D., BILLER, T., KATES, R., HORNUNG, H., WERNER, U., POST, S. & ALLGAYER, H. (2007) Analysis of specific transcriptional regulators as early predictors on independent prognosis relevance in resected colorectal cancer. *Clinical Cancer Research,* 13**,** 1123-1132.

MCCLELLAND, R. A., BARROW, D., MADDEN, T. A., DUTKOWSKI, C. M., PAMMENT, J., KNOWLDEN, J. M., GEE, J. M. & NICHOLSON, R. I. (2001) Enhanced epidermal growth factor receptor signalling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex). *Endocrinology,* 142**,** 2776-88.

MCSHANE, L. M., ALTMAN, D. G., SAUERBREI, W., TAUBE, S. E., GION, M., CLARK, G. M. & FOR THE STATISTICS SUBCOMMITTEE OF THE, N. C. I. E. W. G. O. C. D. (2005) Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK). *Journal of the National Cancer Institute,* 97**,** 1180-1184.

MEDINA, P. J., GOODIN S. (2008) Lapatinib: a dual inhibitor of human epidermal growth factor receptor kinases. *Clinical Theraputics,* 30**,** 1426-1447.

MICHELI, A. (2002) Cancer prevalence in European registry areas. *Annals of Oncology,* 13**,** 840 -865.

MIGONE, T. S., LIN, J. X., CERESETO, A., MULLOY, J. C., O'SHEA, J. J., FRANCHINI, G. & LEONARD, W. J. (1995) Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. *Science,* 269**,** 79-81.

MILLA-SANTOS, A., MILLA, L., RALLO, L. & SOLANA, V. (2000) Anastrazole vs. tamoxifen in hormone dependent advanced breast cancer: a phase II randomized trial. *Breast Cancer Research Treatment,* 64**,** abstract 173.

MONTAGUT, C., TUSQUETS, I., FERRER, B., CORONMINAS, J. M., BELLSILLO, B., CAMPAS, C., SUAREZ, M., FABREGAT, X., CAMPO, E., GASCON, P., SERRANO, S., FERNANDEZ, P. L., ROVIRA, A. & ALBANELL, J. (2006a) Activation of nuclear factor êß is linked to neoadjuvant chemotherapy in breast cancer patients. *Endocrine Related Cancer.,* 13**,** 607-616.

MONTAGUT, C., TUSQUETS, I., FERRER, B., CORONMINAS, J. M., BELLSILLO, B., CAMPAS, C., SUAREZ, M., FABREGAT, X., CAMPO, E., GASCON, P., SERRANO, S., FERNANDEZ, P. L., ROVIRA, A. & ALBANELL, J. (2006b) Activation of nuclear factor kß is linked to neoadjuvant chemotherapy in breast cancer patients. *Endocrine Related Cancer.,* 13**,** 607-616.

MOORE, C. (1867) On the Influence of Inadequate operations on the theory of cancer. *Royal Medical and Chirugical Society London,* 1**,** 244-280.

NAGATA, C., KAWAKAMI, N. & SHIMIZU, H. (1997) Trends in the incidence rate and risk factors for breast cancer in Japan. *Breast Cancer REsearch and Treatment,* 44**,** 75 - 82.

NAKSHATRI, H., BHAT-NAKSHATRI, P., MARTIN, D. A., GOULET, R. J., JR. & SLEDGE, G. W., JR. (1997) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol,* 17**,** 3629-39.

NATOLI, C., SICA, G., NATOLI, V., SERRA, A. & IACOBELLI, S. (1983) Two new estrogen-supersensitive variants of the MCF-7 human breast cancer cell line. *Breast Cancer Research and Treatment,* 3**,** 23-32.

NIETO, Y., NAWAZ, F., JONES, R., SHPALL, E., NAWAZ, S. (2007a) Prognostic significance of over-expression and phosphorylation of epidermal growth factor receptor (EGFR) and the presence of truncated EGFRvIII in locoregionally advanced breast cancer. *Journal of Clinical Oncology,* 25**,** 4405-4413.

NIETO, Y., NAWAZ, F., JONES, R., SHPALL, E., NAWAZ, S. (2007b) Prognostic significance of over-expression and phosphorylation of epidermal growth factor receptor (EGFR) and the presence of truncated EGFRvIII in loco-regionally advanced breast cancer. *Journal of Clinical Oncology,* 25**,** 4405-4413.

NILSSON, S., MAKELA, S. & TREUTER, E. (2001) Mechanisms of estrogen action. *Physiological Reviews,* 81**,** 1535-1565.

O'LONE, R., FRITH, M. C., KARLSSON, E. K. & HANSEN, U. (2004) Genomic targets of nuclear estrogen receptors. *Mol Endocrinol,* 18**,** 1859-75.

PACE, P., TAYLOR, J., SUNTHARALINGHAM, S., COOMBES, R. C. & ALI, S. (1997) Human estrogen receptor beta binds in a manner similar to and dimerizes with ER alpha. *Journal of Biological Chemistry.,* 272**,** 25832-25838.

PAHL, H. L. (1999) Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene,* 18**,** 6853-66.

PAWLOWSKI, V., REVILLION, F., HEBBAR, M., HORNEZ, L. & PEYRAT, J.-P. (2000) Prognostic value of Type I Growth Factor Receptors in a large series and human primary breast cancers quantified with a real time reverse transcription polymerase chain reaction. *Clinical Cancer Research,* 6**,** 4217-4225.

PAWLOWSKI, V. R., F. HEBBAR, M. HORNEZ, L. PEYRAT, J-P. (2000) Prognostic value of Type I Growth Factor Receptors in a large series and human primary breast cancers quantified with a real time reverse transcription polymerase chain reaction. *Clinical Cancer Research,* 6**,** 4217-4225.

PAYNE, S. J., BOWEN, R. L., JONES, J. L. & WELLS, C. A. (2008) Predictive markers in breast cancer - the present. *Histopathology,* 52**,** 82-90.

PAYNE, S. J. B., R.L. JONES, J.L WELLS, C.A. (2008) Predictive markers in breast cancer - the present. *Histopathology,* 52**,** 82-90.

PEROU, C. M., SORLIE, T., EISEN, M. B., VAN DE RIJN, M., JEFFREY, S. S., REES, C. A., POLLACK, J. R., ROSS, D. T., JOHNSEN, H., AKSLEN, L. A., FLUGE, O., PERGAMENSCHIKOV, A., WILLIAMS, C., ZHU, S. X., LONNING, P. E., BORRESEN-DALE, A.-L., BROWN, P. O. & BOTSTEIN, D. (2000) Molecular portraits of human breast tumours. *Nature,* 406**,** 747-752.

PFITZNER E, KLIEM S, BAUS D & CM., L. (2004) The role of STATs in inflammation and inflammatory diseases. *Curr Pharm Dec,* 10**,** 2839-50.

POMPE-KIRN, V., JAPELJ, B. & PRIMIC-ZAKELJ, M. (2000) Future trends in breast, cervical, lung, mouth and pharyngeal cancer incidence in Slovenia. *Cancer Causes Control,* 11**,** 309 - 318.

POOLE, C. J., EARL, H. M., HILLER, L., DUNN, J. A., BATHERS, S., GRIEVE, R. J., SPOONER, D. A., AGRAWAL, R. K., FERNANDO, I. N., BRUNT, A. M., O'REILLY, S. M., CRAWFORD, S. M., REA, D. W., SIMMONDS, P., MANSI, J. L., STANLEY, A., HARVEY, P., MCADAM, K., FOSTER, L., LEONARD, R. C. F. & TWELVES, C. J. (2006) Epirubicin and Cyclophosphamide, Methotrexate, and Fluorouracil as Adjuvant Therapy for Early Breast Cancer. *New England Journal of Medicine,* 355**,** 1851-1862.

PORTER, W., SAVILLE, B., HOIVIK, D. & SAFE, S. (1997) Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol Endocrinol,* 11**,** 1569-80.

