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**The role of IKKalpha, IKKbeta
and NF-kappaB in the
progression of breast cancer**

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BSc(Hons), MSc

**Submitted in fulfillment of the requirements
for the degree of PhD**

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The work presented in this thesis was performed entirely by the author except as acknowledged. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Lindsay Bennett

July 2014

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Summary

Breast cancer is the most common female cancer in the UK and, despite earlier detection and improved treatments, remains the second most common cause of cancer death in women. Although therapies exist for breast cancer, including endocrine therapy for oestrogen receptor (ER) positive tumours, resistance to current treatment remains a major problem. The molecular mechanisms of endocrine resistance have yet to be fully elucidated and in order to improve treatment for patients this needs to be addressed. Clinically breast cancer presents as several distinct diseases with different outcomes and molecular profiles. Over the past decade, through the use of molecular profiling, the number of different subtypes of breast cancer has grown and understanding the pathways driving each subtype may allow a stratified approach to therapy, allowing patients to receive the treatment which will be of most benefit.

The Nuclear Factor kappa B (NF- κ B) pathways regulate the transcription of a wide range of genes involved in the immune response, inflammation, proliferation and apoptosis. Many of these processes are hallmarks of cancer and NF- κ B has been hypothesised to have a role in tumorigenesis. The aim of the current study was to investigate the role of both NF- κ B pathways in the pathogenesis and recurrence of breast cancer.

Immunohistochemistry was employed to assess key components of the canonical and non-canonical NF- κ B pathways on a tissue microarray (TMA) of 544 patients with full clinical follow up and clinical information including ER status, subtype, necrosis, apoptosis and angiogenesis. Nuclear expression of p65 phosphorylated at serine 536 was associated with angiogenesis and shorter recurrence free interval. Cytoplasmic expression of IKK α was associated with cell death (apoptosis and necrosis) and a shorter recurrence free interval was also observed for those with high expression. These observations between phospho-p65/IKK α and recurrence free interval, when subdivided by ER status, remained significant in ER positive tumours but were negated in ER negative tumours. When split further into subtype, a diverging role for each was observed with phospho-p65 associating with recurrence in luminal B tumours and IKK α with luminal A tumours. Other members of the NF- κ B pathways (p65, IKK β , NIK and RelB) were not associated with recurrence free interval. When these results were tested in an independent cohort, IKK α remained significant on recurrence free interval and breast cancer specific survival in ER positive tumours however phospho-p65 was only marginally associated with breast cancer specific survival. Variability of phospho-p65 is a major issue in IHC studies and therefore an alternative marker of the canonical NF- κ B pathway is required. Analysis of expression in

this second cohort also revealed that high levels of IKK α in the cytoplasm were associated with recurrence on tamoxifen. This marker may therefore be able to be employed as a diagnostic tool to predict patients who are likely to display endocrine resistance and may represent a therapeutic strategy in combination with endocrine therapy, or for patients after endocrine resistance has occurred.

Further examination of the pathways in breast cancer cell lines also demonstrated a difference between ER positive and ER negative breast cancer. In ER negative MDA-MB-231 cells phosphorylation of p65 (from the canonical NF- κ B pathway) and phosphorylation of p100 (from the non-canonical NF- κ B pathway) was apparent even in untreated control cells, suggesting constitutive activation. Expression was however found to be inducible in ER positive MCF7 cells.

In order to investigate whether kinases involved in activation of each pathway, IKK β in the canonical pathway and IKK α in the non-canonical NF- κ B pathway, had potential as targets in breast cancer, we examined the phenotypic impact of silencing their expression in breast cancer cell lines. Silencing IKK β induced apoptosis and decreased cell viability in both MCF7 and MDA-MB-231 cells but reduction in expression of IKK α only impacted on cell viability and apoptosis in ER positive MCF7 cells. This data, consistent with results from the clinical specimens, has therefore revealed that inhibitors of IKK α are likely to be most beneficial in the treatment of ER positive tumours.

These results suggest that the NF- κ B pathways are associated with recurrence in patients with ER positive tumours with each pathway possibly associating with recurrence in different subtypes. Additional studies in a larger cohort, including patients receiving aromatase inhibitors are required, accompanied by extensive mechanistic studies to further explore the roles of IKK α and IKK β in breast cancer. These observations highlight that different subgroups of breast cancer may have different signalling pathways driving progression and therefore patients are likely to benefit from different therapeutic strategies.

Publications and presentations

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Abbreviations

AI	Aromatase inhibitors
AIB1	Amplified in breast cancer 1
BRCA	Breast Cancer genes
CDK	Cyclin dependent kinase
CRLF1	Chemokine receptor-like factor 1
CXCL10	Chemokine CXC ligand 10
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
DN	Dominant negative
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
ERE	Oestrogen response elements
FFPE	Formalin-fixed paraffin-embedded
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
IAP	Inhibitor of apoptosis
ICAM	Intracellular adhesion molecule
ICCC	Interclass correlation coefficient
IGFR-1	Insulin-like growth factor receptor 1
IHC	Immunohistochemistry
IKK	I κ B kinase
IL-1 β	Interleukin-1 beta
IQR	Interquartile range
I κ B	Inhibitor of κ B
LTx	Lymphotoxin
MAPK	Mitogen activated protein kinase
mTOR	Mammalian target of rapamycin
NCOR2	Nuclear corepressor 2
NEMO	NF- κ B essential modulator
NES	Nuclear export sequence

NF- κ B	Nuclear Factor kappa B
NIK	NF- κ B-Inducing Kinase
NLS	Nuclear localisation signal
NPI	Nottingham Prognostic Index
NT	Non-targeting siRNA
PCR	Polymerase chain reaction
Pfu	Plaque forming units
PgR	Progesterone receptor
Phospho-p65	Phosphorylation of p65 at serine 536
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative real time PCR
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SERD	Selective oestrogen receptor degraders
SERM	Selective oestrogen receptor modulators
SIGN	Scottish Intercollegiate Guidelines Network
siRNA	Small interfering RNA
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
STWS	Scott's tap water substitute
T-DM1	Trastuzumab emtansine
TAD	Transactivation domain
TBS	Tris buffer saline
TEAM	Tamoxifen Exemestane Adjuvant Multinational trial
TMA	Tissue microarray
TNBC	Triple negative breast cancer
TNF α	Tumor necrosis factor α
TNM	Tumour size, lymph Node involvement, Metastasis
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
uPA	Urokinase-type plasminogen activator
VCAM	Vascular cell adhesion molecule
WST-1	Water-soluble tetrazolium salt 1

Chapter 1:

Introduction

1.1 Breast cancer epidemiology, pathology and prognostic factors

1.1.1 Breast cancer incidence, mortality and survival

Breast cancer is the most common female cancer in the UK with more than 49,500 women diagnosed in 2010 [1]. The number of cases increases by around 1% each year but a peak was seen around 1988 after the introduction of the screening programme due to the detection of undiagnosed cancers (Figure 1.1A). The aim of the breast screening programme is to reduce mortality rates by earlier detection via mammography before any symptoms are apparent. Although earlier detection and improved treatments has resulted in a decrease in the number of deaths (Figure 1.1B) breast cancer still remains the second most common cause of cancer death in women in the UK with nearly 12,000 deaths attributed in 2010 [2].

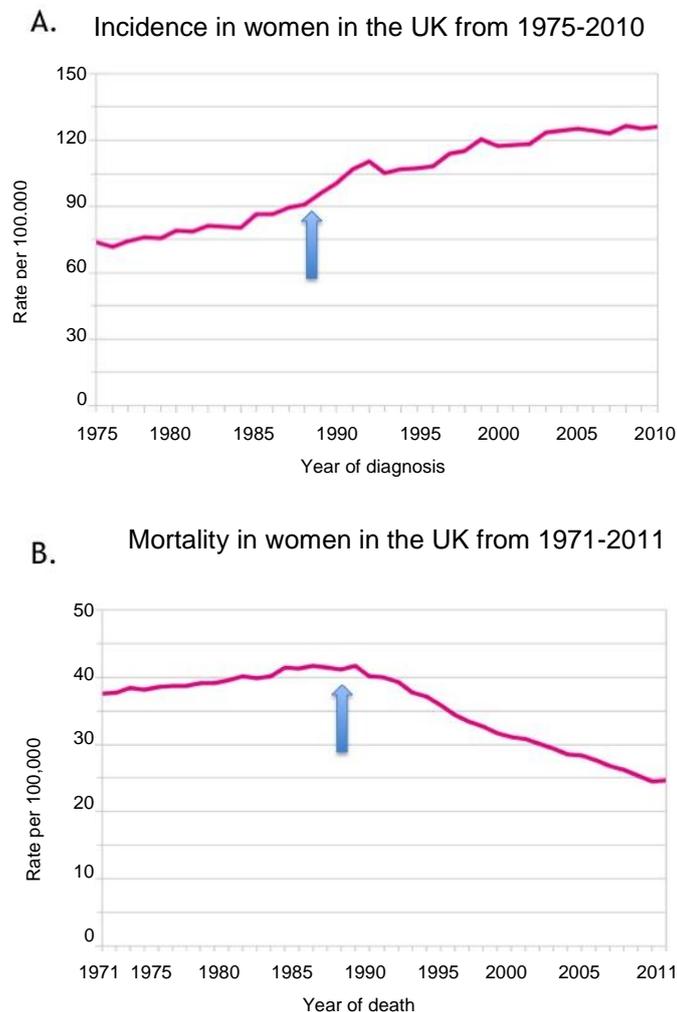


Figure 1.1: Incidence and mortality rates of breast cancer in women in the UK over time. A: Incidence rates per 100,000 women from 1975-2010 [1]. B: Mortality rates per 100,000 women, from 1971 to 2011 [2]. The arrow has been added to indicate the introduction of the screening programme in 1988.

Breast cancer has, however, one of the highest survival rates of the most common cancers in the UK. In Scotland the relative survival of women diagnosed in 1998-2002 after 1 year was 96.1%, after 3 years was 88.1%, for 5 years was 82.8% and the 10 year survival rate was 76.4% [3]. The survival rate varies with age, Table 1.1 shows the relative survival for each age range.

Years survival	Age range (years)					
	15-44	45-54	55-64	65-74	75-84	85-99
1 year	98.1%	98.5%	97.8%	95.4%	88.9%	83.2%
3 year	89.6%	93.3%	93.1%	89.2%	79.3%	65.9%
5 year	82.6%	86.5%	86.7%	78.2%	69.4%	56.3%
10 year	73.9%	80.5%	80.7%	72.0%	63.9%	46.1%

Table 1.1: Survival rates of breast cancer in Scotland by age range. *The percentage survival at 1 and 3 years for patients diagnosed between 2003 and 2007, and the 5 and 10 year survival for patients diagnosed between 1998 and 2002 are shown (Information from [3]).*

1.1.2 Breast cancer risk factors

There are several factors that have been found to increase the risk of breast cancer. Many of these factors are linked to exposure to the hormone oestrogen, which plays a role in the progression of the disease [4].

1.1.2.1 Age

The risk of developing breast cancer increases with age and older age is the largest risk factor other than female gender. Most breast cancers (over 80%) occur in women over the age of 50. For women under the age of 29 the risk is 1 in 2000, the risk increases to 1 in 50 up to age 49, 1 in 22 up to age 59 and 1 in 13 up to age 69 [5].

1.1.2.2 Socioeconomic class and geographical variation

Breast cancer is one of the few cancers where the risk appears to be higher in the more affluent social classes [5]. However, once a woman develops breast cancer, it is those in the

lower socioeconomic classes that have higher cancer mortality rates, as a result of better access to screening and treatment [6-7]. It has been reported that the highest incidence rates of breast cancer were found in Western Europe and lowest in Eastern Africa [8], however, those diagnosed in developed countries have better survival outcome, again likely due to better access to screening and treatment.

1.1.2.3 Puberty and menopause

An increased risk of breast cancer has been reported in women who had earlier menarche (initiation of menses) and earlier onset of regular menses [9]. As well as an increase in risk for every year younger at menarche, every year older at menopause has also been found to independently increase the risk of breast cancer [10]. Oestradiol serum levels at menopause have also been found to influence the risk of breast cancer, with a higher risk in those with elevated levels [11].

Additionally, postmenopausal women who are obese have around a 31% increased risk compared to those with a healthy body mass index [12]. As well as an increased body mass index, larger waist-hip ratio and weight gain in adulthood also result in a greater risk of breast cancer [12].

1.1.2.4 Childbearing age, parity and breastfeeding

Several reproductive factors have been reported as being associated with the risk of breast cancer. Younger age at first child bearing decreases the risk, the parity (number of births) also affected the risk of breast cancer with those with higher parity having a decreased risk [13]. Recent studies have investigated these risk factors in different subgroups of patients depending on hormone receptor status. It was found that earlier time of menarche and longer time between menarche and first full-term childbirth was associated with increased risk in both hormone receptor-positive and hormone receptor-negative groups, however only weakly in the hormone receptor-negative group. Age at first birth was only associated with a decreased risk in the hormone receptor-positive group [14].

1.1.2.5 Hormone replacement therapy

Hormone replacement therapy (HRT) is widely used after the menopause to alleviate symptoms of menopause and prevent osteoporosis [15]. The Collaborative Group on Hormonal Factors in Breast Cancer reanalysed data from several studies and found that for every year of HRT use the risk of breast cancer increases but was limited to women who were currently receiving HRT or had so in the past 5 years [16]. The Million Women Study also

investigated the use of HRT and breast cancer incidence and again found that current users of HRT were more likely to develop breast cancer than women who had never used HRT [17]. There was an increased risk for women prescribed oestrogen only HRT, but the highest risk was with oestrogen-progestagen combination. This study estimated there were 20,000 extra cases of breast cancers in the UK that decade due to HRT, 15,000 of which were associated with oestrogen-progestagen [17].

1.1.2.6 Family history

One of the most well known risk factors for breast cancer is family history. If a woman has a mother or sister with breast cancer before the age of 50 this increases her risk 2 fold or more and if there are multiple affected relatives this increases further. It is thought up to 10% of all breast cancers are due to an inherited mutation [18].

For decades it has been known that in families with a strong history of breast cancer, in particular those arising in young women, this disease clustering is likely due to the inheritance of a highly penetrant dominant susceptibility allele which confers a high risk of developing breast cancer [19]. The first susceptibility gene was mapped to the q arm of chromosome 17 in 1990 [20] and the candidate gene identified 4 years later [21]. *BRCA2* was discovered in 1994 by genomic linkage in families with suspected familial breast cancer but without *BRCA1* mutation. This second breast cancer susceptibility gene was mapped to the q arm of chromosome 13 [22]. The contribution of both these genes to inherited breast cancer was estimated at 52% for *BRCA1* and 32% for *BRCA2* with 16% of familial breast cancers not being linked to either of these genes [23]. Mutations in both *BRCA1* and *BRCA2* also increase the risk of ovarian cancer. In families with a history of breast and ovarian cancer 81% were linked to a mutation in *BRCA1* and 14% to a mutation in *BRCA2*. Mutations in *BRCA2* also increase the risk of male breast cancer, with 76% of families with male and female breast cancer being due to mutations in *BRCA2* [23].