PRITCHARD, K. I. S., L.E. O'MALLEY, F. P. ANDRULIS, I. L. TU, D. BRAMWELL, V. H. MARK, N. (2006) HER2 and responsiveness of breast cancer to adjuvant chemotherapy. *N Engl J Med,* 354**,** 2103-2111.

PROWELL, T. M. A., D.K. (2006) Selecting endocrine therapy for breast cancer: what role does HER-2/neu status play? *Seminars in Oncology,* 33**,** 681-687.

PTASHNE, M. & GANN, A. (1997) Transcriptional activation by recruitment. *Nature,* 386**,** 569-577.

PURDIE, C. A., BAKER, L., ASHFIELD, A., CHATTERJEE, S., JORDAN, L. B., QUINLAN, P., ADAMSON, D. J. A., DEWAR, J. A. & THOMPSON, A. M. (2010) Increased mortality in HER2 positive, oestrogen receptor positive invasive breast cancer: a population-based study. *Br J Cancer,* 103**,** 475-481.

QIAN, L., CHEN, L., SHI, M., YU, M., JIN, B., HU, M., XIA, Q., ZHANG, X., SHEN, B. & GUO, N. (2006) A novel cis-acting element in Her2 promoter regulated by Stat3 in mammary cancer cells. *Biochemical & Biophysical Research Communications,* 345**,** 660-668.

RAKHA, E. A., EL-REHIM, D. A., PAISH, C., GREEN, A. R., LEE, A. H. S., ROBERTSON, J. F., BLAMEY, R. W., MACMILLAN, D. & ELLIS, I. O. (2006) Basal phenotype identifies a poor prognostic subgroup of breast cancer of clinical importance. *European Journal of Cancer,* 42**,** 3149-3156.

RAMPAUL, R. S., PINDER, S.E., NICHOLSON, R.L., (2005) Clinical value of epidermal growth factor expression in primary breast cancer. *Advances in Anatomical Pathology.,* 12**,** 271-273.

RAVDIN, P. M., GREEN, S. & DORR, T. M. (1992) Prognostic significance of progesterone receptor levels in estrogen receptor positive patients with metastatic breast cancer treated with tamoxifen; results of a prospective Southwest oncology group study. *Journal of Clinical Oncology,* 10**,** 1284-1291.

RAZANDI, M., OH, P., PEDRAM, A., SCHNITZER, J. & LEVIN, E. R. (2002) ERs associate with and regulate the production of caveolin: implications for signalling and cellular actions. *Mol Endocrinol,* 16**,** 100-15.

RAZANDI, M., PEDRAM, A., PARK, S. T. & LEVIN, E. R. (2003) Proximal events in signalling by plasma membrane estrogen receptors. *J Biol Chem,* 278**,** 2701-12.

REAL, P. J., SIERRA, A., DE JUAN, A., SEGOVIA, J. C., LOPEZ-VEGA, J. M. & FERNANDEZ-LUNA, J. L. (2002) Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. *Oncogene,* 21**,** 7611-7618.

REBBECK, T. R., FRIEBEL, T., LYNCH, H. T., NEUHAUSEN, S. L., VAN Â'T VEER, L., GARBER, J. E., EVANS, G. R., NAROD, S. A., ISAACS, C., MATLOFF, E., DALY, M. B., OLOPADE, O. I. & WEBER, B. L. (2004) Bilateral Prophylactic Mastectomy Reduces Breast Cancer Risk in BRCA1 and BRCA2 Mutation Carriers: The PROSE Study Group. *journal of clinical oncology,* 22**,** 1055-1062.

REBBECK, T. R., KAUFF, N. D. & DOMCHEK, S. M. (2009) Meta-analysis of Risk Reduction Estimates Associated With Risk-Reducing Salpingo-oophorectomy in BRCA1 or BRCA2 Mutation Carriers. *Journal of the National Cancer Institute*.

REES, M. (1995) The age of menarche. *Orgyn***,** 2-4.

REINER, A., NEUMEISTER, B., SPONA, J., REINER, G., SCHEMPER, M. & JAKESZ, R. (1990) Immunocytochemical localisation of estrogen and progesterone receptor and prognosis in human primary breast cancer. *Cancer Research,* 50**,** 7057-7061.

RHODES, A., JASANI, B., ANDERSON, E., DODSON, A. R. & BALATON, A. J. (2002) Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumours. *American Journal of Clinical Pathology,* 118**,** 408-417.

RICCA, A., BIROCCIO, A., DEL BUFALO, D., MACKAY, A. R., SANTONI, A. & CIPPITELLI, M. (2000) bcl-2 over-expression enhances NF-kappaB activity and induces mmp-9 transcription in human MCF7(ADR) breast-cancer cells. *Int J Cancer,* 86**,** 188-96.

RIGGINS, R. B., ZWART, A., NEHRA, R. & CLARKE, R. (2005) The nuclear factor kappa B inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells. *Mol Cancer Ther,* 4**,** 33-41.

RING, A. & DOWSETT, M. (2004) Mechanisms of tamoxifen resistance. *Endocrine-Related Cancer,* 11**,** 643-658.

ROBERT, S. A., STROMBOM, I., TRENTHAM-DIETZ, A., HAMPTON, J. M., MCELROY, J. A., NEWCOMB, P. A. & REMINGTON, P. L. (2004) Socioeconomic risk factors for breast cancer: distinguishing individual- and community-level effects. *Epidemiology,* 15**,** 442-50.

ROMANO D., B. E., BORACCHI P., GRECO M., RETSKY M. W. (2008) Recurrence dynamics does not depend upon the recurrence site. *Breast Cancer Research,* 10.

ROMASHKOVA, J. A. & MAKAROV, S. S. (1999) NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature,* 401**,** 86-90.

ROMOND, E. H., PEREZ, E. A. & BRYANT, J. (2005) Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med,* 353**,** 1673-1684.

ROUZIER, R., PEROU, C. M., SYMMANS, W. F., IBRAHIM, N., CRISTOFANILLI, M., ANDERSON, K., HESS, K. R., STEC, J., AYERS, M., WAGNER, P., MORANDI, P., FAN, C., RABIUL, I., ROSS, J. S., HORTOBAGYI, G. N. & PUSZTAI, L. (2005) Breast Cancer Molecular Subtypes Respond Differently to Preoperative Chemotherapy. *Clinical Cancer Research,* 11**,** 5678-5685.

SÃ¸RLIE, T., PEROU, C. M., TIBSHIRANI, R., AAS, T., GEISLER, S., JOHNSEN, H., HASTIE, T., EISEN, M. B., VAN DE RIJN, M., JEFFREY, S. S., THORSEN, T., QUIST, H., MATESE, J. C., BROWN, P. O., BOTSTEIN, D., LÃ¸NNING, P. E. & BÃ¸RRESEN-DALE, A.-L. (2001) Gene expression patterns of breast carcinomas distinguish tumour subclasses with clinical implications. *Proceedings of the National Academy of Sciences,* 98**,** 10869-10874.

SAFE, S. & ABDELRAHIM, M. (2005) Sp transcription factor family and its role in cancer. *European Journal of Cancer,* 41**,** 2438-2448.

SAFFER, J. D., JACKSON, S. P. & ANNARELLA, M. B. (1991) Developmental expression of Sp1 in the mouse. *Mol. Cell. Biol.,* 11**,** 2189-2199.

SAVILLE, B., WORMKE, M., WANG, F., NGUYEN, T., ENMARK, E., KUIPER, G., GUSTAFSSON, J. A. & SAFE, S. (2000) Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *J Biol Chem,* 275**,** 5379-87.

SCHECTER, A. C., STERN, B. F. & VAIDYANATHAN, L. (1984) The neu oncogene: An erb-B related gene encoding a 185,000Mr tumour antigen,. *Nature,* 312**,** 513-516.