1.1.2.7 Previous breast disease

Women who have previously had certain benign breast diseases have a higher risk of breast cancer. Non-proliferative lesions are not associated with an increased risk but proliferative lesions increase the risk. If these lesions are without atypia, there is a 2 times higher risk and women with previous atypical hyperplasia have a 4 times higher risk than those with no proliferative change [4].

1.1.3 Breast cancer pathology

1.1.3.1 Anatomy of the breast

The breasts (or mammary glands) consist mainly of fat and each breast has up to 20 lobes each with many smaller lobules (Figure 1.2, [24]). These are connected to the nipple by ducts and supported by the surrounding fat and connective tissue. Breast tissue leads to the axilla (armpit), where a network of lymph nodes exists.

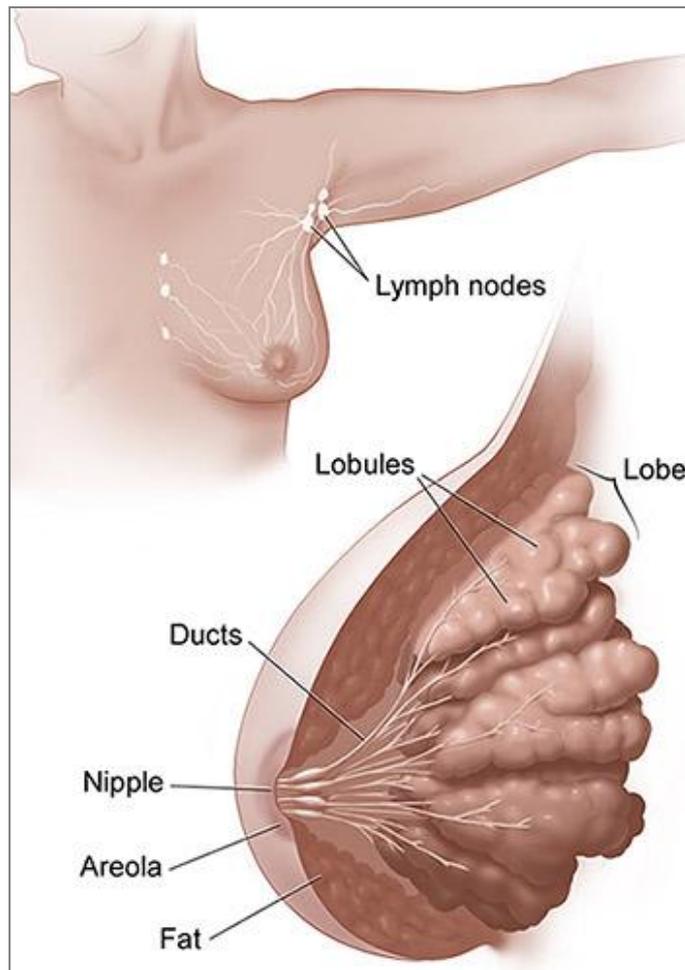


Figure 1.2: Anatomical structure of the breast. *The breast consists mainly of fat and contains up to 20 lobes. These lobes are made of smaller lobules and are connected to the nipple by ducts (Image from [24]).*

1.1.3.2 Breast cancer histopathology

Breast tumours are mainly adenocarcinomas, which arise from epithelial cells that line the ducts and lobules. These cells proliferate in the absence of external stimuli and uncontrolled growth occurs. Normal breast tissue forms structured glands (Figure 1.3A) and this structure is lost in invasive carcinoma (Figure 1.3B). The development of invasive breast cancer may be preceded by ductal or lobular carcinoma *in situ*, which are confined to the site of origin (ducts or lobules) and do not spread beyond the basement membrane [25].

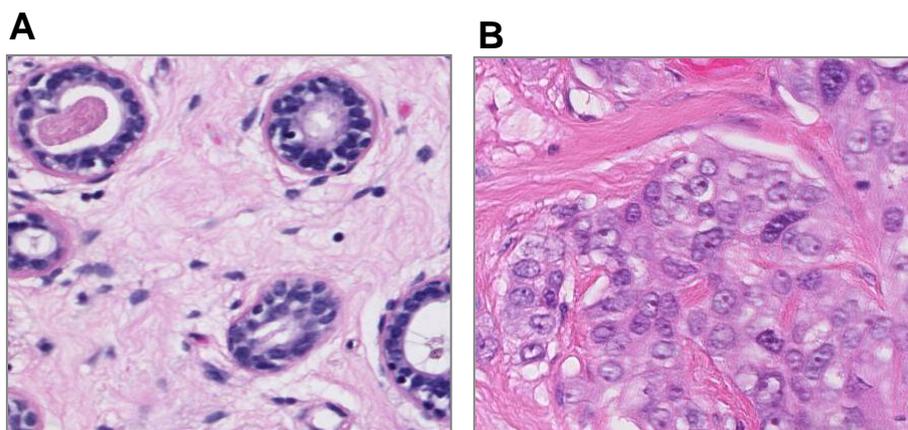


Figure 1.3: Histopathology of normal breast tissue and invasive carcinoma. Normal tissue (A) forms structured glands and this structured appearance is lost in invasive cancer (B).

The most common invasive breast cancer (around 70% of cases), is ductal carcinoma not otherwise specified and it is this group that typically carry the worst prognosis. The normal ductal structures form solid nests and in some cases solid sheets of cancer cells [25]. Several other special types of ductal carcinoma exist, such as tubular and mucinous carcinoma. These are much less common and have a better prognosis. Invasive lobular carcinoma, although more likely to occur bilaterally, has a better prognosis than invasive ductal carcinoma not otherwise specified and accounts for 5-15% of invasive breast cancers [26]. The cancerous cells form rows of cells that infiltrate the stroma [25]. Around 5% of cancers are classed as mixed with areas of both ductal and lobular [26].

This thesis focuses on invasive cancer and therefore does not include any patients with carcinoma *in situ*.

1.1.4 Pathological prognostic markers

There are several pathological markers of breast cancer relating to the appearance of the tumour and how advanced the disease is. These are used diagnostically to stage tumours and identify tumours with different prognoses, allowing the selection of optimal treatment.

1.1.4.1 Tumour Size

Tumour size is categorised into three groups: <2, 2 - 5cm and >5cm. Tumour size is an independent prognostic factor for both overall survival and disease free survival [27].

1.1.4.2 Histological Grade

The grade of tumour depends on its histological appearance and differentiation. Grading of breast tumours is based on tubule formation (% of cancer cells composed of tubular structures), nuclear pleomorphism (changes in cell size and uniformity) and mitotic index (number of dividing cells) [28]. Each of these is scored from 1 to 3 and all are added to give a final score of 3-9. If there is tubule formation in more than 75% of the tumour it is scored 1 point, if 10-64% it is scored 2 points and if less than 10% of the tumour has tubular formation it is scored 3 points. For nuclear pleomorphism, if nuclei show minimal variation in size and shape the tumour is scored 1 point, moderate variation 2 points and marked variation 3 points. The mitotic index is measured as the number of mitoses per 10 fields, the number used to assign to 1, 2 or 3 varies depending on the objective and microscope used.

The final score of 3-9 defines the differentiation status of the tumour. Tumours with scores of 3-5 are considered as Grade 1 and well differentiated. Grade 2 tumours that score a final count of 6-7 are considered moderately differentiated. Poorly differentiated Grade 3 tumours are assigned a final score of 8-9. The higher the grade, the poorer the prognosis, meaning Grade 3 patients have the worst prognosis [28].

1.1.4.3 Nodal Status

The locoregional lymph node status is one of the most useful prognostic factors in early stage breast cancer. Patients with negative lymph node have a 15-30% risk of recurrence compared to 70% in those with lymph node involvement [29]. The higher the number of nodes involved, the worse the prognosis [30-31]. Nodal status is graded from N0-3 with N0 meaning no involvement, N1 meaning <4 positive lymph nodes, N2 meaning 4-9 positive lymph nodes and N3 ≥ 10 [32].

1.1.4.4 Metastasis

The presence of metastases, spread to other organs, is observed in less than 10% of newly diagnosed breast cancers and around 30% of patients with early breast cancer will develop metastasis [33]. The most common distal site is bone, the second is lung, closely followed by brain and finally liver. After the development of metastases, treatment is palliative and aims to prolong survival while managing symptoms and maximising quality of life [33].

1.1.5 Pathological grading systems

1.1.5.1 The TNM staging system

Breast cancer is commonly classified using the TNM system. TNM staging integrates the tumour size (T), whether there is lymph node involvement (N) and if metastasis has occurred (M). These are grouped into 4 different stages with various substages, and prognosis worsens the higher the stage (Table 1.2).

Stage	Size	Lymph Node	Metastases	5 year survival
I	T1 (<2cm)	N0 (no LN)	M0 (no metastases)	92%
IIa	T0 (no evidence of tumour)	N1 (<4 LN)	M0	82%
	T1	N1	M0	
	T2 (2-5cm)	N0	M0	
IIb	T2	N1	M0	65%
	T3 (>5cm)	N0	M0	
IIIa	T0-T2	N2 (4-9 LN)	M0	47%
	T3	N1-2	M0	
IIIb	T4 (grown into chest wall or skin)	Any N	M0	44%
	Any T	N3 (≥ 10 LN)	M0	
IV	Any T	Any N	M1 (distant metastases)	14%

Table 1.2: TNM staging of breast cancer. *The stage of cancer is based on the size of the tumour, lymph node involvement and if any metastases are present. 5 year survival (%) decreases with increased stage. LN = number of lymph nodes involved (Table adapted from similar in [34]).*

1.1.5.2 Nottingham prognostic index (NPI)

The Nottingham prognostic index (NPI) is used to stratify patients with primary operable breast cancer into prognostic groups. It uses tumour size, grade and lymph node involvement, calculated by:

(Tumour size in cm x 0.2) + tumour grade (1 = good, 2 = moderate, 3 = poor) + tumour stage (based on lymph node status, 1 = no involvement, 2 = 1-3 axillary lymph node or 1 internal mammary lymph node involved, 3 = ≥ 4 axillary lymph nodes or an axillary and internal mammary lymph node involved).

Based on the NPI there are three prognostic categories. Patients with a score of <3.4 have a good prognosis, a score of 3.4 - 5.4 results in a moderate prognosis and patients with a score of >5.4 have a poor prognosis [35-36].

1.1.5.3 Clinical stages of breast cancer

Breast cancer can present at several stages at diagnosis from early breast cancer to locally advanced breast cancer and advanced metastatic breast cancer. The clinical stage at presentation is an important factor in deciding treatment options. The majority of women diagnosed with breast cancer present with early breast cancer that is stage I-II [37]. These tumours are typically treated surgically. These patients also often receive radiotherapy and in many cases receive systemic adjuvant therapy, as discussed later.

Around 6% of breast cancers are stage III at diagnosis and described as locally advanced, which includes tumours that are large and those that have spread to the lymph nodes or into other tissues around the breast [38]. Standard treatment of locally advanced breast cancer is typically systemic therapy and this may enable surgery when tumours were previously inoperable due to their size. Inflammatory breast cancer, which typically conveys a poor prognosis, accounts for 2.5% of breast cancers and is treated with chemotherapy followed by mastectomy and radiotherapy [39]. Less than 10% of women newly diagnosed breast cancer present with metastatic disease, stage IV. As previously mentioned, when metastases are present treatment is palliative with systemic treatment which aims to prolong survival and alleviate symptoms to maximise quality of life [33].

The tissue cohorts studied in this thesis include patients with early invasive breast cancer and some patients with locally advanced disease.

1.1.6 Molecular prognostic factors

As well as pathological prognostic markers relating to the appearance and stage of the tumour, there are also molecular markers that can be employed in diagnostic laboratories. These molecular markers, which are based on the presence of certain proteins in the tumour, contribute to the patient's prognosis and when present allows selection of appropriate targeted therapy.

1.1.6.1 Oestrogen receptor

The female sex hormones (oestrogen and progesterone) are important in development and growth of the breast and as previously described many risk factors associated with breast cancer are linked to oestrogen. Around 75% of breast cancers express oestrogen receptor α (ER), with their main growth stimulus being oestradiol [40]. ER functions as a ligand activated transcription factor and controls the transcription of many genes involved in cell proliferation and survival, which in breast cancer results in growth and progression. Oestrogen binds ER causing the formation of a homodimer that binds to oestrogen response elements (EREs) in target genes and controls their expression. The presence of co-regulators is important in the precise cellular response. ER also exhibits crosstalk with several growth factor receptor and cell signalling pathways [41]. There are 2 isoforms, ER α and ER β , but only ER α is tested for and used as a prognostic biomarker for breast cancer [42].

The main testing strategy for ER is immunohistochemistry (IHC) and this is scored via the Allred scoring method, which uses both the proportion of positive cells and staining intensity. Tumours are scored on a scale of 0-5 for percentage positive (0 = no positive, 1 = < 1/100 positive, 2 = 1/100 to 1/10, 3 = 1/10 to 1/3, 4 = 1/3 to 2/3, and 5 = \geq 2/3 cells positive) and 0-3 for intensity (0 = negative, 1 = weak, 2 = intermediate, 3 = strong). The two scores are combined and the tumour is given a score of 0 or 2-8. A score of 3 or more is considered ER positive. The use of immunohistochemistry for ER testing is superior to the previously used ligand-binding assay [43].

1.1.6.2 Progesterone receptor

The progesterone receptor (PgR) is another steroid hormone receptor and may be a useful marker in breast cancer to predict how likely it is a tumour will respond to endocrine therapy. ER regulates expression of PgR and it is thought to represent a functional ER pathway. Around 55% of tumours are ER+ PgR+, 22% are ER- PgR-, 20% are ER+ but PgR- and 3% are PgR+ but ER- [40]. The presence of PgR makes endocrine therapy more effective [44] and some patients with ER- PgR+ breast cancer may respond to endocrine therapy.

1.1.6.3 Human Epidermal Growth Receptor 2

Around 15-20% of breast tumours overexpress Human Epidermal Growth Factor Receptor 2 (HER2, also known as ERBB2), and are described as HER2 positive. These tumours tend to be faster growing with a poor prognosis; however therapies targeting HER2 signalling can be used [45]. IHC can be used to test for overexpression, and is scored with a semiquantitative method dividing expression into 4 groups:

- 0, negative = 10% of cells or less show membrane staining
- 1+, negative = a faint partial staining of the membrane in more than 10% of cells
- 2+, borderline = weak to moderate staining in more than 10% of cells
- 3+, positive = strong staining of whole cell membrane is observed in over 30%.

Tumours are considered HER2 positive if 30% of cells have staining of the complete cell membrane. Those tumours with a 2+ score are borderline and should be tested for amplification of HER2 using fluorescent *in situ* hybridisation [46].

1.1.6.4 Ki67

The proliferation rate of a tumour is important in the prognosis of breast cancer. Ki67 is a nuclear antigen found when cells are proliferating (in G1, S, G2 or M phase of the cell cycle) but not in cells that are in G0 phase and therefore not proliferating. The development of a monoclonal antibody, MIB-1, which detects Ki67 has allowed immunohistochemical studies to use this marker to assess tumour proliferation. Expression of Ki67 correlated with clinical outcome in several studies [47].

For any marker to be used in routine testing, it is important guidelines and protocols are established to allow for reproducibility and standardisation of results, and appropriate cut off values must be defined. Guidelines for Ki67 have still to be established, however an expert panel at the St. Gallen International Breast Cancer Conference in 2011 supported the use of Ki67 staining to routinely split the luminal subtypes once guidelines are available [48].

1.1.7 Breast cancer subtypes

Gene expression analyses of over 8000 genes in human breast tumours have been used to categorise them into subtypes based on these molecular profiles. Hierarchical clustering grouped expression patterns of genes correlating to proliferation, oestrogen receptor, HER2 signalling and genes expressed in basal epithelial breast cells [49]. The breast cancer samples were then grouped based on the gene expression patterns of the clusters of genes from each category into four subtypes. These are the ER+/luminal-like subtype, the basal-like subtype,

HER2-enriched subtype and normal breast-like subtype [49]. Since this initial study additional subtypes have been added with the division of the luminal subtype into luminal A and B, and the inclusion of the claudin-low subtype [50-51].

Both the luminal A and B subtypes show expression of ER, PgR or ER associated genes such as GATA3 and some genes expressed in luminal epithelial cells such as cytokeratins 8 and 18. Luminal A is the most common subtype, accounting for around 40% of breast cancers. Around 20% of tumours are luminal B and can be distinguished from luminal A due to higher expression of proliferation- and/or HER2-related genes and lower expression of ER-related genes. The HER2-enriched group accounts for 15-20% of breast cancers. These tumours exhibit high expression of HER2-related genes such as those involved in HER2 signalling as well as neighbouring genes which are overexpressed with HER2 due to gene amplification. The basal-like and claudin-low groups do not express ER, PgR or HER2 and are closely associated to triple negative breast cancers. Basal-like tumours are categorised using expression of basal epithelial genes such as cytokeratin 5, 14 and 17, and HER1. This subtype also shows high expression of genes involved in proliferation. The claudin-low subtype exhibits expression of genes related to cell adhesion and interaction. In the original study by Perou *et al* [49] about 5-10% of breast tumours were placed in the normal breast group, however many believe it is a technical artefact due to contamination with normal tissue.

Gene expression profiling is continuing to evolve with genetic drivers for each subtype being investigated. The Cancer Genome Atlas Network has recently identified many copy number variations and genetic mutations associated with the different subtypes [52].

The development of different subtypes highlights the variable profiles in breast cancer and the need to consider it as not one disease but a collection of molecularly distinct breast diseases. The different molecular profiles mean tumours are likely to respond differently to various therapies and this should be considered as much as possible when deciding on treatment.

1.1.8 Tests for molecular profile of breast cancer

The aim of translational medicine is to gain insight into mechanisms of disease in order to develop new therapeutics and identify which current treatments will be of most benefit to the patient. Understanding what drives the progression of breast cancer, and identifying a marker that can be tested for, may mean treatment can be tailored for each patient.

Several tests have been developed to generate gene expression profiles for individual patients to help predict which therapy they will respond to and prevent the use of chemotherapy in those for whom it is unnecessary. Pam50 is a real time polymerase chain reaction (PCR) based assay that measures expression of 50 genes to give a gene signature and classify tumours into the four intrinsic subtypes (luminal A, luminal B, HER2 enriched and basal-like). Oncotype DX is a real time PCR based assay for 21 genes, which gives a recurrence score to ER positive, node-negative patients, to predict recurrence at 10 years. Another assay, MammaPrint, uses microarray technology to assess expression of 70 genes to categorise patients with stage I or II cancer which is node negative and <5cm into those with good and poor prognosis [53].

Gene expression assays provide large amounts of data on multiple genes, however, these are not yet routinely used and a method of classifying breast cancer into subtypes using simpler methods is required. Cheang *et al* proposed using an immunohistochemical analysis of ER/PgR, HER2 and Ki67 to identify the subtypes (as in Table 1.3), and an expert panel at the St. Gallen International Breast Cancer Conference in 2011 supported this method [48, 54]. This allows the classification of tumours based on markers that are already routinely tested for with the exception of Ki67, which is used to subdivide the luminal subtypes. The St. Gallen panel suggested that if Ki67 index is unavailable another measure could be used such as a histological grade [48]. Although several studies have included a basal marker, which is positive in the basal-like subtype, such as cytokeratin 5/6, staining is not sufficiently reproducible and the inclusion of these markers for clinical decision-making was not supported [48].

Subtype	Markers	Prognosis	Frequency
Luminal A	ER+ and/or PgR+, HER2-, low Ki67	Good	~40%
Luminal B	ER+ and/or PgR+, HER2+ or high Ki67	Intermediate/ poor	~20%
HER2 enriched	ER-, PgR-, HER2+	Poor	~20-30%
Triple negative	ER-, PgR-, HER2-	Poor	~15%

Table 1.3: Subtypes of breast cancer based on routine IHC markers. For each subtype the IHC markers used to distinguish them, prognosis and approximate frequency of breast cancers are shown. (adapted from similar in [48], with addition of percentages from [55]).

1.2 Treatment of breast cancer

The National Institute for Health and Clinical Excellence (NICE) and Scottish Intercollegiate Guidelines Network (SIGN) have both published guidelines to improve and standardise breast cancer treatment in the UK [56]. The treatment of breast cancer varies depending on the characteristics of the tumour such as size and the presence of certain markers for targeted/hormonal therapy.

1.2.1 Surgery

Women with invasive breast cancer may undergo breast conservation surgery or mastectomy, the choice of which is dependant on a number of tumour and patient specific factors.

1.2.1.1 Mastectomy

Mastectomy, surgical removal of the breast, remains the primary treatment of invasive breast cancer when patients present with large tumours and also in some patients who decide to undergo mastectomy after being fully informed of all options.

1.2.1.2 Breast conserving surgery

Many women now have the option of breast conserving surgery instead of a complete mastectomy. This surgery removes the tumour and some surrounding tissue [57]. Radiotherapy is given after surgery and patients may require further surgery if radial tumour margins are not clear [56]. Breast conserving surgery tends to be more cosmetically and psychologically preferable to patients.

1.2.1.3 Axillary surgery

If there is no evidence of axillary involvement, sentinel lymph node biopsy is the recommended management. Dye is injected into breast tissue and the first 1 to 4 nodes are removed to evaluate if any local metastasis has occurred. If metastases are present, axillary lymph node dissection or radiotherapy of the axilla is then performed [56-57].

1.2.2 Chemotherapy

Chemotherapy is an effective adjuvant treatment for many women, reducing recurrence in women under 50 by 35% and 50-69 by 20%, and mortality in the under 50s by 27% and 11% in 50-69 year olds [58]. However, certain types of breast cancer are likely to be unresponsive to chemotherapy and patient selection is therefore extremely important to avoid unnecessary risks and side effects for patients who are unlikely to benefit from the treatment. ER positive tumours tend to be less responsive than ER negative tumours, however patients with luminal B tumours are often chemosensitive [59]. There is a great need to find a way to identify those patients with microscopic disease that will be sensitive to chemotherapy therefore increasing disease free and overall survival and reducing side effects from unnecessary treatment [60]. The Optimal Personalised Treatment of early Breast Cancer using Multiparameter Analysis (OPTIMA) trial began in the UK in 2012 with the aim of testing whether standard therapy (chemotherapy and endocrine therapy for all patients) or test-directed therapy (chemotherapy given only to patients with high risk scores, and endocrine therapy to all) is more effective. It also aims to identify which test, e.g. Oncotype DX, Pam50, MammaPrint, IHC4, is most cost-effective and appropriate [60].

Chemotherapy can also be offered as neoadjuvant therapy to patients with inoperable breast cancer to downsize large tumours. In these patients often the only surgical option is mastectomy and neoadjuvant therapy may shrink the tumour meaning breast conservation could become an option [56].

1.2.3 Radiotherapy

Radiotherapy is recommended after breast conserving surgery and after mastectomy in patients with a high or intermediate risk of recurrence [56]. The Standardisation of Breast Radiotherapy (START) trials began in 1999 to investigate the effectiveness of different radiotherapy schedules (number of doses and size of dose) following surgery. Lower overall doses delivered in fewer doses of a larger size (hypofractionation) were as safe and effective as the standard high dose previously given [61].

1.2.4 Targeted therapy

Presence or over-expression of certain markers can subdivide breast cancer and guide treatment. ER positive tumours are treated with hormonal therapy (discussed later) and HER2 positive patients can receive therapies that target HER2.

The development of targeted therapies for HER2 positive breast cancer has greatly improved the outcome of patients with these tumours, which tend to be faster growing. Trastuzumab (branded as Herceptin) is a monoclonal antibody that is directed against the extracellular domain of HER2 receptor and interferes with HER2 signalling, inhibiting cell proliferation [62]. The first trial of this therapy showed that in metastatic breast cancer overexpressing HER2, the addition of trastuzumab in combination with chemotherapy resulted in longer time to disease progression and longer survival when compared to chemotherapy alone [62]. In the HERA (Herceptin Adjuvant) trial of early breast cancer patients with HER2 positive tumours, the use of trastuzumab after chemotherapy improved the disease free survival in comparison to chemotherapy alone [63].

In addition to trastuzumab, additional HER targeted therapies are being developed. Lapatinib, a type 1 tyrosine kinase inhibitor, blocks both HER1 and HER2 and therefore disrupts signalling to downstream mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways. Advanced breast cancer following progression on trastuzumab is reported to respond better to lapatinib in combination with chemotherapy compared to chemotherapy alone [64]. Additionally, the use of lapatinib and trastuzumab together has been observed to be more effective than lapatinib alone [65-66]. Dual targeting allows synergistic effects and overcomes the primary or acquired resistance to either of the agents. Recently an antibody-drug conjugate, trastuzumab emtansine (T-DM1) was developed, which has HER2 anti-tumour action of trastuzumab with the addition of cytotoxic properties of derivative of maytansine (DM1), a microtubule inhibitory agent. This allows more cytotoxic HER2 targeting and reduces exposure in normal tissue [67]. The EMILIA study was designed to investigate the safety and efficacy of T-DM1 in HER2 positive advanced breast cancer patients who previously were treated with trastuzumab and chemotherapy. Patients treated with T-DM1 had delayed time to progression compared with patients treated with lapatinib and chemotherapy [67].

Unfortunately there are no effective targeted therapies for triple negative breast cancer (TNBC). These tumours do not express the markers currently used for targeted therapies (ER/PgR and HER2) meaning endocrine therapies and HER2 targeted therapies are

ineffective. TNBC has relatively poor prognosis and the only adjuvant therapy available is chemotherapy. Several targets are being investigated, such as the PI3K pathway [68].

1.2.5 Hormonal therapy

Many tumours depend on oestrogen for their growth, and hormonal (or endocrine) therapy is used to treat tumours that are ER positive, either as adjuvant therapy following surgery for early cancer or as neoadjuvant treatment to shrink tumours before surgery. There are three main categories of endocrine therapy with different mechanisms of action (shown in Figure 1.4):

- 1) Selective oestrogen receptor modulators (SERM) prevent oestrogen from binding to ER.
- 2) Selective oestrogen receptor degraders (SERD) stimulate degradation of ER.
- 3) Aromatase inhibitors (AI) decrease the levels of estrogen by binding to the aromatase enzyme.

1.2.5.1 SERMs

For several decades the anti-oestrogen tamoxifen has been the gold standard endocrine therapy for ER positive breast cancer patients. Tamoxifen is a selective oestrogen receptor modulator (SERM) that binds to the ER, altering the conformational form. This interferes with the interaction with nuclear transcriptional co-activators and results in altered downstream effects. SERMs have mixed agonist and antagonist activity which depending on the target organ. In the breast it has antagonistic activity and therefore acts as an anti-oestrogen. Analysis of randomised trials of tamoxifen show that when used as an adjuvant therapy, it increases overall and disease free survival and reduces mortality [69]. Although tamoxifen is better tolerated than chemotherapy, it has several adverse effects such as an increase in the risk of endometrial cancer [70]. An additional challenge is that many patients do not respond to this therapy despite being ER positive and a high proportion of tumours, which initially respond, develop resistance.

1.2.5.2 SERDs

Selective oestrogen receptor degraders (SERD) are another class of anti-oestrogens that act to degrade the ER and therefore block ER dependent signalling. One example of a SERD is fulvestrant (FaslodexTM), which enhances ubiquitination of ER α , preventing dimerisation and therefore inhibiting oestrogen dependent gene transcription. It is often used for patients after the development of tamoxifen resistance and trials have shown it to be as effective as tamoxifen when used as a first line treatment [71-72]. The major benefit of this treatment

when compared to tamoxifen is that it acts as a pure antagonist and therefore does not have agonistic effect in the endometrium [73].

1.2.5.3 Aromatase inhibitors

Aromatase inhibitors (AIs) are now the first choice treatment for post-menopausal breast cancer patients. They bind to the aromatase enzyme and inhibit the conversion of androgens (testosterone and androstenedione) into oestrogens (oestradiol and oestrone). There are two classes, Class I (e.g. exemestane) bind irreversibly and Class II (e.g. letrozole and anastrozole) bind reversibly, both of which decrease circulating levels of oestrogen [74]. AIs are most effective in post-menopausal women as most of the circulating oestrogen is produced by peripheral aromatase. However in pre-menopausal women where the ovaries remain active, significant toxicity is observed with AIs, so tamoxifen remains the endocrine therapy of choice in these patients [74].