SCHINZINGER, A. (1889) Uber Carcinoma Mammae. *Zentralbl Chir,* 16**,** 55-56.

SCHNITT S.J., G. A. J. (2004) Pathology of invasive breast cancer. IN HARRIS J., L. M., MORROW M., OSBORNE C. (Ed.) *Diseases of the Breast.* third ed. Philadelphia, Lippincott Williams & Wilkins.

SCHNITT, S. J. & GUIDI, A. J. (2004) Pathology of invasive breast cancer. IN HARRIS J., L. M., MORROW M., OSBORNE C. (Ed.) *Diseases of the Breast.* third ed. Philadelphia, Lippincott Williams & Wilkins.

SCHULTZ, J. R., PETZ, L. N. & NARDULLI, A. M. (2003a) Estrogen receptor alpha and Sp1 regulate progesterone receptor gene expression. *Mol Cell Endocrinol,* 201**,** 165-75.

SCHULTZ, J. R., PETZ, L. N. & NARDULLI, A. M. (2003b) Estrogen receptor alpha and Sp1 regulate progesterone receptor gene expression. *Mol Cell Endocrinol,* 201**,** 165-7.

SELANDER, K. S., LI, L., WATSON, L., MERRELL, M., DAHMEN, H., HEINRICH, P. C., MULLER-NEWEN, G. & HARRIS, K. W. (2004a) Inhibition of gp130 Signalling in Breast Cancer Blocks Constitutive Activation of Stat3 and Inhibits in Vivo Malignancy

10.1158/0008-5472.CAN-03-2516. *Cancer Res,* 64**,** 6924-6933.

SELANDER, K. S., LI, L., WATSON, L., MERRELL, M., DAHMEN, H., HEINRICH, P. C., MULLER-NEWEN, G. & HARRIS, K. W. (2004b) Inhibition of gp130 Signalling in Breast Cancer Blocks Constitutive Activation of Stat3 and Inhibits in Vivo Malignancy

10.1158/0008-5472.CAN-03-2516. *Cancer Res,* 64**,** 6924-6933.

SETHI, G., SUNG, B. & AGGARWAL, B. B. (2008) Nuclear Factor-{kappa}B Activation: From Bench to Bedside. *Exp. Biol. Med.,* 233**,** 21-31.

SHI, Q., LE, X., ABBRUZZESE, J. L., PENG, Z., QIAN, C.-N., TANG, H., XIONG, Q., WANG, B., LI, X.-C. & XIE, K. (2001) Constitutive Sp1 Activity Is Essential for Differential Constitutive Expression of Vascular Endothelial Growth Factor in Human Pancreatic Adenocarcinoma. *Cancer Research,* 61**,** 4143-4154.

SINGLETARY, S. E. (2002) Surgical margins in patients with early-stage breast cancer treated with breast conservation therapy. *American journal of surgery,* 184**,** 383-393.

SJORGEN, S., INGANAS, M., LINDGREN, A., HOLMBERG, L. & BERGH, J. (1998) Prognostic and predictive value of c-erbB-2 over expression in primary breast cancer, alone and in combination with other prognostic markers. *Journal of Clinical Oncology,* 16**,** 462-469.

SJORGEN, S. I., M. LINDGREN, A. HOLMBERG, L. BERGH, J. (1998) Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. *Journal of Clinical Oncology,* 16**,** 462-469.

SLAMON, D. J., LEYLAND-JONES, B. & SHAK, S. (2001) Use of chemotherapy plus a monclonal antibody directed against HER2 for metastatic breast cancer that over-expresses HER2. *N. Engl. J. Med.,* 344**,** 809-820.

SMITH, C. L. & TOFT, D. O. (1993) Steroid receptors and their associated proteins. *Molecular Endocrinology,* 4**,** 4-11.

SMITH, I., PROCTOR, M. & GELBER, R. D. (2007) 2-year follow-up of trastuzumab following adjuvant chemotherapy in HER2-positive breast cancer: a randomised control trial. *Lancet,* 369**,** 29-36.

SONG, H., JIN, X. & LIN, J. (2004) Stat3 upregulates MEK5 expression in human breast cancer cells. *Oncogene,* 23**,** 8301-9.

SONG, J. L. & GRANDIS, J. R. (2000) STAT signalling in head and neck cancer. *Oncogene,* 19**,** 2489-2495.

SOULE, H. D., VASQUEZ, J., LONG, A., ALBERT, S., BRENNEN, M. (1973) A Human Cell Line From A Pleural Effusion Derived From A Breast Cancer. *Journal of the National Cancer Institute,* 51**,** 5421-5424.

SPEIRS, V., MALONE, C., WALTON, D. S., KERIN, M. J. & ATKIN, S. L. (1999) Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res,* 59**,** 5421-4.

SPEIRS, V., MALONE, C., WALTON, D. S., KERIN, M. J., ATKIN, S. L. (1999) Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res,* 59**,** 5421-4.

STATISTICS, O. F. N. (2005) Cancer Statistics registrations: Registrations of cancer diagnosed in 2002, England. *MB1 no 33.* London., National Statistics.

STATISTICS, O. O. N. (2008) Cancer Statistics Registrations; Registrations of cancers diagnosed in 2005. London, National Statistics.

STEWART, H. J. (1991) Adjuvant systemic therapy for operable breast cancer. *Br Med Bull,* 47**,** 343-356.

STOICA, G. E., FRANKE, T. F., MORONI, M., MUELLER, S., MORGAN, E., IANN, M. C., WINDER, A. D., REITER, R., WELLSTEIN, A., MARTIN, M. B. & STOICA, A. Effect of estradiol on estrogen receptor-[alpha] gene expression and activity can be modulated by the ErbB2//PI 3-K//Akt pathway. *Oncogene,* 22**,** 7998-8011.

STOUTJESDIJK, M. J., BOETES, C., JAGER, G. J., BEEX, L., BULT, P., HENDRIKS, J. H. C. L., LAHEIJ, R. J. F., MASSUGER, L., VAN DIE, L. E., WOBBES, T. & BARENTSZ, J. O. (2001) Magnetic Resonance Imaging and Mammography in Women With a Hereditary Risk of Breast Cancer. *Journal of the National Cancer Institute,* 93**,** 1095-1102.

STRUEWING, J., HARTGE, P., WACHOLDER, S., BAKER, S. M., BERLIN, M., MCADAMS, M., TIMMERMAN, M. M., BRODY, L. C. & TUCKER, M. A. (1997) The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *New England Journal of Medicine,* 336**,** 1401-8.

SWEENEY, C., BLAIR, C. K., ANDERSON, K. E., LAZOVICH, D. & FOLSOM, A. R. (2004) Risk factors for breast cancer in elderly women. *Am J Epidemiol,* 160**,** 868-75.

TAKEDA, K., NOGUCHI, K., SHI, W., TANAKA, T., MATSUMOTO, M., YOSHIDA, N., KISHIMOTO, T. & AKIRA, S. (1997) Targeted disruption of the mouse STAT3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A,* 94**,** 3801-3804.

TAYLOR, I. W., HODSON, P. J., GREEN, M. D. & SUTHERLAND, R. L. (1983) Effects of Tamoxifen on Cell Cycle Progression of Synchronous MCF-7 Human Mammary Carcinoma Cells. *Cancer Res,* 43**,** 4007-4010.

TEHARD, B. & CLAVEL-CHAPELON, F. (2006) Several anthropometric measurements and breast cancer risk: results of the E3N cohort study. *Int J Obes (Lond),* 30**,** 156-63.

TEHARD, B., FRIEDENREICH, C. M., OPPERT, J. M. & CLAVEL-CHAPELON, F. (2006) Effect of physical activity on women at increased risk of breast cancer: results from the E3N cohort study. *Cancer Epidemiol Biomarkers Prev,* 15**,** 57-64.

TERRY, M. B., ZHANG, F. F., KABAT, G., BRITTON, J. A., TEITELBAUM, S. L., NEUGUT, A. I. & GAMMON, M. D. (2006) Lifetime alcohol intake and breast cancer risk. *Ann Epidemiol,* 16**,** 230-40.

TREMPE, G. L. (1976) Human breast cancer in culture. *Recent Results Cancer Research,* 21**,** 33-41.

TUBBS, R., HICKS G, COOK J, DOWNS-KELLY E, PETTAY J, HARTKE M, HOOD L, NEELON R, MYLES J, BUDD GT, MOORE HC, ANDRESEN S & JP., C. (2007) Fluorescence in situ hybridization (FISH) as primary methodology for the assessment of HER2 Status in adenocarcinoma of the breast: a single institution experience.

. *Diagnostic Mol Patholo.,* 16**,** 207-2010.

TURKSON, J. & JOVE, R. (2000) STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene,* 19**,** 6613-26.

VAN LAERE, S. J., VAN DER AUWERA, I., VAN DEN EYNDEN, G. G., VAN DAM, P., VAN MARCK, E. A., VERMEULEN, P. B. & DIRIX, L. Y. (2007a) NFêß activation in inflammatory breast cacner is associated with oestrogen receptor downregulation, secondary to EGFR and/or ErbB2 overexpression and MAPK hyperactivation. *British Journal of Cancer.,* 97**,** 659-669.

VAN LAERE, S. J., VAN DER AUWERA, I., VAN DEN EYNDEN, G. G., VAN DAM, P., VAN MARCK, E. A., VERMEULEN, P. B. & DIRIX, L. Y. (2007b) NFkß activation in inflammatory breast cancer is associated with oestrogen receptor down-regulation, secondary to EGFR and/or ErbB2 over expression and MAPK hyperactivation. *British Journal of Cancer.,* 97**,** 659-669.

WALKER, R. A. (2008) Immunohistochemical markers as predictive tools for breast cancer. *Journal of Clinical Pathology,* 61**,** 689-696.

WALKER, R. A., BARTLETT, J. M. S., DOWSETT, M., ELLIS, I. O., HANBY, A. M., JASANI, B., MILLER, K. & PINDER, S. E. (2008) HER2 testing in the UK: further update to recommendations. *Journal of Clinical Pathology,* 61**,** 818-824.

WANG, L., GUAN, X., GONG, W., YAO, J., PENG, Z., WEI, D., WU, T. T., HUANG, S. & XIE, K. (2005) Altered expression of transcription factor Sp1 critically impacts the angiogenic phenotype of human gastric cancer. *Clinical and Experimental Metastasis,* 22**,** 205-213.

WANG, L., WEI, D., HUANG, S., PENG, Z., LE, X., WU, T. T., YAO, J., AJANI, J. & XIE, K. (2003) Transcription Factor Sp1 Expression Is a Significant Predictor of Survival in Human Gastric Cancer. *Clinical Cancer Research,* 9**,** 6371-6380.

WANG, L. H., YANG, X. Y., MILHALIC, K., XIAO, W., LI, D. & FARRAR, W. L. (2001) Activation of estrogen receptor blocks interleukin-6-inducible cell growth of human multiple myloma involving molecular cross-talk between estrogen receptor and STAT3 mediated by co-regulator PIAS3. *Journal of Biological Chemistry.,* 276**,** 31839-31844.

WANG, Z., JIANG, B. & BRECHER, P. (2002) Selective inhibition of STAT3 phosphorylation by sodium salicylate in cardiac fibroblasts. *Biochemical Pharmacology,* 63**,** 1197-1207.

WANG, Z., ZHANG, X., SHEN, P., LOGGIE, B. W., CHANG, Y. & DEUEL, T. F. (2006) A variant of estrogen receptor-alpha, hER-Alpha 36: Transduction of estrogen and anti-estrogen-dependant membrane-initiated mitogenic signalling. *Proceedings of the National Academy of Sciences USA,* 103**,** 9063-9068.

WATANABE, N., OKOCHI, E., MOCHIZUKI, M., SUGIMURA, T. & USHIJIMA, T. (2001) The Presence of Single Nucleotide Instability in Human Breast Cancer Cell Lines. *Cancer Research,* 61**,** 7739-7742.

WEN, Z., ZHONG, Z. & DARNELL, J. E. (1995) Maximal activation of STAT1 and STAT3 requires both tyrosine and serine phosphorylation. *Cell,* 82**,** 241-250.

WHEATER, P. R. (2000) *Wheater's Functional Histology.,* London, Churchill Livingstone.

WIDSCHWENDTER, A., TONKO-GEYMAYER, S., WELTE, T., DAXENBICHLER, G., MARTH, C. & DOPPLER, W. (2002) Prognostic significance of signal transducer and activator of transcription 1 activation in breast cancer. *Clin Cancer Res,* 8**,** 3065-74.

WITTON, C. J. R., J.R. GOING, J.J. COOKE, T.G. BARTLETT, J.M. (2003) Expression of the HER1-4 family of tyrosine kinase in breast cancer. *Journal of Pathology,* 200**,** 290-297.

WOLFF, A. C., HAMMOND, E. H., JARED, N., SCHWARTZ, K. L., HAGERTY, D., ALLRED, C., COTE, R. J., DOWSETT, M., FITZGIBBONS, P. L., HANNA, W. M., LANGER, A., MCSHANE, L. M., PAIK, S., PEGRAM, M. D., PEREZ, E. A., PRESS, M. F., RHODES, A., STURGEON, C., TAUBE, S. E., TUBBS, R., VANCE, G. H., VAN DE VIJVER, M., WHEELER, T. M. & HAYES, D. F. (2007) American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. *Journal of Clinical Oncology,* 25**,** 118-145.

WONG, C. W., MCNALLY, C., NICKBARG, E., KOMM, B. S. & CHESKIS, B. J. (2002a) Estrogen receptor-interacting protein that modulates its non-genomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci U S A,* 99**,** 14783-8.

WONG, C. W., MCNALLY, C., NICKBARG, E., KOMM, B. S. & CHESKIS, B. J. (2002b) Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci U S A,* 99**,** 14783-8.

XIE, T.-X., WEI, D., LIU, M., GAO, A. C., ALI-OSMAN, F., SAWAYA, R. & HUANG, S. Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. *Oncogene,* 23**,** 3550-3560.

YAMAMOTO, T., MATSUDA, T., JUNICHO, A., KISHI, H., SAATCIOGLU, F. & MURAGUCHI, A. (2000a) Cross-talk between signal transducer and activator of transcription 3 and estrogen receptor signalling. *FEBS Lett,* 486**,** 143-8.

YAMAMOTO, T., MATSUDA, T., JUNICHO, A., KISHI, H., SAATCIOGLU, F. & MURAGUCHI, A. (2000b) Cross-talk between signal transducer and activator of transcription 3 and estrogen receptor signalling. *FEBS Lett,* 486**,** 143-8.

YAMAUCHI, H., STEARNS, V. & HAYES, D. F. (2001) When is a tumour marker ready for prime time? A case study of c-erbB-2 as a predictive factor in breast cancer. *Journal of Clinical Oncology,* 19**,** 2334-2356.

YAZIJI, H., GOLDSTEIN, L. C., BARRY, T. S., WERLING, R., HWANG, H., ELLIS, G. K., GRALOW, J. R., LIVINGSTON, R. B. & GOWN, A. M. (2004) HER-2 Testing in Breast Cancer Using Parallel Tissue-Based Methods. *JAMA: The Journal of the American Medical Association,* 291**,** 1972-1977.

YEH, Y. T., YANG, F. O., CHEN, I. F., YANG, S. F., WANG, Y. Y., CHUANG, H. Y., SU, J. H., HOU, M. F. & S.F., Y. (2006a) STAT3 ser727 phosphorylation and its association with negative estrogen receptor status in breast infiltrating ductal carcinoma. *International Journal of Cancer,* 118**,** 2943-2947.