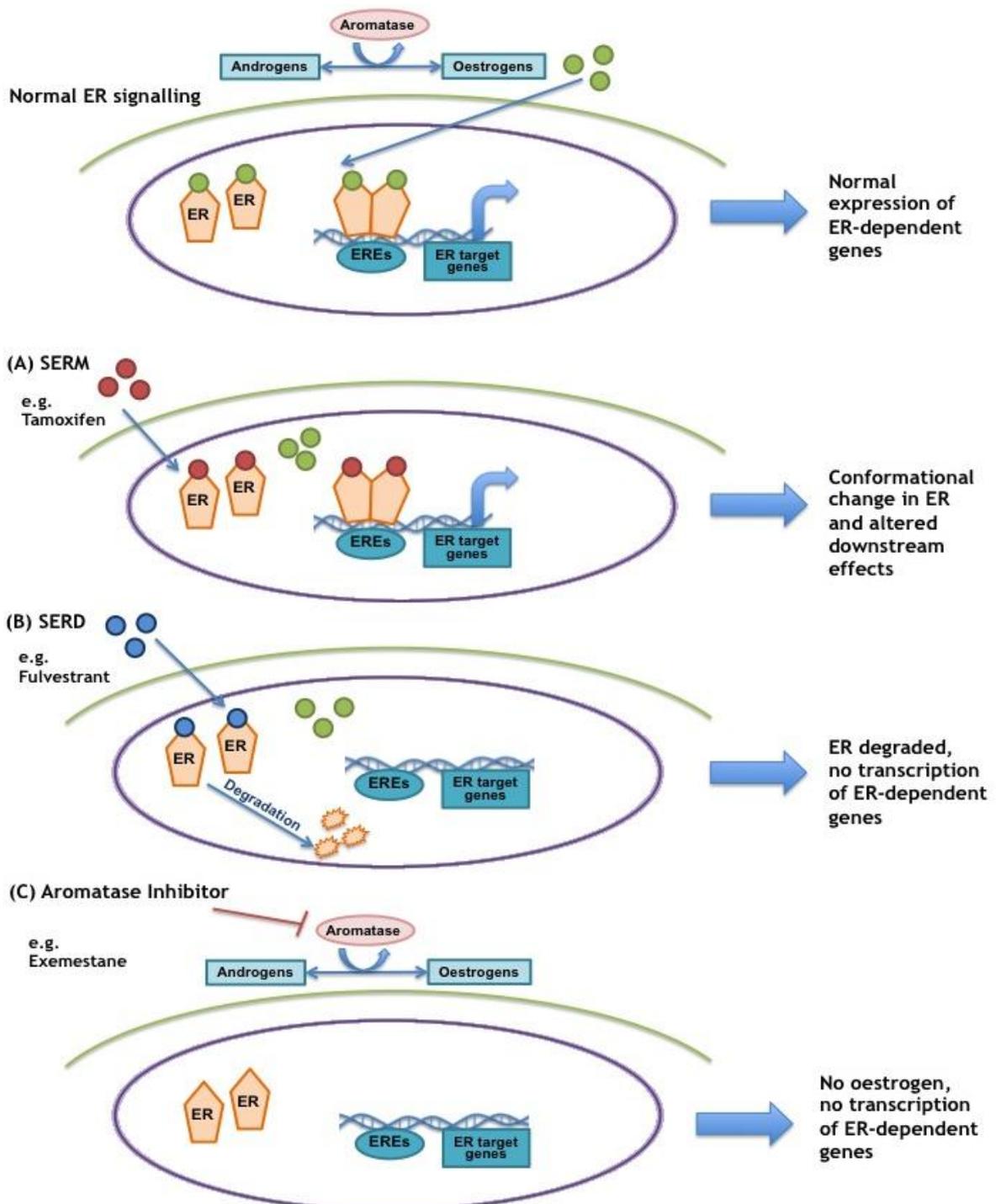


Figure 1.4: Mechanisms of action of different endocrine therapies. *In normal ER signalling androgens are converted to oestrogens by aromatase, oestrogen binds ER, which dimerises and binds to oestrogen responsive elements (EREs) resulting in transcription of ER-dependent genes. (A) Selective oestrogen receptor modulators (SERM) competitively bind to ER causing a conformational change and alterations downstream. (B) Selective oestrogen receptor degraders (SERD) enhance ubiquitin-mediated degradation of ER and oestrogen is therefore unable to bind. (C) Aromatase inhibitors (AI) stop the production of oestrogen, preventing dimerisation of ER and ER dependent gene transcription is blocked. (Adapted from [74]).*

1.2.6 Endocrine resistance

Although endocrine therapy is highly effective and beneficial to ER positive patients, a major problem is endocrine resistance. Resistance can be *de novo* (existing before treatment) or acquired (developed during therapy) and several mechanisms have been proposed for resistance.

Lack of ER expression is the primary mechanism of *de novo* resistance. Other causes of *de novo* resistance have been identified such as mutations in cytochrome P450 2D6 enzyme, which metabolises tamoxifen into its active form, endoxifen [75]. Mutations have been classified into three groups: silent mutations which result in a fully functioning enzyme, those which result in intermediate metabolism due to reduction in enzyme activity and finally those with poor metabolism as a result of no protein expression or expression of a protein without enzymatic activity. Patients with less functioning enzyme have poorer metabolism and therefore lower rate of conversion of tamoxifen to endoxifen [75].

There have been several mechanisms that are thought to contribute to acquired (and some possibly also to *de novo*) resistance, where patients initially respond to therapy but subsequently relapse. Loss of ER expression after tamoxifen treatment was found to occur in 17% of tumours [76]. However many resistant tumours are responsive to other endocrine treatments after recurrence. These tumours retain ER expression and it has been hypothesised that the cell finds an escape pathway. Epidermal growth factor receptor (EGFR) and HER2 expression and downstream signalling such as PI3K and MAPK pathways have been suggested as escape pathways by providing an alternative survival pathway. In matched tissue from before and after recurrence on tamoxifen, ER and HER2 initially showed inverse correlation but in the tamoxifen resistant tumours this was lost. Instead, a correlation between ER and phosphorylation of MAPK was observed [76]. An increase in phosphorylation of Akt, which is downstream of PI3K signaling, is observed in tamoxifen resistance breast cancer cells [77]. Combination treatments targeting both ER and growth factor signalling and therefore blocking crosstalk between the pathways and eliminating escape routes may be effective at reducing resistance to therapy. Everolimus has been developed as an inhibitor of mammalian target of rapamycin (mTOR), a downstream component of PI3K signalling activated by Akt. The use of everolimus and aromatase inhibitors in combination has been found to result in synergistic induction of apoptosis and inhibition of proliferation [78]. The Breast Cancer Trials of Oral Everolimus 2 (BOLERO-2) trial was designed to compare the use of everolimus and the aromatase inhibitor exemestane to exemestane alone in metastatic breast cancer. Patients who received everolimus plus exemestane exhibited improved

progression-free survival compared to patients treated with exemestane alone [79]. Another trial in post-menopausal patients with metastatic breast cancer tested the effectiveness of combining tamoxifen plus everolimus (TAMRAD) in resensitising tumours to endocrine therapy. This study found that this combination was effective in patients with acquired endocrine resistance resulting in a reduction in mortality risk and an overall increase in clinical benefit rate and time to disease progression [80].

Coregulatory proteins have also been implicated in resistance to tamoxifen. Amplified in breast cancer 1 (AIB1, also known as SRC3) is an ER coactivator, which can be activated by HER2. High expression of AIB1 reduces the antagonist effects of tamoxifen in tumours that also overexpress HER2, resulting in poor outcome in these patients [81]. In MCF7/HER2 cells, which express high levels of HER2 and AIB1, tamoxifen has agonistic effects and it is thought that this results in *de novo* resistance [82]. Tamoxifen may therefore stimulate growth of the tumour in patients with high AIB1 and HER2. Downregulation of corepressors are also documented in endocrine resistance. The loss of corepressor NCoR results in loss of antagonist activity of tamoxifen [83]. The expression or activity of corepressors and coactivators appears to modulate the agonist and antagonist activity of tamoxifen and therefore are likely to be of importance in resistance.

The complex network of ER signalling, with its crosstalk with growth factor pathways and interaction with coregulatory proteins, is important in mediating the progression of breast cancer. Further investigation is needed into these mechanisms and the possibility of combination therapy. Patients that will benefit most from dual targeting need to be identified. Although tamoxifen and other endocrine therapies are effective in ER positive tumours, endocrine resistance remains a clinical problem and the mechanisms behind progression are not yet fully understood. The Gap Analysis Working Group identified molecular mechanisms driving resistance to treatment as one of the top 10 gaps in research that would make the biggest clinical impact if filled [84].

1.2.7 Summary on breast cancer treatment

The primary treatment of breast cancer remains to be surgery, either mastectomy or lumpectomy. Adjuvant therapy after surgery includes chemotherapy, radiotherapy, anti-HER2 targeted therapy in HER2 overexpressing tumours and endocrine therapies in ER positive tumours. These therapies may also be used in the neoadjuvant setting before surgery. Different subtypes of breast cancer are more responsive to certain therapies and treatment is tailored accordingly (as detailed in Table 1.4). Despite the development of many effective therapies, there remains a need for novel therapeutics for TNBC and for patients who have developed endocrine resistance. In order to do so, the mechanism of recurrence in different subtypes of patients requires further investigation to predict what patients will benefit most from certain treatments and to identify novel targets.

Subtype		Therapy
Luminal A (ER/PgR+, HER2-, low Ki67)		Endocrine - tamoxifen, or AIs (postmenopausal) - not many require chemotherapy
Luminal B (ER/PgR +)	HER2 -, high Ki67	Endocrine ± chemotherapy
	HER2 +	Chemotherapy + HER2 targeted + endocrine
HER2 enriched (ER/PgR- HER2+)		Chemotherapy + HER2 targeted
Triple negative (ER-, PgR-, HER2-)		Chemotherapy

Table 1.4: Effective therapies for the different breast cancer subtypes (adapted from [48]).

1.3 The NF- κ B pathways

1.3.1 Cell growth mechanisms and signalling pathways in cancer

There are many different pathways involved in the regulation of cellular proliferation and impaired signal transduction of many of these has been implicated in cancer. Targeting of these pathways may be an appropriate therapeutic option in many cancers, including breast cancer.

Mutations in the PI3K pathway have been found to be frequent in cancer and dysregulation of MAPK signalling has also been suggested to play an important role in many cancers [85-86]. Aberrations in genes involved in cell cycle regulation, such as cyclins and cyclin-dependent kinases (CDKs), are also evident [87]. An inhibitor of CDK4/6, which form a complex with cyclin D1, has been demonstrated to inhibit proliferation in ER positive and HER2-amplified breast cancer cells and may therefore be beneficial in combination with endocrine or anti-HER2 therapy [88].

Other pathways that have an emerging role in cancer progression and development are the NF- κ B pathways. This family of transcription factors regulates the transcription of many genes involved in cell growth and apoptosis and may therefore be involved in oncogenesis [89].

The mammalian Nuclear Factor kappa B (NF- κ B) was first identified in 1986 as a transcription factor that binds a specific DNA sequence within the immunoglobulin kappa light chain gene in mature B cells [90]. It has since been found to regulate transcription in many different cells by binding sequences known as κ B sites in the promoters or enhancers of many genes. There have been two main NF- κ B pathways identified, the canonical and non-canonical pathways [91].

1.3.2 Members of the NF- κ B family

There are five different NF- κ B family members, which form homo/heterodimers to function as transcription factors: NF- κ B1 (or p50 and its precursor p105), NF- κ B2 (or p52 and its precursor p100), RelA (p65), RelB and c-Rel. All family members contain a conserved N-terminal 300 amino acid Rel homology region that is responsible for homo- and hetero-dimerisation, interaction with inhibitor proteins and contains the nuclear localisation signal (NLS) [92]. RelB, c-Rel and p65 also contain a transactivation domain, which is essential for transcriptional activity. Homo- and hetero-dimers of the NF- κ B family members are present in most cells and normally, when in an inactive state, inhibitory proteins (I κ Bs) bind to NF- κ B dimers, masking the NLS and holding them in the cytoplasm. There have been 8 inhibitory proteins identified: I κ B α , I κ B β , I κ B ϵ , I κ B ζ , BCL-3, precursor proteins p100 and p105 and most recently I κ B η [93]. These inhibitory proteins are characterised by a motif of 5-7 ankyrin repeats in their C-terminal region. This motif is also present in the precursor proteins p100 and p105, which also inhibit nuclear localisation, and therefore transcriptional activity, of NF- κ B. I κ B α and I κ B β also contain a PEST domain, rich in proline, glutamine, serine and threonine, which regulates turnover [94]. Upon phosphorylation of I κ B by an I κ B kinase (IKK), the I κ B protein is ubiquitinated and subsequently degraded by the proteasome. This releases the NF- κ B dimer allowing it to translocate to the nucleus where it binds to κ B sites, along with co-activators or co-repressors, and regulates the transcription of target genes. Transcriptional output differs depending on the context, with the binding of different subunits, co-regulators, and crosstalk with other transcription factors [89]. Target genes are involved in many processes including proliferation, apoptosis, inflammation and immune response.

1.3.3 The canonical NF- κ B pathway

The stimulus of the canonical pathway is usually pro-inflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin-1 β (IL-1 β). Binding of the ligand to its receptor results in recruitment of the IKK complex, which consists of catalytic subunits IKK β and IKK α and the regulatory subunit NF- κ B essential modulator (NEMO) (see Figure 1.5). Activation of the IKK complex occurs either by trans-autophosphorylation or via an IKK kinase such as TAK1 [95]. Experiments with knockout mice have shown that IKK β is the predominant IKK in the canonical pathway [96]. IKK β phosphorylates the I κ B, which is typically I κ B α bound to p65/p50 dimers. The p50 subunit is encoded as a precursor of 105kDa that is constitutively processed to form p50 [97]. Crystallographic structures of I κ B α bound to p65/p50 demonstrate that it is only the p65 NLS that is covered and the NLS of p50 remains unmasked [98-99]. The NLS of p50 and the nuclear export sequence (NES) of I κ B α both being accessible means it is possible for shuttling of the I κ B α /p65/p50 complex between the nucleus and cytoplasm to occur, but steady state localisation of the complex is in the cytoplasm [100-101]. Phosphorylation and subsequent ubiquitination of I κ B results its degradation, releasing the NF- κ B dimer. IKK β , as well as other kinases, phosphorylates the p65 subunit at serine residue 536 (S536) in the transactivation domain (TAD). This is important for the transcriptional activation of the NF- κ B dimer [102]. Degradation of I κ B α means there is no longer a NES in the complex and the NLS of p65 and p50 are unmasked, meaning localisation is then nuclear. The NF- κ B dimer, once translocated to the nucleus, regulates transcription of various genes. One of these genes is I κ B α and there has therefore been a negative feedback mechanism identified. Binding of the p65/p50 dimer results in I κ B α resynthesis and I κ B α is then exported back to the cytoplasm, binding NF- κ B and inactivating the pathway. Protein phosphatase 2A is a key regulator of this feedback loop as it dephosphorylates IKK β allowing for the stabilisation of the newly synthesised I κ B α in the cytoplasm, which is then able to bind the NF- κ B dimer [103]. An amplifying loop also exists as cytokines such as TNF α and IL-1 β are also among the genes transcribed, as well as anti-apoptotic genes and others involved in innate immunity such as adhesion molecules intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [104].