YEH, Y. T., YANG, F. O., CHEN, I. F., YANG, S. F., WANG, Y. Y., CHUANG, H. Y., SU, J. H., HOU, M. F. & S.F., Y. (2006b) STAT3 ser727 phosphorylation and its association with negative estrogen receptos status in breast infilatrating ductal carcinoma. *International Journal of Cancer,* 118**,** 2943-2947.

YOSHIDA T, HANADA T, TOKUHISA T, KOSAI K, SATA M, KOHARA M & A, Y. (2002) Activation of STAT3 by the Hepatitis C Virus Core Protein Leads to Cellular Transformation. *J Exp Med,* 195**,** 641-653.

YU, H. & JOVE, R. (2004) The STATs of cancer - new molecular targets come of age. *National Review of Cancer.,* 4**,** 97-105.

ZACHARIAS, L. & SCHATZOFF, M. (1970) Sexual maturation in contemporary American girls. *American Journal of Obstetrics and Gynaecology*
108**,** 833-846.

ZANNETTI, A., DEL VECCHIO, S., CARRIERO, M. V., FONTI, R., FRANCO, P., BOTTI, G., DÂ€™AIUTO, G., STOPPELLI, M. P. & SALVATORE, M. (2000) Coordinate Up-Regulation of Sp1 DNA-binding Activity and Urokinase Receptor Expression in Breast Carcinoma. *Cancer Research,* 60**,** 1546-1551.

ZEILLINGER, R., KURY, F., SPEISER, P., SLIUTZ, G., CZERWENKA, K. & KUBISTA, E. (1993) EGF-R and Steroid receptors in breast cancer; A comparison with tumour grading, tumour size, lymph node involvement and age. *Clinical Biochemistry,* 26**,** 221-227.

ZEILLINGER, R. K., F. SPEISER, P. SLIUTZ, G. CZERWENKA, K. KUBISTA, E. (1993) EGF-R and Steroid receptors in breast cancer; A comparasion with tumout grading, tumour size, lymph node involvement and age. *Clinical Biochemistry,* 26**,** 221-227.

ZENON RATER, J. M. (2003) *Medical Therapy of Breast Cancer,* Cambridge, Cambridge Univerity Press.

ZHANG, J., ZHU, Z. G., JI, J., YUAN, F., YU, Y., LIU, B. & LIN, Y. (2005a) Tramscripton factor Sp1 expresion in gastric cancer and its relationship to long term prognosis. *World Journal of Gasroenterology,* 11**,** 2213-2217.

ZHANG, J., ZHU, Z. G., JI, J., YUAN, F., YU, Y., LIU, B. & LIN, Y. (2005b) Transcription factor Sp1 expression in gastric cancer and its relationship to long term prognosis. *World Journal of Gastroenterology,* 11**,** 2213-2217.

ZHONG, Z., WEN, Z. & DARNELL, J. E. (1994) Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science,* 264**,** 95-98.

ZHOU, Y., EPPENBERGER-CASTORI, S., MARX, C., YAU, C., SCOTT, G. K., EPPENBERGER, U. & BENZ, C. C. (2005) Activation of nuclear factor êß (NFêß) identifies a high -risk subset of hormone-dependent breast cancers. *International Journal of Biochemistry and Cell Biology,* 37**,** 1130-1144.

ZHOU, Y., YAU, C., GRAY, J. W., CHEW, K., DAIRKEE, S. H., MOORE, D. H., EPPENBERGER, U., EPPENBERGER-CASTORIA, S. & BENZ, C. C. (2007) Enhanced NFêß and AP-1 transcriptional activity associated with anti-estrogen resistant breast cancer. *BMC Cancer,* 7**,** 4880-4895.

ZIEGLER, R. G. (1993) Migration patterns and breast cancer risk in Asian-American women. *Journal of the National Cancer Institute,* 85**,** 1819 - 27.

ZOLL, B., KYNAST, B. & CORRELL, B. (1992) Alterations of the c-erbB2 gene in human breast cancer. *Journal of Cancer Research and Clinical Oncology.,* 118**,** 468-473.

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This thesis is dedicated to my wonderful wife Stephanie whose support I relied on throughout the past 4 years. Without this support I would have never have completed this project.

Appendix.

Contents

Sample size/power calculation formulae

T Chadwick 20/03/09

I have revised the calculation to look for the power available to detect the observed differences rather than what could be obtained with 80% power as this is a calculation made after the experiment.

To illustrate we consider the alpha-ostrogen receptor with a sample group of 140 patients :

70% of the sample is positive on this (of which approximately 83% survive) and 30% are negative.

We need to define the following terms:

Group 1: Postive

m – sample size in group (98 [70% of 140])

p1 – survival proportion in group (0.83 – taken as the [rounded] midpoint of the range provided)

Group 2: Negative

n – sample size in group (42)

p2 – survival (0.68)

General

phi = n/m (i.e. the ratio of group sizes = 3/7 = 0.429)

delta = abs(p2-p1) (the absolute value of the difference in survival = 0.15)

alpha = 0.05 (significance level)

beta = 1 – power

z_(1-alpha/2) = 1.96 (from normal distribution)

p = (p1 + phi*p2)/(1+phi)

Now to obtain the power which was available to detect the observed difference at the given significance level we use the following formula:

z_(1-beta) =

[delta*sqrt(m*phi) – z_(1-alpha/2)*sqrt(p*(1-p)*(1+phi))]/[sqrt(phi*p1*(1-p1)+p2*(1-p2))]

and we then obtain the value of beta from normal distribution tables (from the z-score).

[Note: to obtain a sample size first calculate m as shown below and then n = phi*m

m =

{[z_(1-alpha/2)*sqrt(p*(1-p)*(1+phi))+z_(1-beta)*sqrt(phi*p1*(1-p1)+p2*(1-p2))]/[delta*sqrt(phi)]}^2]

Results

Receptor	***p1 (m)***	***p2 (n)***	***z_(1-beta)***	***Power***
alpha-o	*0.83 (98)*	*0.68 (42)*	*0.0184258*	*51%*
EGFR	*0.75 (70)*	*0.63 (70)*	*-0.4285806*	*33%*
HER2	*0.75 (35)*	*0.63 (105)*	*-0.703246*	*24%*

Using rearrangements of the formula it can be shown that:

alpha-o:

There would be 80% power to detect a difference between 0.83 & 0.58 (or 0.68 & 0.91) – around 25% difference in survival [worst case]

EGFR:

There would be 80% power to detect a difference between 0.75 & 0.51 (or 0.63 & 0.85) – around 24% difference in survival [worst case]

HER2:

There would be 80% power to detect a difference between 0.75 & 0.47 (or 0.63 & 0.89) – around 28% difference in survival [worst case]

Data Sheet for pSTAT3 antibody.

BD™ Phosflow Technical Data Sheet PE Mouse Anti-Stat3 (pY705)

Product Information Material Number: 612569

Size: 50 tests

Vol. per Test: 20 µl

Clone: 4/P-STAT3

Immunogen: Phosphorylated Human Stat3 (Y705) Peptide

Isotype: Mouse IgG2a, .

QC Testing: Human

Tested in Development: Mouse

Storage Buffer: Aqueous buffered solution containing BSA and =0.09% sodium azide.

Description

Stat proteins function as both cytoplasmic signal transducers and activators of transcription. Stat3 has been reported to be a 92kDa protein that is activated as a DNA binding protein through cytokines, such as IL-6, and growth factors, such as EGF. Stat3 activation occurs via tyrosine phosphorylation at Tyr-705. Tyrosine phosphorylation in response to cytokine stimulation is generally mediated by JAK1. Upon activation,Stat3 dimerizes, translocates to the nucleus, and binds DNA response elements thereby regulating gene expression. It has been reported that Stat3 binds to DNA as a homodimer, but it is also capable of binding as a heterodimer with Stat1. In addition to tyrosine phosphorylation, Stat3 is also phosphorylated at Ser-727 via the MAPK pathway. Stat3 is widely expressed and can bind to the sis-inducible element (SIE) site from the c-fos promoter. This site is similar to the GAS element that is present in IFN-. induced genes. Thus, phosphorylation of Tyr-705 in Stat3 occurs in response to growth factors and cytokines, and is essential for normal transcription activity.