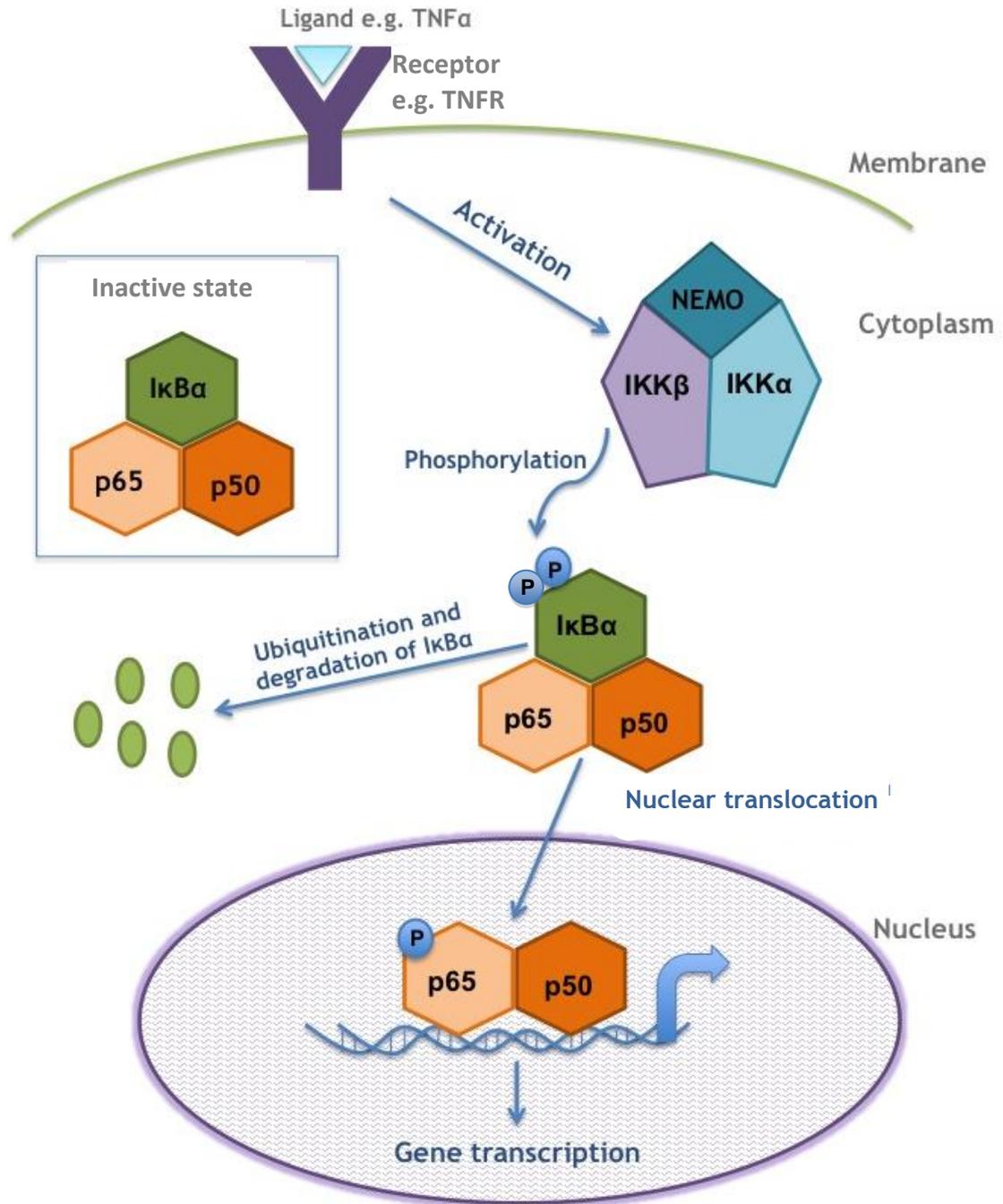


Figure 1.5: The canonical NF- κ B pathway. In an inactive state, I κ B α holds NF- κ B dimers of p65/p50 in the cytoplasm. Upon binding of the ligand (such as TNF α) to its receptor the IKK complex, consisting of IKK α , IKK β and NEMO, is activated. IKK β phosphorylates I κ B α , which is ubiquitinated and degraded, unmasking the NLS of p65 and the dimer translocates to the nucleus. Phosphorylation of p65 also occurs in the TAD, which is important for transcriptional activation. In the nucleus the p65/p50 dimer binds to κ B sites of certain genes and regulates their transcription.

1.3.4 The non-canonical NF- κ B pathway

A second pathway, the non-canonical or alternate NF- κ B pathway, was identified in 2001 (Figure 1.6). This requires IKK α kinase activity and unlike the NF- κ B canonical pathway is independent of IKK β and NEMO [105-106]. This non-canonical NF- κ B pathway is activated by ligands such as the B Cell-activating factor, CD40 and lymphotoxin β . Dimers of IKK α are activated by the upstream NF- κ B-Inducing Kinase (NIK). Another difference between the non-canonical and canonical pathway is that the processing of p105 to p50 is constitutive, but processing of p100 to p52 is controlled by activation of the non-canonical pathway. In an inactive state, RelB is sequestered in the cytoplasm by p100, but upon activation of the pathway, p100 is phosphorylated and processed into p52. The processing of p100 to p52 results in the loss of the ankyrin repeats in the C-terminus and allows translocation of the RelB/p52 dimers into the nucleus [107]. Similar to the canonical pathway, a negative feedback loop has been suggested, involving NIK phosphorylation by IKK α . This results in NIK stabilisation and a decrease in non-canonical signalling, therefore preventing over activation [108]. The non-canonical NF- κ B pathway regulates genes with a role in lymphoid organ development and adaptive immunity [104].

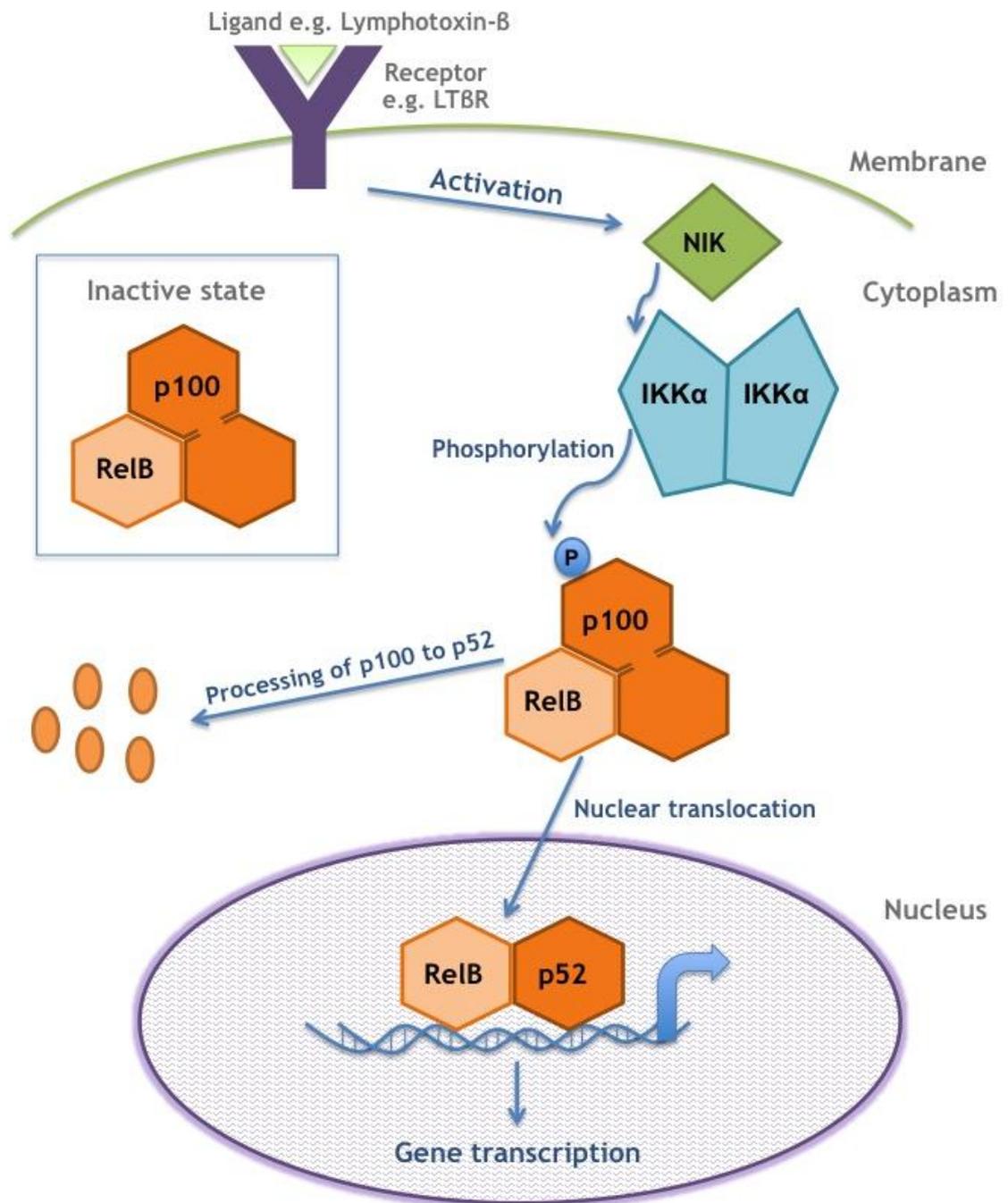


Figure 1.6: The non-canonical NF- κ B pathway. In an inactive state, p100 holds RelB in the cytoplasm. Upon binding of the ligand (such as lymphotoxin β) to its receptor the pathway is activated and NIK phosphorylates IKK α , which is found as a homodimer in the non-canonical pathway. IKK α phosphorylates p100, resulting in its processing to p52. Dimers of p52/RelB translocate to the nucleus, bind to κ B sites of certain genes and regulate their transcription.

1.3.5 Functions of the IKKs

In the NF- κ B pathways, the IKKs serve to induce I κ B degradation and release NF- κ B dimers, as well as being involved in post-translational modification of the NF- κ B subunits. There have also been other functions of each IKK identified, which are independent of NF- κ B signalling [91].

1.3.5.1 IKK β

IKK β has been found to be important for normal development and IKK β knockout mice die after embryonic day 12 [109]. Further investigation by histological examinations revealed apoptosis of the liver to be the cause of death in these mice embryos [109]. This is likely due to canonical NF- κ B signalling as p65 knockout mice also died at a similar time in development [110]. It was shown that in IKK β deficient mouse embryonic fibroblasts, IKK α remained expressed but I κ B α was not degraded and NF- κ B was not activated by TNF α [109]. This demonstrates that IKK β , but not IKK α , is essential for canonical signalling. IKK β is involved in regulating inflammation and cell survival through the activation of NF- κ B dependent gene transcription. In cells expressing a dominant-negative form of IKK β , TNF α failed to induce expression of inflammatory genes such as IL-8 as well as ICAM-1 and VCAM-1 [111-112].

1.3.5.2 IKK α

Despite sharing 52% identity, IKK α and IKK β have distinct roles, both NF- κ B dependent and independent. Like IKK β , IKK α knockout mice are not viable however they die perinatally around 30 minutes - 4 hours after birth [113-114]. These mice showed various birth defects with shorter limbs and tails, which was thought to be due to decreased expression of the transcription factor Twist. They also showed skin defects with taut, shiny skin with no visible wrinkles [113-114]. When stained with Ki67, as a marker of proliferation, there was much higher expression in the basal cell layer of the epidermis of knockout mice than the wild-type. There was also increase in expression of Keratin K14 in the knockout mice. Fillagrin and involucrin (late and early markers of keratinocyte differentiation), also showed altered expression [113]. It has since been shown that the role of IKK α in formation of the epidermis is independent of NF- κ B and is instead dependent on regulation of the secretion of an autocrine factor that induces keratinocyte differentiation [115].

IKK α , unlike IKK β , has an NLS and is therefore able to move into the nucleus to phosphorylate certain proteins. IKK α has been found to have a nuclear role in regulating chromatin structure by inducing histone H3 phosphorylation, a process that is required for the

activation of NF- κ B-directed gene expression [116]. The phosphorylation of histone H3 at Ser10 is also promoted by IKK α in an NF- κ B-independent context. In EGF-stimulated cells IKK α is recruited to the promoter of c-fos and phosphorylates histone H3, and therefore regulates expression [117].

The nuclear co-repressor silencing mediator for retinoid or thyroid-hormone receptors (SMRT) is phosphorylated by IKK α and subsequently removed from chromatin with HDAC3 followed by exportation from the nucleus to the cytoplasm. These events are essential for optimal expression of several NF- κ B-dependent genes including cellular inhibitor of apoptosis 2 (cIAP2) and IL-8 and also for cell survival [118]. Formation of a complex of IKK α , AIB1 and ER α results in regulation of transcription of oestrogen genes such as cyclin D1 and c-Myc. IKK α enhances oestrogen-mediated expression of these genes and increases cell proliferation [119].

Additionally, IKK α and IKK β both have functions in β -catenin-dependent transcriptional activity with IKK β negatively regulating its activity and IKK α increasing β -catenin-dependent transcriptional activation. IKK α therefore also induces cyclin D1 transcription through β catenin [120]. Cyclin D1 levels were decreased in IKK α deficient mouse embryonic fibroblasts. IKK α is also required in the mammary gland during pregnancy for lactation, and mice with an IKK α mutant that is unable to perform its kinase activity have a reduction in proliferation and are therefore unable to lactate [121]. A different study however found that cyclin D1 was phosphorylated by IKK α , resulting in its degradation [122]. A complex crosstalk is therefore evident between the IKKs and pathways involving cyclin D1.

1.3.6 NF- κ B/IKKs and cancer

The NF- κ B pathways regulate the transcription of a wide range of genes involved in the immune response, inflammation, proliferation and apoptosis. Many of these processes are hallmarks of cancer [123] and NF- κ B has been hypothesised to be a link between inflammation and tumourigenesis.

Aberrant NF- κ B signalling has been detected in some leukaemias and lymphoma. This results in dysregulation of expression of genes involved in the regulation of growth and proliferation of lymphocytes such as the cytokines interleukin-2 and interleukin-6. Constitutively active NF- κ B signaling, due to loss or mutation of *I κ B α* , has been observed in Hodgkin's lymphoma [124-125]. Constitutive activity of IKK α has also been reported in Hodgkin's lymphoma [126]. Chronic lymphocytic leukaemia patients have been observed to have constitutively high NF- κ B activity [127] and constitutive activation of the non-canonical NF- κ B pathway has been linked to pathogenesis of multiple myeloma [128].