This antibody is routinely tested by flow cytometric analysis. Other applications were tested at BD Biosciences Pharmingen during antibody development only or reported in the literature.

Flow cytometric analysis of Stat3 (pY705). Human wholeblood was collected in the presence of heparin. Whole blood was either left unstimulated (unshaded) or stimulated (shaded) with recombinant human IL-6 (MN 550071) at 100 ng/mL for 15 min at 37 °C. Cells were lysed and fixed in a single step using BD™ Phosflow Lyse/Fix buffer (MN 558049) for 10 min at 37 °C. Cells were then permeabilized in BD™ Phosflow Perm Buffer III (MN 558050) for 30 min on ice or overnight at -20 °C. Cells were then washed twice in BD Pharmingen™ Stain Buffer (MN 554656) and stained with PE mouse anti-Stat3 (pY705) antibody (MN 612569) for 30 min at room temperature. Samples were analyzed on a BD FACSCalibur™ instrument.

Preparation and Storage ; Store undiluted at 4° C and protected from prolonged exposure to light. Do not freeze.

The monoclonal antibody was purified from tissue culture supernatant or ascites by affinity chromatography.

The antibody was conjugated with R-PE under optimum conditions, and unconjugated antibody and free PE were removed by gel filtration chromatography.

Application

Intracellular staining (flow cytometry) Routinely Tested 612569 Rev. 7 Page 1 of 2

Suggested Companion Products

Catalog Number Name Size Clone

554656 Stain Buffer (FBS) 500 ml (none)

558050 Perm Buffer III 125 ml (none)

558049 Lyse/Fix Buffer 5X 250 ml (none)

550071 Recombinant Human IL-6 10 µg (none)

558595 PE Mouse IgG2a, . Isotype Control 50 tests MOPC-173

Product Notices

1.This reagent has been pre-diluted for use at the recommended Volume per Test. We typically use 1 X 10e6 cells in a 100-µl experimental sample (a test).

2. Source of all serum proteins is from USDA inspected abattoirs located in the United States. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

3. This conjugated product is sold under license to the following patents: US Patent Nos. 4,520,110; 4,859,582; 5,055,556; European Patent No. 76,695; and Canadian Patent No. 1,179,942.

4. For fluorochrome spectra and suitable instrument settings, please refer to our Fluorochrome Web Page at www.bdbiosciences.com/pharmingen/colors.

5. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.

References

Bromberg J, Darnell JE. The role of STATs in transcriptional control and their impact on cellular function. Oncogene. 2000; 19:2468-2473.(Biology)

Kazuroni I, Leonard WJ. The Jak-STAT pathway. Mol Immunol. 2000; 37:1-11.(Biology)

Liu KD, Gaffen SL, Goldsmith MA. JAK/STAT signaling by cytokine receptors. Curr Opin Immunol. 1998; 10(3):271-278.(Biology) 612569 Rev. 7 Page 2 of 2

Table Showing the transcription factor IHC results.

Pt number	STAT3 status	SP1 status	NFkB status
99H00004	2	3	0
99H00016	1	2	0
99H00038	1	2	0
99H00089	1	2	1
99H00205	3	3	0
99H00207	2	1	0
99H00495	3	2	0
99H00506	2	2	1
99H00508	1	3	0
99H00631	1	1	0
99H00695	3	2	0
99H00716	2	2	0
99H00740	0	2	0
99H00741	2	2	0
99H00838	2	1	0
99H00856	1	2	0
99H00947	3	3	0
99H00979	2	3	0
99H01129	2	2	0
99H01170		3	
99H01171	1	2	0
99H01289	3	2	1
99H01518	1	1	1
99H01587	0	3	0
99H01588	2	3	1
99H01591	2	2	1
99H01687	2	1	1
99H01730	2	3	1
99H01749	1	2	0
99H01823	1	3	1
99H01824	1	2	0
99H01825	1	2	0
99H01857	1	2	2
99H01955	1	0	0
99H01958	2	3	1
99H01983	2	3	1
99H01984	3	3	0
99H02067	2	3	0
99H02072	3	2	2

99H02089	3	3	1
99H02180	1	3	2

Pt number	STAT3 status	SP1 status	NFkB status
99H02181	1	0	2
99H02242	3	1	1
99H02261	3	2	0
99H02287	2	2	2
99H02320	3	3	2
99H02321		3	2
99H02326	2	3	1
99H02463	1	3	1
99H02483	1	3	2
99H02506	3	1	1
99H02705			0
99H02707	3	2	1
99H02726	3	1	2
99H02728	1	1	1
99H02752	0	1	0
99H02798	1	1	1
99H02926	1	1	1
99H03091	3	2	1
99H03144	2	3	0
99H03173	1	3	1
99H03175	1	3	1
99H03188	2	2	1
99H03299	2	2	1
99H03312	1	2	0
99H03392	2	2	1
99H03450	3	2	2
99H03464	1	2	1
99H03467	1	3	2
99H03508	1	3	0
99H03512	2	2	1
99H03557	2	2	0
99H03566	3	3	1
99H03660	2	2	0
99H03747	2	1	2
99H03769	3	3	2
99H03962	1	2	1
99H03972	1	1	0
99H03973	1	1	2

99H03979	0	1	0
99H03989	1	2	0
99H04208	3	3	1
99H04294	2	3	0
Pt number	STAT3 status	SP1 status	NFkB status
99H04345	1	2	1
99H04346	3	3	2
99H04347	1	3	2
99H04439	2	2	0
99H04440	1	3	1
99H04533	3	2	1
99H04542	2	2	0
99H04545	1	1	1
99H04613	1	1	0
99H04642	1	2	2
99H04737	1	3	0
99H04746	2	3	0
99H04750	1	2	0
99H04776	3	1	2
99H04840	1	3	0
99H04943	2	3	1
99H05020	3	3	0
99H05032	3	2	2
99H05033	1	2	0
99H05071	1	2	1
99H05222	3	2	1
99H05234	3	3	1
99H05235	2	3	2
99H05275	2	3	0
99H05281	3	3	1
99H05342			
99H05475	3	2	1
99H05478	1	2	0
99H05479	1	1	2
99H05480	2	2	1
99H05481	2	3	0
99H05482	1	2	0
99H05616	1	3	0
99H05655	1	2	2
99H05698	1	2	0
99H05710	2	2	0
99H05746	1	2	0
99H05747	2	2	1

99H05798	1	3	1
99H05910	2	1	1
99H05925	1	2	2
99H05983	1	3	1
99H05984	1	1	0
Pt number	STAT3 status	SP1 status	NFkB status
99H05993	2	3	0
99H06041			
99H06087	2	2	0
99H06108	3	1	0
99H06119	1	2	1
99H06204	1	2	0
99H06205	0	3	1
99H06210	0	2	1
99H06292	3	0	2
99H06322	2	1	1
99H06435	2	1	1
99H06449	2	3	1
99H06500	1	1	1
99H06514	3	3	1
99H06515	1	0	0
99H06675	3	2	1
99H06689	3	3	0
99H06720	3	2	0
99H06863	2	3	0
99H06870	2	1	1
99H06933	1	2	0
99H07104	1	2	0
99H07106	1	1	0
99H07170	2	2	1
99H07474	3	2	2
99H07648	3	2	0
99H07680	1	3	0
99H07681	3	2	1
99H07785		3	
99H07917	3	2	2
99H07923	1	3	0
99H07968	2	3	0
99H08028	1	2	0
99H08029	2	3	0
99H08143	2	1	1
99H08179	2	1	1
99H08242	2	0	2