High levels of NF- κ B activity have also been observed in gastrointestinal cancers. NF- κ B and IKK are constitutively active in pancreatic adenocarcinoma cells. Inhibition decreases expression of NF- κ B regulated genes, which have been implicated in pancreatic cancer such as cyclooxygenase-2 [129]. Colorectal cancer tumours have increased activity of the p65 subunit compared to normal colorectal tissue. This was reported to be associated with higher stage and in colon cancer cells NF- κ B activation correlated with an increase in proliferation [130].

NF- κ B pathways are implicated in prostate cancer, which is also hormonally controlled and develops resistance to anti-hormone therapy. An increase in NF- κ B activity and higher levels of phosphorylated I κ B α were observed in highly invasive PC3 cells compared to PC3 cells with lower invasive potential [131]. A faster turnover of I κ B α has been reported in androgen-independent prostate cancer cells and kinase assays show constitutive activation of IKK in these cells [132]. Tissue studies with prostate cancer patients have demonstrated that activation of the canonical NF- κ B pathway is associated with poor outcome [133], metastasis to the lymph nodes [134] and progression to castrate resistant disease [135]. Additionally, nuclear localisation of RelB is associated with higher-grade tumours [136].

1.3.7 NF- κ B/IKKs and breast cancer

Expression and activity of NF- κ B has been reported to change in the mammary epithelium during pregnancy, lactation and involution [137] and IKK α has an essential role in the proliferation of mammary epithelium during post-natal development [121]. Considering this, along with its role in the regulation of proliferation and apoptosis, it is not surprising that aberrant NF- κ B signalling has been reported in breast cancer.

Elevated expression of NF- κ B and increased DNA binding activity have been reported in breast cancer cell lines, in rat mammary tumours when compared to normal tissue, and in human primary breast tumours [138]. Nuclear expression of the p65 subunit (and therefore activation of the canonical pathway) was found to correlate with higher proliferation index, higher tumour grade and ER negativity in breast tumours [138]. DNA binding activity of p65 and p50 strongly correlated in breast cancer tumour samples, although p50 was more abundant [139]. Kaplan-Meier curves for disease free survival showed that patients with tumours displaying higher p50 DNA binding activity had poorer disease free survival than those with lower activity [139].

Subunits involved in both the NF- κ B pathways have been investigated. Both the p50 subunit from the canonical NF- κ B pathway and the p52 subunit from the non-canonical NF- κ B pathway are expressed in the nucleus of breast cancer tumours. When immunohistochemistry was employed on breast cancer tissue and adjacent normal tissue, expression of the p50 and p52 subunits were significantly higher in the cancer tissue. Western blots of nuclear fractions extracted from cancerous and adjacent normal breast tissue also showed an increase in p52 in breast cancer [140]. This was accompanied by an increase in mRNA levels of κ B dependent genes including p50, p52, inhibitory proteins IkB α and Bcl-3 and cyclin D1, which likely represents an increase in transcription of genes regulated by NF- κ B [140]. In mice that transgenically overexpress p100/52, a delay in mammary gland development is observed as well as an increase in cyclin D1, MMP2, MMP9 and COX-2 expression. These mice developed multiple tumours and although during mammary gland development p100/52 levels were low, during tumourigenesis expression of p100/52 was elevated [141].

Several studies have shown a correlation of NF- κ B activity with oestrogen independence. In breast cells lines, elevated levels of NF- κ B DNA binding activity were observed in ER negative cells in comparison to ER positive cells [142]. Additionally, rat mammary adenocarcinoma cells with a hormone-independent phenotype had a two-fold increase in

DNA binding activity compared to those who had not progressed to hormone-independence [142]. The absence of ER also correlated with increased NF- κ B DNA binding in a series of primary breast tumours [142]. This is consistent with other evidence showing an inverse relationship between ER and NF- κ B with tumours with lower ER content displaying higher NF- κ B DNA binding activity [139].

Increased NF- κ B activity has also been reported in cells that display endocrine resistance. Expression of NF- κ B was compared between MCF7 cells and MCF7/LCC9 cells, which are tamoxifen and fulvestrant resistant. The p65 subunit displayed increased expression in MCF7/LCC9 cells and the addition of parthenolide, an inhibitor of NF- κ B activation, inhibited proliferation in these cells. This restored fulvestrant sensitivity and combination of parthenolide and fulvestrant acted synergistically, further reducing cell growth and increasing apoptosis [143]. Subsequent studies using these cells have shown that inhibition of NF- κ B also restores sensitivity to tamoxifen. A decrease in BCL2 expression was observed, which has anti-apoptotic activity, and induction of apoptosis via caspase-8 [144].

Crosstalk between other pathways and NF- κ B has been reported in breast cancer and there is evidence to suggest that this contributes to endocrine resistance. HER2 has been reported to activate NF- κ B [145-146] and overexpression of HER2 potentiates the apoptotic response to TNF α via NF- κ B [147]. In breast cancer cell lines with different ER and HER2 expression, ER negative cells (both HER2 positive and negative) showed higher p50 DNA binding activity than ER positive, HER2 negative cells (MCF7 and T47D) and ER positive, HER2 positive cells showed intermediate activity (BT474 and MCF7/HER2). Higher p65 activity was observed in ER negative, HER2 negative cells (MDA-MB-231) compared to ER positive cells (HER2 positive and negative) and also ER negative, HER2 positive SkBr3 cells [139]. Treatment with parthenolide was found to restore tamoxifen sensitivity in MCF7/HER2 cells and BT474 cells, both of which are ER and HER2 positive, supporting the hypothesis that HER2 induces activation of NF- κ B and this contributes to resistance to tamoxifen [139].

NF- κ B p65/p50 dimers have been found to display higher DNA-binding activity in ER negative tumours, especially those that also express HER2. NF- κ B activation was stimulated by heregulin (which stimulates HER2) in SkBr3 cells, which are ER negative with HER2 amplification. The addition of herceptin then inhibited NF- κ B activity, as did the NEMO-binding domain peptide, resulting in blocked cell cycle progression and proliferation and induction of apoptosis [146].

ER positive MCF7 cells transfected with a plasmid conferring constitutive activation of Akt (myrAkt1 MCF7 cells) displayed tamoxifen resistance and addition of eicosapentaenoic acid, which inhibits Akt, sensitised the cells to tamoxifen treatment [148]. Further investigation using these tamoxifen-resistant myrAkt1 MCF7 cells, in comparison to normal MCF7 cells, showed they had elevated levels of phosphorylated I κ B α and NF- κ B DNA binding and increased transcriptional activity [149]. Addition of the NF- κ B inhibitor parthenolide or a non-degradable I κ B, to inhibit NF- κ B activity in these cells, restored sensitivity to tamoxifen [149].

Furthermore, urokinase-type plasminogen activator (uPA) (which regulates cell migration) is activated by interaction between NF- κ B and activator protein 1 (AP-1), a transcription complex which is also involved in regulation of genes involved in many processes including proliferation and apoptosis [150-151]. DNA binding activity of the NF- κ B canonical pathway was found to correlate with levels of HER2 in ER positive breast tumours [139]. Activation of the p50 subunit correlated with the activity of AP-1 and expression of uPA [139]. High expression of uPA has been demonstrated to result in decreased disease free survival in early breast cancers [152] and NF- κ B may therefore be involved in invasive potential and recurrence of breast cancer.

A relationship between NF- κ B and ER is evident and the NF- κ B pathways have been demonstrated to play a role in the progression of breast cancer. It is therefore likely that they make attractive therapeutic targets and inhibitors may be beneficial in ER positive cancers following development of endocrine resistance. One strategy is to target the IKKs in order to inhibit their function and downstream NF- κ B signalling as well as NF- κ B independent functions. At present there are no IKK α selective inhibitors available and IKK β inhibitors that have been developed have exhibited adverse effects such as cellular toxicity and immunosuppression [91]. Selective IKK α inhibitors and effective IKK β inhibitors are therefore required.

A current collaboration between the University of Glasgow and the University of Strathclyde with the aim of developing IKK α and IKK β selective inhibitors is ongoing and several compounds have shown promising preclinical results in various cancer backgrounds. This may represent a future targeted approach in breast and other cancers. There is also a need to further explore the role of the NF- κ B pathways in different subtypes, due to evidence presented above, as crosstalk with other pathways and expression of ER and HER2 appear to impact on NF- κ B activity.

1.4 Research aims and hypothesis

This thesis investigated the role of the canonical and non-canonical NF- κ B pathways in the progression of breast cancer. Using archival tissue from breast cancer patients, as well as breast cancer cell lines, the expression of key components of each pathway were assessed. Additionally, we observed the effect of knock down of the upstream kinases from the canonical and non-canonical NF- κ B pathways, the IKKs, in breast cancer cell lines in order to investigate their potential as novel targets in breast cancer.

This tested the hypothesis that high expression of the NF- κ B pathways would result in poorer outcome in breast cancer patients and also due to evidence from the literature that these pathways may be involved in the development of endocrine resistance. It was also speculated that inhibition of the IKKs would reduce the viability of breast cancer cells. Therefore, the aim of this study was to establish if members of the NF- κ B pathway were associated with clinical outcome in patients with invasive breast cancer and whether these pathways were attractive therapeutic targets. In order to address this and test the hypotheses, the main objectives were to:

- Further develop a clinical database with linked tissue by assessing levels of apoptosis and proliferation within the tumours and establishing tumour subtype as classified by the St. Gallen guidelines.
- Establish if expression of NF- κ B family members could be employed as prognostic or predictive breast cancer markers.
- Validate markers identified as prognostic/predictive in an independent cohort of breast cancer patients.
- Establish markers of downstream signalling associated with IKK α or IKK β in breast cancer.
- Assess the phenotypic impact of modification/manipulation of IKK signalling in breast cancer cell lines to establish if further investigation was warranted into the IKKs as therapeutic targets in breast cancer.

Chapter 2:

Materials and methods

2.1 Tissue studies

To test whether the NF- κ B pathways were associated with clinical outcome and other clinical parameters, expression of members of the pathways were assessed using IHC on a tissue microarray (TMA) of breast cancer patients. All antibodies were validated before use in the patient cohort to ensure the antibody was specific to the antigen of interest.

2.1.1 Antibody validation

This was demonstrated by a single band of the correct size on a western blot, followed by blocking peptide experiments and/or appropriate expression in stimulated or silenced cell pellets (see Table 2.1). Antibodies for p65 and phospho-p65 S536 were validated as part of this thesis. The remaining antibodies had previously used in the laboratory and had already been validated: IKK α , IKK β , RelB and NIK antibodies by Mr Lewis McKenzie and Dr Antonia Roseweir and p65 NLS by Dr Pamela McCall.

Protein	Antibody	Validation method
p65	Santa Cruz sc-8008	<ul style="list-style-type: none"> Validated in breast and prostate cancer cell lines Single band on western blot ~65kDa Expression in TNFα stimulated cell pellets
phospho-p65 S536	Abcam ab28856	<ul style="list-style-type: none"> Validated in breast and prostate cancer cell lines Single band on western blot ~65kDa Expression in TNFα stimulated cell pellets
p65 NLS	Millipore MAB3026	<ul style="list-style-type: none"> Validated in prostate cancer cell lines Single band on western blot ~65kDa Expression in TNFα stimulated cell pellets
IKK β	Abcam ab32135	<ul style="list-style-type: none"> Validated in prostate cancer tissue/cell lines Single band on western blot ~87kDa IHC on IKKβ and IKKα silenced cell pellets Specific loss of staining when mixed with IKKβ protein (but not IKKα or p65 protein) in IHC
IKK α	Genway GWB-662250	<ul style="list-style-type: none"> Validated in prostate cancer cell lines Single band on western blot ~85kDa Western blot of IKKα and IKKβ silenced lysates IHC on IKKα and IKKβ silenced cell pellets
NIK	Epitomics S2922	<ul style="list-style-type: none"> Validated in prostate cancer cell lines Single band on western blot ~125kDa IHC on cell pellets transfected with NIK virus
RelB	Cell Signaling 4954	<ul style="list-style-type: none"> Validated in prostate cancer cell lines Single band on western blot ~70kDa Cellular location in cell pellets after lymphotoxin treatment

Table 2.1: Antibody validation. Protein of interest, antibody used and validation method are detailed, as well as the tumour types that antibodies were validated in.

2.1.2 Patient TMA

Formalin-fixed paraffin-embedded tissue (FFPE) blocks were retrieved from Pathology archives and used to construct a TMA, which was available for use in this thesis. Haematoxylin and eosin stained sections were used to identify tumour rich areas from each block, which were marked by Dr Elizabeth Mallon (Consultant Pathologist at the Department of Pathology, Southern General Hospital). Marked slides were matched to the FFPE blocks and three different 0.6mm² cores were lifted from each donor block and placed into three separate recipient paraffin blocks.

The use of TMAs has several benefits. Firstly, only 3 small cores from each paraffin block are used and the remaining block can be returned to the patient archives. All tissue can be stained in one run as 544 patients could be analysed in triplicate on just 9 slides. This means the internal consistency of the staining is greater and it also saves on reagents and time. There are however some issues when considering intra-tumour heterogeneity. The analysis of a single core might not be representative of the whole tumour and may not allow for the detection of varied levels of protein in different areas. To overcome this, three cores were used to allow a more representative result. Ethical approval for the use of this tissue was granted by the Research Ethics Committee of the North Glasgow University Hospitals NHS Trust (West of Scotland REC 4, Molecular profiling and clinical validation of Breast Cancer biological subtypes, REC reference: 07/S0704/61).

This study was conducted and is reported in accordance with REMARK criteria, with the exception of point 9 [153]. There was no power calculation used to determine the number of patients included in this study, as patients were included based on availability of tissue from retrospective cohorts. However, as detailed in the following sections, the events for each end point were around 100 and therefore this should allow enough events for statistical significance to compare high/low expression and also for analysis after stratification of the cohorts.

2.1.2.1 Cohort 1: 1800-Bre-TMA

This retrospective TMA of breast cancer patients was constructed by Clare Orange (TMA and Image Analysis Unit Manager, University Department of Pathology, Southern General Hospital). The cohort included 544 breast cancer patients (identified by Ms Julie Doughty, consultant breast surgeon, Western Infirmary) presenting with invasive breast cancer in the West of Scotland (at Glasgow Royal Infirmary, Glasgow Western Infirmary and Stobhill

Hospital), between 1995 and 1998. From an initial list of 1800 patients, the first block was constructed using ER positive patients from this list, followed by an ER negative TMA and a further ER positive TMA. These were constructed in this manner to allow for ER positive and ER negative specific studies and this selection accounts for the slightly skewed proportion of ER negative cases included in the cohort. A database was available with clinico-pathological data including age, tumour grade, tumour size, lymph node status, and any therapy the patient received.

Dr Zahra Mohammed performed staining for ER, PgR, HER2 and Ki67. Additionally, microvessel density was available due to the study by Dr Zahra Mohammed. Large vessels and single stained endothelial cells that were separate from adjacent microvessels and tumour cells as well as any other elements of connective tissue were counted as a single microvessel and branching structures were also considered. This was completed both on full sections by visually counting the number of vessels in three fields per section and using an average score and in TMAs by counting the number of vessels in each core. There was good correlation between scores obtained using whole sections and TMAs, with an ICC of 0.87 [154].

Updated clinical follow up was available thanks to Dr Jennifer Campbell. Patients were excluded if there was incomplete follow up or had insufficient tumour tissue (as determined by the pathologist). Clinical end points used were the recurrence free interval [155] (which included locoregional and distant recurrences), and breast cancer specific survival (which included deaths due to breast cancer and deaths from other causes were censored).

Median follow up time for breast cancer specific survival was 12.4 years, with an interquartile range (IQR) of 5.9 to 13.9 years. For recurrence free interval the median follow up was 5.7 years (IQR = 4.9 to 6.7 years). At the last follow up 101 patients had died of their disease and 102 had died of other causes. Follow up of recurrence status found 120 patients had relapsed.

2.1.2.2 Cohort 2: ST-Bre-TMA (validation)

In order to confirm results from the first cohort, a second cohort of patients was stained for IKK α and phospho-p65 S536. This TMA and clinical database was retrospectively constructed by Dr Sian Tovey and included 456 patients presenting with operable breast cancer between 1980 and 1999. Patients were included in this cohort based on ER positive pathology reports. However when this was verified using current technologies it was found although the majority of cases were ER positive (392), 20 were found to be ER negative and 44 were of unknown ER status. All patients selected for inclusion in this cohort were treated with adjuvant tamoxifen and time on tamoxifen was available in the database, as well as recurrence and survival follow up.

Clinical end points used were again recurrences or deaths due to breast cancer. The recurrence free interval was defined as the time from surgery to recurrence at any site. Breast cancer specific survival was defined as the time from surgery to death due to breast cancer and deaths from other causes were censored. Additionally, recurrence on tamoxifen was used with recurrence at any site whilst receiving tamoxifen as events.

The follow up for breast cancer specific survival was 7.3 years (IQR = 5.1 to 10.0 years). For recurrence free interval average follow up was 6.2 years (IQR = 4.3 - 9.0 years) and for recurrence on tamoxifen was 5.0 (IQR = 4.0 - 5.0 years). At the last follow up 97 patients had died of their disease and 78 had died of other causes. Recurrence follow up found 111 patients had relapsed, 85 of which were during tamoxifen treatment.

There was no overlap in patients included in this cohort and the previous cohort. A comparison of both cohorts is detailed in Table 2.2. Both cohorts were similar in terms of percentage in each group for age, size, invasive grade, nodal status and NPI. However the ST-Bre-TMA, which is mainly ER positive, differed in terms of treatment with less patients receiving chemotherapy and radiotherapy but all patients were treated with tamoxifen. This cohort also had a higher proportion of PgR positive and less HER2 positive than the 1800-Bre-TMA.

		1800-Bre-TMA (N=544)	ST-Bre-TMA (N=456)
Age	≤50 years	29%	18.5%
	>50 years	71%	81.5%
	missing	-	13
Size	≤20 mm	60.1%	52.5%
	21-50 mm	36.7%	42%
	>50 mm	3.1%	5.5%
	missing	2	37
Invasive grade	I	20.1%	24.7%
	II	43.5%	48.9%
	III	36.3%	26.4%
	missing	2	43
Nodal status	Negative	57.4%	53.5%
	Positive	42.6%	46.5%
	missing	6	52
NPI	<3.4	31.1%	37.5%
	3.4 - 5.4	51.7%	45.5%
	>5.4	17.3%	17.1%
	missing	-	93
ER	ER-	35.9%	4.9%
	ER+	64.1%	95.1%
	missing	4	44
PgR	PgR-	54.5%	41.5%
	PgR+	45.5%	58.5%
	missing	6	49
HER2	HER2-	85.3%	91.4%
	HER2+	14.7%	8.6%
	missing	21	24
Chemotherapy	No	59%	76.3%
	Yes	41%	23.7%
	missing	3	13
Radiotherapy	No	55.3%	69.5%
	Yes	44.7%	30.5%
	missing	3	-
Endocrine therapy	No	25.6%	0%
	Yes	74.4%	100% All received tamoxifen
	missing	9	-

Table 2.2: Comparison of the two cohorts used. *The percentage of patients in each group for each factor of the cohort is detailed for both the 1800-Bre-TMA and the ST-Bre-TMA. Missing indicates the number of patients for which this was unknown.*

2.1.2.3 Control tissue

TMA of breast cancer patients without linked clinical data were available for optimisation of antibodies. These were included as positive and negative controls in IHC experiments. Positive controls were included in every step of the IHC process and incubated in identical solutions as to the patient TMAs. Negative controls were included in every step with the exception of primary antibody where antibody was omitted and slides were instead incubated in antibody diluent for the duration of primary antibody incubation.

2.1.3 Immunohistochemistry

IHC was performed on the tissue to assess protein levels of members of the NF- κ B pathways. This uses a primary antibody that recognises the specific antigenic site and is recognised by EnVision™ (K5007, Dako, Glostrup, Denmark). The EnVision™ reagent has a polymer backbone to which a large number of horseradish peroxidase (HRP) molecules and secondary antibody molecules have been coupled. The secondary antibody reacts with both rabbit and mouse immunoglobulins. Following incubation with secondary antibody the tissue is incubated with a substrate solution, which consists of 3,3'-diaminobenzidine (DAB) chromogen and hydrogen peroxide. The HRP molecules on the EnVision™ interact with the substrate solution to produce a brown end product, representing the antigen of interest, which can be viewed using a light microscope.

2.1.3.1 Preparation of slides

Once antibodies were validated as specific and had been fully optimised, sections from patient TMAs were requested from Clare Orange (TMA and Image Analysis Unit Manager, University Department of Pathology, Southern General Hospital) and stored at 4°C for up to 1 month before use. TMAs were cut into 2.5 μ m thick paraffin wax sections and mounted onto slides. Slides were then incubated at 56°C overnight and stored at 4°C. Immediately before use in IHC the slides were baked again for 15 minutes at 56°C to minimise risk of lost cores.

2.1.3.2 Dewaxing and rehydration of tissue

Tissue was dewaxed by immersing in xylene twice for 5 minutes, and rehydrated through a series of graded alcohols (2x 100% for 3 minutes; 1x 90% for 3 minutes, 1x 70% for 3 minutes) before being washed in running water.

2.1.3.3 Antigen retrieval

In FFPE tissue the fixation process can often mask antigenic sites meaning it is necessary to perform antigen retrieval to break the protein crosslinks and uncover these sites to allow antibody binding. This was performed by heat induced antigen retrieval in a solution of either Citrate Buffer pH6 or TE Buffer pH9, depending on what was optimal for each antibody (see Table 2.2). TE Buffer pH9 contained 1mM Ethylenediaminetetraacetic acid (EDTA) (27285, Sigma-Aldrich, St Louis, USA) and 5mM Tris Base (BP152-1, Fisher Scientific, Loughborough, UK) in 1L distilled water. Citrate Buffer pH6 was prepared using 2mM Sodium Citrate (S/3320/53, Fisher Scientific, Loughborough, UK) and 8mM Citric Acid (27109, Sigma-Aldrich, St Louis, USA) in 1L distilled water.

Buffer solutions were preheated in the microwave in a pressure cooker for 13.5 minutes before slides were placed into the solution and the lid secured. This was heated for around 1.5 minutes until under pressure, then heated for a further 5 minutes once under pressure, before being cooled for 20 minutes.

2.1.3.4 Blocking endogenous peroxidase

Due to the presence of endogenous peroxidase activity in some tissue, it is necessary to block peroxidase activity to prevent background staining. This was achieved by incubating the tissue in 3% hydrogen peroxide for 10 minutes.

2.1.3.5 Blocking non-specific binding

Non-specific background staining can occur if hydrophobic bonds are formed between the antibody and other tissue proteins. This is blocked by incubation at 25°C in either 5% horse serum (S-2000, Vector Laboratories, Burlingame, CA, USA) for 30 minutes (diluted in Tris Buffer Saline (TBS)) or 1x Caesin (SP-5030, Vector Laboratories, Burlingame, CA, USA) for 1 hour (diluted in distilled water). Sections were circled with the Dako delimiting pen (S2002, Dako, Copenhagen, Denmark) to create a hydrophobic barrier around the tissue and ensure solutions remained covering the tissue, helping to achieve more uniform staining and reducing the amount of reagent required. Each antibody was used with whichever blocking solution was found to be optimal (see Table 2.3).

Protein of interest	Buffer solution	Blocking conditions	Antibody dilution & incubation conditions
p65	Citrate pH6	5% Horse serum 30 mins	1:25 dilution, 2 hours 25°C (Santa Cruz, sc-8008)
phospho-p65 S536	Tris-EDTA pH9	1x Caesin 1 hour	1:25 dilution, overnight 4°C (Abcam, ab28856)
p65 NLS	Tris-EDTA pH9	5% Horse serum 30 mins	1:200 dilution, overnight 4°C (Millipore, MAB3026)
IKK β	Citrate pH6	5% Horse serum 30 mins	1:500 dilution, overnight 4°C (Abcam, ab32135)
IKK α	Citrate pH6	5% Horse serum 30 mins	1:1000 dilution, overnight 4°C (Genway, GWB-662250)
NIK	Citrate pH6	1x Caesin 1 hour	1:250 dilution, overnight 4°C (Epitomics, S2922)
RelB	Citrate pH6	5% Horse serum 30 mins	1:75 dilution, overnight 4°C (Cell Signaling, 4954)
Ki67	Tris-EDTA pH9	5% Horse serum 30 mins	1:150 dilution, overnight 4°C (Dako, M7240)

Table 2.3: Antibody optimal conditions. *The antigen retrieval buffer, blocking solution and antibody conditions for each protein of interest are listed.*

2.1.3.6 Incubation with primary antibody

Immediately after incubation in the blocking solution, tissue was then incubated in primary antibody. Antibodies were diluted in antibody diluent (Dako, Copenhagen, Denmark) and the mixture added to slides. This was either incubated at 25°C for either 1 or 2 hours or overnight at 4°C. Optimal dilution and incubation conditions were assessed on practice tissue before being applied to the cohort.

2.1.3.7 Incubation with secondary antibody

Following incubation with the primary antibody, slides were washed twice in TBS for 5 minutes. Slides were then incubated for 30 minutes at 25°C in Dako EnVision™ (K5007, Dako, Copenhagen, Denmark), which detects both mouse and rabbit primary antibodies before being washed twice with TBS Buffer for 5 minutes.

2.1.3.8 Detection and visualisation

The chromagen used to visualise the presence of the protein of interest was 3,3'-diaminobenzidine (DAB). A DAB peroxidase substrate kit was used (SK-4100, Vector Laboratories, Burlingame, CA, USA). To make the solution, 5ml of distilled water was mixed with 2 drops of DAB Buffer solution, 2 drops of DAB Substrate solution, and 1 drop of Hydrogen Peroxidase solution. This was added to the tissue and left for 5 minutes until brown staining developed, and slides were then washed in running water.

2.1.3.9 Counterstaining of the slides

Counterstaining was performed by immersing slides in Harris Haematoxylin (HHS128, Sigma, UK) for 1 minute until tissue appeared red, then quickly dipping in 1% Acid Alcohol to remove excess Haematoxylin. Tissue was then blued in Scott's Tap Water Substitute (STWS) for 45 seconds. Acid Alcohol (1%) was prepared using 396ml 70% Ethanol + 4ml Hydrochloric acid. STWS contained 80mM Magnesium sulphate (25165.260, VWR, Poole, UK) and 40mM Sodium hydrogen carbonate (102475W, BDH, Poole, UK) in 2L dH₂O.

2.1.3.10 Dehydrating and mounting of the slide

After counterstaining, tissue was dehydrated in a series of alcohol washes with increasing percentage: 1x 70% for 1 minute, 1x 90% for 1 minute, 2x 100% for 1 minute and 2x Xylene for 1 minute. Slides were then mounted with coverslips using DPX mountant (06522, Sigma-Aldrich, St Louis, USA).

2.1.4 TUNEL assay

The amount of apoptotic cells in each core was assessed using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100, Millipore, Billerica, MA, USA). This uses the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay method. One hallmark of apoptosis is DNA fragmentation, which causes nicks in DNA. The enzyme TdT catalyses the addition of dUTPs to the 3'hydroxyl DNA ends to form a heteropolymer which anti-digoxigenin antibody binds to. As in the previously described, this antibody has been labelled with peroxidase and on the addition of DAB created a brown end product which allows visualisation. The kit was used following manufacturers guidelines. Tissue was dewaxed and rehydrated as normal but antigen retrieval was achieved by incubation in 20µg/ml Proteinase K Solution (539480, Millipore, Billerica, MA, USA). Slides were washed in distilled water twice for 2 minutes then endogenous

peroxidase was quenched in 3% hydrogen peroxide for 5 minutes before slides were washed twice for 5 minutes. Equilibration buffer was then applied to the tissue for 10 minutes. Positive slides were then incubated in a 70:30 ratio of Reaction buffer and TdT Enzyme for 1 hour at 37°C. Negative slides remained in equilibration buffer during this time. Slides were then washed in stop/wash buffer, first agitating the solution for 15 seconds then washing for 10 minutes. Slides were then washed 3x for 1 minute in TBS and anti-digoxigenin conjugate was applied for 30 minutes. After washing slides 4 times in TBS for 2 minutes, working strength peroxidase substrate (1:50 DAB substrate:DAB dilution buffer) was then added to the slides and the brown colour allowed to develop for 10 minutes. Slides were then washed for 10 minutes in running water before being counterstained with Harris haematoxylin, blued in STWS then dehydrated and mounted with coverslips and DPX mountant.

2.1.5 Scoring of IHC

2.1.5.1 Histoscore

Protein expression of each core was assessed with the scorers blinded to clinical end point or any information on stage or hormone receptor status and other markers. This was scored using the weighted histoscore method as described in Kirkegaard *et al* [156]. This grades staining intensity as negative (0) weak (1), moderate (2) and strong (3), multiplied by the percentage of tumour cells in each category giving a histoscore ranging from a minimum of 0 to a maximum of 300. One observer blindly scored all cores present, having previously found to correlate with an experienced scorer. A second observer then independently scored 10% of cores, blinded to the previous observers score as well as outcome and clinico-pathological factors, and statistical analysis was performed to measure consistency between scores using the interclass correlation coefficient (ICCC). An ICC of >0.7 was classed as having good correlation. Patients were then split into low and high expressors. Those with a score above the median were classed as having high expression and under as low.