99H08345	3	3	0
99H08405	1	1	1
99H08416	1	1	1
99H08417	3	1	
99H08428	1	0	0
99H08494	3	3	0
Pt number	STAT3 status	SP1 status	NFkB status
99H08512	3	0	1
99H08758	1	0	1
99H08780	1	1	0
99H08790	2	2	
99H08791	1		0
99H08893	2	0	0
99H08908	2	1	0
99H08909	3	1	0
99H08988			
99H09139	3	2	1
99H09157	1	0	0
99H09318	2	2	1
99H09334	0	0	1
99H09335	3	3	1
99H09336	3	2	0
99H09344	1	0	1
99H09354	1	2	1
99H09357	1	2	2
99H09530	0	2	0
99H09567	3	1	0
99H09633	2	2	0
99H09650	3	2	0
99H09662	2	3	1
99H09749	3	0	0
99H09754		3	0
99H09796	2	3	0
99H09797	2	2	0
99H09798	1	3	0
99H09799	1		1
99H09892	3	0	
99H09909	3		1
99H09992	1	2	1
99H10000	2		1
99H10049	2	0	1
99H10050	1	1	1
99H10066	0	0	1

99H10069	3	2	1
99H10080	3	3	
99H10223	2	0	0
99H10254	1	2	1
99H10607	2	3	0
99H10731	2	1	1
99H10757	3	0	1
Pt number	STAT3 status	SP1 status	NFkB status
99H10805	1	2	2
99H10820	3	3	1
99H10993	2		2
99H10995	3	3	2
99H11009	1	2	1

Scoring:

STAT3/SP1 0 = no stain.

1 = weak cytoplasmic

2 = strong cytoplasmic

3 = nuclear stain

NFkB 0 = negative.

1 = cytoplasmic.

2 = nuclear.

MCF7 cell model results.

Table showing cell cycle results for MCF7 model at 24 hr

MCF7 24hr cell cycle

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
G1 %	64.87	.55	65.77	1.00	65.20	1.05	61.57	1.74	43.00	2.91	54.67	.81	43.13	1.08
S1 %	25.03	1.31	25.23	1.44	24.43	1.64	26.33	.25	41.00	3.83	31.83	1.06	40.97	.50
G2 %	9.89	1.07	9.23	1.07	10.82	1.12	11.60	1.25	17.53	1.46	12.63	.67	15.47	.74

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
G1 %	E F G	E F G	E F G	E F G		E G	
S1 %					A B C D F	A B C D	A B C D F
G2 %					A B C D F	B	A B C D

Table showing cell cycle results for MCF7 model at 48 hr

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
G1 %	75.73	.81	75.87	1.59	75.20	1.71	74.03	1.66	63.10	3.03	70.30	.78	58.83	.68
S1 %	14.67	.51	14.30	1.25	15.50	.69	15.57	1.62	24.33	1.29	19.47	.68	27.33	.55
G2 %	9.18	.97	9.98	.75	9.79	1.09	10.12	.42	11.76	1.72	10.09	.79	12.03	.74

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
G1 %	E F G	E F G	E G	E G		E G	
S1 %					A B C D F	A B C D	A B C D F
G2 %							

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

Table showing cell cycle results for MCF7 model at 72 hr

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
G1 %	78.97	1.31	83.10	2.25	76.00	2.72	79.20	1.35	57.57	.86	68.87	.31	59.90	1.95
S1 %	14.17	1.07	10.90	.61	15.30	1.61	13.57	.75	28.77	1.24	19.80	.82	25.80	.52
G2 %	6.46	3.19	6.35	1.89	9.10	.63	7.96	1.26	12.73	.84	11.83	.90	13.63	1.77

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
G1 %	E F G	C E F G	E F G	E F G		E G	
S1 %	B		B		A B C D F	A B C D	A B C D F
G2 %					A B	A B	A B D

Table showing cell number results for MCF7 cell model at 24 hrs.

	Treatment													
	control		ETOH		HCl		ETOH&HCl		E2		EGF		E2&EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Total cell number	250507.30	9609.77	211684.20	32534.48	246017.37	29489.04	237636.53	20391.91	340886.63	25414.40	221182.13	27871.51	318000.47	45185.67
Live cell numbers	216364.07	16707.30	192566.23	28506.76	218421.47	23217.06	208670.10	17879.77	306571.97	27599.18	183134.50	23390.85	282468.03	34087.29
Apoptotic cell number	23717.67	11387.04	11391.50	1068.31	19562.43	5275.38	21511.23	7111.63	26435.83	4152.21	28061.10	3765.39	22548.20	4646.52
Necrotic cell numbers	10425.57	2181.43	7726.47	3706.75	8033.43	2115.38	7455.17	1405.81	7878.83	2005.49	9986.50	1814.27	12984.23	6633.12

Comparisons of Column Means[a]

	Treatment						
	control	ETOH	HCl	ETOH&HCl	E2	EGF	E2&EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Total cell number					A B C D F		B F
Live cell numbers					A B C D F		B F
Apoptotic cell number							
Necrotic cell numbers							

Table showing cell number results for MCF7 cell model at 48 hrs.

Cell number experiment MCF7 cell model 48 hrs.

	Treatment													
	control		ETOH		HCl		ETOH&HCl		E2		EGF		E2&EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Total cell number	302353.57	17179.11	231227.30	17489.02	292603.33	17641.71	294220.43	11772.73	359475.43	3424.97	321437.23	24100.95	395157.90	44842.96
Live cell numbers	249450.20	8992.41	194783.20	14087.03	229105.40	9484.81	234436.17	14625.57	297432.13	7119.80	237296.40	19756.67	306846.70	37463.95
Apoptotic cell number	39763.20	7405.86	25847.13	4609.12	51715.63	10608.31	49061.10	3696.05	48761.73	4246.18	70070.30	5504.11	69058.70	3907.42
Necrotic cell numbers	13140.13	3717.57	10597.00	1732.78	11782.30	972.20	10723.17	853.40	13281.53	440.20	14070.57	1184.55	19252.57	7920.10

Comparisons of Column Means[a]

	Treatment						
	control	ETOH	HCl	ETOH&HCl	E2	EGF	E2&EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Total cell number	B				B	B	A B C D F
Live cell numbers					B C D F		A B C D F
Apoptotic cell number			B	B	B	A B D E	A B D E
Necrotic cell numbers							

Table showing cell number results for MCF7 cell model at 48 hrs.

Cell number experiment MCF7 cell model 72 hrs.

	Treatment													
	control		ETOH		HCl		ETOH&HCl		E2		EGF		E2&EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Total cell number	362706.97	33721.44	320731.73	14603.48	406544.90	30109.14	268498.87	13584.50	519558.67	60619.59	338801.20	32127.14	390903.87	14674.72
Live cell numbers	334375.03	33057.23	285957.50	17567.77	362757.80	34477.82	242801.07	15787.51	488513.47	62125.13	296671.63	29766.71	357767.50	10179.01
Apoptotic cell number	19724.77	2505.02	29279.70	8214.56	36001.93	6516.10	19321.30	2947.57	23913.17	1404.66	36307.97	7805.45	26539.40	6348.80
Necrotic cell numbers	8607.13	1419.83	5494.50	1133.15	7785.20	2521.21	6376.47	1283.53	7131.97	231.72	5821.63	285.88	6597.00	466.47

Comparisons of Column Means[a]

	Treatment						
	control	ETOH	HCl	ETOH&HCl	E2	EGF	E2&EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Total cell number			D		A B C D F G		D
Live cell numbers			D		A B C D F G		D
Apoptotic cell number							
Necrotic cell numbers							

Table Showing pSTAT3 median channel shift at 12 hr time point MCF7 cell model.

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	1081.33	35.92	1007.33	53.16	829.00	222.54	948.00	142.52	1491.33	412.54	1579.67	146.65	1324.33	187.40

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift					C	C D	

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

a. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

Table Showing pSTAT3 median channel shift at 24 hr time point MCF7 cell model.

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	1791.00	198.20	1689.67	590.14	1719.33	569.83	1429.00	237.99	1739.00	371.69	2165.33	185.77	1827.00	229.97

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift							

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

a. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

Table Showing pSTAT3 median channel shift at 48 hr time point MCF7 cell model.

48 hr median channel shift.

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	327.00	169.68	824.00	103.32	525.00	238.70	1227.67	170.50	1669.33	420.64	1968.67	295.06	1558.67	163.40

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift				A	A B C	A B C D	A C

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

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Table Showing pSTAT3 median channel shift at 72 hr time point MCF7 cell model.

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	199.00	90.93	1503.33	211.52	1368.67	133.72	1271.67	191.28	928.33	203.04	1432.33	336.85	1544.67	311.07

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift		A	A	A	A	A	A

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

a. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

SKBr3 Results

Table showing Cell cycle results of SKBr3 cell model 24 hrs.

	treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
G1 %	76.80	4.94	73.06	3.64	71.09	3.82	76.72	2.75	73.86	3.29	80.53	3.98	82.34	2.54
S %	15.81	3.85	14.58	1.56	16.50	8.26	15.01	2.58	17.32	2.37	10.64	5.00	10.74	1.67
g2per	7.39	1.72	12.36	3.97	12.41	6.33	8.27	3.21	8.83	3.01	8.82	2.91	6.92	1.33

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
G1 %							C
S %							
g2per							

Table showing Cell cycle results of SKBr3 cell model 48 hrs.

	treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
G1 %	75.13	3.98	74.98	3.49	76.08	2.30	75.61	1.28	74.95	3.34	62.79	3.43	68.93	5.14
S %	18.15	3.12	16.53	3.88	16.11	4.13	18.56	4.24	20.84	2.41	30.05	5.04	26.87	5.39
g2per	6.72	3.17	8.48	2.18	7.81	2.00	5.84	3.86	4.22	1.11	7.17	2.55	4.20	1.58

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
G1 %	F	F	F	F	F		
S %						B C	
g2per							

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

Table showing Cell cycle results of SKBr3 cell model 72 hrs.

	treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
G1 %	75.65	8.52	72.16	1.23	72.15	2.99	70.29	2.40	73.28	.97	66.73	2.37	64.20	.81
S %	16.37	5.35	14.90	4.05	17.32	4.79	18.10	4.75	15.28	4.45	20.90	3.04	22.58	5.03
g2per	7.99	3.75	12.94	2.95	10.52	2.26	11.61	3.25	11.45	4.45	12.37	2.68	13.22	4.88

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
G1 %	G						
S %							
g2per							

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

Table showing Cell number results of SKBr3 cell model 24 hrs.

	Treatment													
	control		ETOH		HCl		ETOH&HCl		E2		EGF		E2&EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Total cell number	56248.86	3444.04	66674.99	8420.38	50226.10	3606.74	69801.20	18321.43	71441.13	17146.10	83110.74	8774.53	89106.84	9511.83
Live cell numbers	34344.13	2107.95	45619.15	1000.61	25876.87	3967.23	46946.58	13185.64	45574.93	21099.68	56487.98	9281.08	69804.20	8891.96
Apoptotic cell number	4427.36	507.13	7761.66	7062.48	8471.04	3230.62	11949.05	11134.96	13976.91	8383.77	13956.02	9820.20	6580.49	827.29
Necrotic cell numbers	17477.37	5443.51	13294.18	2608.79	15878.19	1972.48	10905.57	2926.49	11889.29	2685.08	12666.75	2416.87	12722.16	943.98

Comparisons of Column Means[a]

	Treatment						
	control	ETOH	HCl	ETOH&HCl	E2	EGF	E2&EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Total cell number							C
Live cell numbers							A C
Apoptotic cell number							
Necrotic cell numbers							

Table showing Cell number results of SKBr3 cell model 48 hrs.

	Treatment													
	control		ETOH		HCl		ETOH&HCl		E2		EGF		E2&EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Total cell number	78402.38	22123.94	101230.00	22694.97	81870.08	12245.07	86239.26	3400.86	86125.53	14226.44	80602.23	2389.74	105195.51	17858.58
Live cell numbers	46320.63	10940.14	67113.66	11801.91	51691.18	11979.73	62561.31	5796.85	62130.96	14181.12	55666.12	2643.13	80864.52	11887.86
Apoptotic cell number	21166.50	9817.26	20540.11	8775.43	17776.25	4904.28	14740.21	5083.95	16601.96	641.42	11988.40	2509.13	8269.80	2591.63
Necrotic cell numbers	10915.25	1605.86	13576.23	2837.98	12402.65	1817.04	8937.74	384.71	7392.60	725.93	12947.71	4347.79	16061.18	4921.03

Comparisons of Column Means[a]

	Treatment						
	control	ETOH	HCl	ETOH&HCl	E2	EGF	E2&EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Total cell number							
Live cell numbers							A
Apoptotic cell number							
Necrotic cell numbers							

Table showing Cell number results of SKBr3 cell model 72 hrs.

	Treatment													
	control		ETOH		HCl		ETOH&HCl		E2		EGF		E2&EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Total cell number	53498.71	5678.67	67042.75	3159.85	48724.23	5880.16	62576.76	3522.12	85311.62	12330.50	95236.67	8728.56	99208.98	10007.34
Live cell numbers	44885.28	4016.57	55357.45	4759.58	35323.70	4778.49	50958.14	2549.43	61657.69	14894.20	77514.55	9117.51	81494.60	9310.35
Apoptotic cell number	2779.83	379.61	4679.78	3037.71	6938.54	1120.13	4341.51	822.05	14599.80	3651.71	7475.17	1000.08	5642.46	809.49
Necrotic cell numbers	5833.60	2538.72	7005.53	604.77	6461.99	1270.99	7277.11	569.38	9054.13	667.33	10246.95	2908.28	12071.93	2166.27

Comparisons of Column Means[a]

	Treatment						
	control	ETOH	HCl	ETOH&HCl	E2	EGF	E2&EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Total cell number					A C	A B C D	A B C D
Live cell numbers					C	A C D	A B C D
Apoptotic cell number					A B C D F G		
Necrotic cell numbers							A C

Table showing SKBr3 cell model pSTAT3 induction median channel shift 12 hr.

SKBr3 pSTAT median channel shift 12 hr

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	1063.00	65.82	879.00	53.51	798.33	20.55	714.33	94.94	751.67	133.81	904.67	166.49	822.33	146.77

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift	D						

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

a. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

Table showing SKBr3 cell model pSTAT3 induction median channel shift 24 hr.

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	1239.67	242.92	1267.67	255.63	1153.67	10.97	878.33	150.04	1661.33	204.71	1806.00	15.62	1448.67	72.70

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift					C D	A B C D	D

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

a. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

Table showing SKBr3 cell model pSTAT3 induction median channel shift 48 hr.

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	817.33	181.51	1048.67	65.90	967.33	35.53	994.00	203.03	1136.33	95.09	1203.33	167.16	910.00	162.08

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift							

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

a. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

Table showing SKBr3 cell model pSTAT3 induction median channel shift 72 hr.

SKBr3 pSTAT median channel shift 72 hr

	Treatment													
	Control		ETOH		HCl		ETOH & HCl		E2		EGF		E2 & EGF	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Median Channel Shift	494.00	38.11	606.00	50.32	569.33	170.37	541.00	138.43	513.67	40.46	961.33	71.84	946.00	148.16

Comparisons of Column Means[a]

	Treatment						
	Control	ETOH	HCl	ETOH & HCl	E2	EGF	E2 & EGF
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Median Channel Shift						A B C D E	A B C D E

Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean.

a. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.