2.1.5.2 Ki67 and TUNEL scoring

Ki67 and TUNEL expression was scored using the percentage of positive nuclei. The total number of nuclei and the number of positive nuclei were counted and the percentage was calculated using:

$$\frac{\text{Number of positive nuclei}}{\text{Total number of nuclei}} \times 100$$

For the 1800-Bre-TMA cohort Ki67 was already available, both manual and automated scoring. Scores obtained by manual scoring were used for analysis in this thesis. TUNEL was scored using the Slidepath Tissue IA system to automatically count the positive and negative nuclei. Similar to the histoscore, 10% was manually scored and ICCC calculated to assess correlation between automated and manual scoring. For the ST-Bre-TMA cohort, Ki67 and TUNEL were both manually counted due to discordance with automated counting. Ki67 expression was categorised as high if the value was $\geq 15\%$ [157] and TUNEL was split using the median score, as no cut off value has previously been established.

2.1.6 Statistical Analysis

Analysis was performed using SPSS version 18 (IBM, New York, USA). Kaplan-Meier curves were plotted to assess breast cancer specific survival, recurrence free interval and recurrence on tamoxifen. Chi squared tests were conducted to test the associations between markers of the pathway and clinico-pathological factors. Hazard ratios were calculated using Cox regression with 95% confidence intervals. Cox's multivariate analysis (Cox regression) was also performed with the inclusion of known predictive factors, such as size, grade and nodal status. Values of $P < 0.05$ were reported as statistically significant, and a more stringent significance value of $P < 0.01$ was also considered. Where multiple comparisons were made with more than two groups in a Kaplan Meier curve, a more stringent P value is required using Bonferroni's correction. This was the case with the 1800-Bre-TMA subtype breast cancer specific survival analysis. In this analysis there were 6 possible comparisons: (1) luminal A versus luminal B, (2) luminal A versus triple negative, (3) luminal A versus HER2 enriched, (4) luminal B versus triple negative, (5) luminal B versus HER2 enriched and (6) triple negative versus HER2 enriched. As there were 6 possible comparisons a P value of 0.008 is required to consider the result statistically significant (as 0.05 divided by 6 is 0.00833). All statistical analysis was performed by myself and repeated by a second (and senior) investigator (Joanne Edwards) in order to validate methods used and results obtained.

2.2 *In vitro* studies

2.2.1 Culturing of breast cancer cell lines

MCF7 (ER positive) and MDA-MB-231 (ER negative) breast cancer cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) (10313-021, Life Technologies, Paisley, UK) with 10% Fetal Bovine Serum (FBS) (F9665, Sigma-Aldrich, St Louis, USA), 10 Units/ml Penicillin/Streptomycin (15070-063, Life Technologies, Paisley, UK) and 1x GlutaMAX™ (35050-038, Life Technologies, Paisley, UK). Cells were grown in T-75 flasks and maintained in 5% CO₂ at 37°C, with media changed twice per week and cells passaged once a confluency of around 70% was reached. To passage cells, flasks were washed once with 1.5 ml of trypsin to remove traces of serum. This was removed and 1ml trypsin was added to detach cells from the flask and then incubated at 37°C in 5% CO₂ until cells detached. Once cells had detached, DMEM was added to inactivate the trypsin. Cells were gently pipetted on the side of the flask to break any clusters, before being split 1:6 into new T-75 flasks with fresh DMEM. Cells were routinely tested for mycoplasma twice a year.

Stocks of cells were stored in liquid nitrogen at -196°C. To freeze cells the procedure was as follows: after trypsinisation cells were centrifuged at 1200 rpm for 5 minutes and media removed before resuspension in 1ml of culture media with 10% DMSO, which is used as a cryoprotectant. Cells were then immediately placed in a Mr Frosty™ freezing container (5100-0001, Thermo Scientific Nalgene) and stored in -80°C overnight before being transferred to liquid nitrogen (-196°C) for storage. When new cells were required, aliquots were retrieved from liquid nitrogen, cells were defrosted by warming in a 37°C water bath and added to a T-75 flask with 10ml DMEM immediately due to the toxicity of DMSO. Cells were passaged twice before use in experiments. Stocks of MCF7 cells were P11-14 and passages used in experiments were P13-20. MDA-MB-231 cell stocks were P9-11 and experiments used P11-20.

2.2.2 Stimulation of the NF-κB pathways in breast cancer cells

In order to activate the pathways and stimulate expression of certain pathway members, various ligands were applied to the cells. Protein was then extracted to observe expression of the pathway members, or a functional assay was performed to observe the effect of stimulation on cell growth/survival.

Ligands:

- Tumour necrosis factor alpha (TNF α):

The canonical pathway was stimulated in MCF7 and MDA-MD-231 cells using 20ng/ml TNF α (SRP3177, Sigma-Aldrich, St Louis, USA) at a range of time points (5 minutes, 15 minutes, 30 minutes, 60 minutes and 120 minutes).

- Interleukin-1 beta (IL-1 β):

20ng/ml IL-1 β (10-1012-C, Insight Biotechnology, Wembley, UK) was also used to stimulate the canonical pathway at a range of time points (5 minutes, 15 minutes, 30 minutes, 60 minutes and 120 minutes).

- Lymphotoxin $\alpha_1\beta_2$:

For stimulation of the non-canonical pathway, 20ng/ml Lymphotoxin $\alpha_1\beta_2$ (LTx) (L5162, Sigma-Aldrich, St Louis, USA) was used for 4 hours, 8 hours and 24 hours.

For use in western blot, cells were seeded in 12 well plates at 1×10^5 cells/well, grown to 70% confluency before being serum starved in DMEM media with no FBS for 24 hours followed by stimulation with the ligand at the appropriate concentration and time point before protein was lysed (section 2.6.1).

For use in functional assays, cells were seeded in 96 well plates (as described in section 2.3), then serum starved for 24 hours and stimulated with TNF α or LTx (both at 20ng/ml) then the functional assay of interest performed.

2.2.3 siRNA knockdown of IKK α and IKK β in breast cancer cells

In order to observe the effect the loss of the IKKs has on the cells, MCF7 and MDA-MB-231 cells were transfected with siRNA for IKK α and IKK β . Expression of mRNA was knocked down using ON-TARGET $plus$ siRNA (Thermo Scientific, Waltham, MA, USA) specific to *CHUK* (IKK α) and *IKBKB* (IKK β). Target sequences are shown in Table 2.4.

Each siRNA was made to a stock concentration of 20uM by resuspension in 1ml of siRNA buffer (B-002000-UB-100, Thermo Scientific, Waltham, MA, USA; diluted to from 5x to 1x in RNase free water) and placed in an orbital mixer for 30 minutes

Gene	ON-TARGET PLUS siRNA	Target Sequence
<i>CHUK</i> (IKK α)	CHUK (1147) Cat#: J-003473-09	GCGUGAAACUGGAAUAAAU
<i>IKBKB</i> (IKK β)	IKBKB (3551) Cat#: J-003503-13	GAGCUGUACAGGAGACUAA
Non-targeting (negative control)	Non-targeting #1 Cat#: D-001810-01-20	UGGUUUACAUGUCGACUAA

Table 2.4: siRNA information. *The gene, siRNA used and target sequence are shown.*

Lipofectamine® RNAiMAX (13778150, Life Technologies, Paisley, UK) was used as the transfection reagent to deliver the siRNA to the cells. Lipofectamine® and siRNA were both diluted in Opti-MEM® (11058-021, Life Technologies, Paisley, UK), then both mixed together at a 1:1 ratio and left to incubate at room temperature for 20 minutes to allow the formation of complexes. Cells were meanwhile incubated in Opti-MEM® for 20 mins before the siRNA mixture was added drop by drop to the cells. Volumes for each component for 6 well, 12 well and 96 well plates are shown in Table 2.5. After 6 hours media was replaced with the usual 10% DMEM.

- 6 well plates were seeded at 2×10^5 cells/well and used to make protein lysates. These were used to establish the optimal concentration (100nM, 200nM and 300nM) and time (48 or 72 hours) for each siRNA in both MCF7 and MDA-MB-231. Once optimal concentration/time point established, 12 well plates were seeded at 1×10^5 cells/well and used to make lysates for each functional assay to observe siRNA knockdown efficiency for each assay.
- 96 well plates were seeded at 5×10^3 cells/well (as described in section 2.6.1/2) and once the correct confluency was reached, cells were silenced using 200nM siRNA for 48 hours before the functional assay was performed.

Well/flask format	OptiMEM® volume	siRNA mixture volumes		Lipofectamine® mixture volumes	Total volume
6 well	1.5ml (2ml in control)	100nM	5µl + 245µl OptiMEM®	10µl + 240µl OptiMEM®	2ml
		200nM	10µl + 240µl OptiMEM®		
		300nM	15µl + 235µl OptiMEM®		
12 well	750µl (1ml in control)	5µl siRNA + 125µl OptiMEM® (200mM)		5µl + 125µl OptiMEM®	1ml
96 well	150µl (200µl in control)	1µl siRNA + 24µl OptiMEM® (200mM)		1µl + 24µl OptiMEM®	200µl

Table 2.5: siRNA/Lipofectamine® dilution volumes for each size of plate.

2.2.4 DN-IKK β adenovirus infection in breast cancer cells

Breast cancer cells were infected with dominant negative IKK β adenovirus in order to overexpress dominant negative (DN) IKK β , which is catalytically inactive. This is an alternative approach to silencing to inhibit activation of the pathways, allowing the protein to perform its scaffolding role but preventing it acting as a kinase.

The adenoviral construct used was supplied by Dr Andrew Paul, University of Strathclyde. Cells were seeded in 6 well plates at 2×10^5 cells/well and once around 50% confluence was reached one well was trypsinised and counted using a haemocytometer. Adenovirus (of known titre) was then added to cells (in normal growth medium) using the appropriate volume calculated using the formula:

Number of cells x concentration (pfu)

Titre of the adenovirus

A range of concentrations of adenovirus, measured by plaque forming units per cell (pfu/cell), was applied to establish which was most appropriate including 25, 50, 100 and 200 pfu/cell. Cells were also infected with 200 pfu/cell of adenovirus containing β -galactosidase (β -gal) as a negative control to ensure changes in expression were not due to the infection procedure. After 40 hours incubation, protein was lysed (described in 2.3.1) and western blotting performed to assess protein levels (described in 2.3.2 - 2.3.4).

2.3 Western blotting

2.3.1 Lysis of protein

Cells were exposed to appropriate agonist/vehicle or siRNA for the relevant period of time and they were then placed on ice to stop the reaction and prevent protein degradation or dephosphorylation. Cells were then washed twice with 750µl ice cold PBS before adding 250µl (for a 12 well plate) or 500µl (for a 6 well plate) of pre-heated laemmli's sample buffer (63mM Tris-HCl pH6.8, 2mM Na₄P₂O₇, 5mM EDTA, 10% glycerol, 2% SDS, 50mM DTT, 0.007% bromophenol blue). Cells were then scraped and pushed through a 21 gauge needle to shear the chromosomal DNA. Samples were then transferred to Eppendorf tubes and boiled for 5 minutes to denature the proteins and stored at -20°C until use.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gels were prepared containing 0.1% SDS, 0.375M Tris base (pH8.8), 3% glycerol, 10% Ammonium persulfate (APS), the appropriate amount of 30% acrylamide/bisacrylamide stock depending on the size of the protein of interest, and distilled water to 12mls. Most proteins were suitable to be separated on 10% gels (4mls of 30% acrylamide/bisacrylamide in 12ml total volume), but due to the size and lack of specificity of the phospho-p100 antibody, a 7.5% (3mls of 30% acrylamide/bisacrylamide in 12ml total volume) gel was required to allow separation of the fragments. The addition of 0.05% tetramethylethylenediamine (TEMED) then began the polymerisation process. Glass plates were set up on a Mini-PROTEAN casting chamber and the solution was poured between the 2 plates with 200µl of 0.1% SDS added on top. Following gel polymerisation the SDS solution was removed and a stacking gel containing 10% acrylamide, 0.1% SDS, 10% APS and 0.05% TEMED was poured on top of the resolving gel, and a comb was inserted. After polymerisation, the comb was carefully removed and gels were assembled in a Bio-Rad Mini-PROTEAN electrophoresis tank filled with electrophoresis buffer (25mM Tris, 129mM glycine, 0.1% SDS). 15µl of sample was loaded per well using a Hamilton microsyringe. A prestained marker of known molecular weights was run on the same gel in order to identify the protein of interest by size and ensure transfer to membrane was successful. Samples were electrophoresed at a constant voltage of 120V, until the bromophenol blue dye had reached the bottom of the gel (usually around 1.5 hours).

2.3.3 Protein transfer

Proteins were transferred from the gel to nitrocellulose membranes by electrophoretic blotting in wet conditions. A sandwich of sponge, filter paper, gel, membrane, filter paper and sponge was immersed in transferred buffer (25M Tris, 19mM glycine, 20% methanol) and stacked on the Bio-Rad cassette as in Figure 2.1. This was slotted into a Bio-Rad Mini-PROTEAN electrophoresis tank and a constant current of 300mA was applied for 1h and 45min with an ice pack included to cool the tank. The presence of SDS gives the protein a negative charge, so the cassette was oriented with the membrane towards the anode meaning protein would move towards this positive electrode and bind to the membrane.

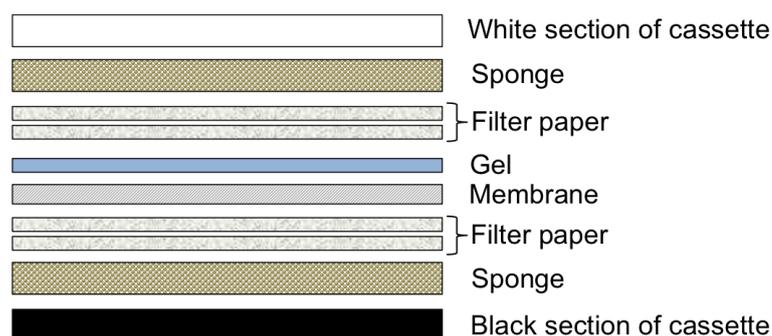


Figure 2.1: Assembly of the sandwich for western blot transfer. *Diagram showing how the sandwich of sponge, filter paper, membrane and gel were stacked for transfer.*

2.3.4 Blocking, staining and visualisation

Following transfer, the membrane was removed and non-specific binding blocked by incubation in a solution of 3% or 4% BSA in NaTT buffer for 90 minutes on an orbital shaker. Membranes were then incubated overnight, either at room temperature or 4°C, in primary antibody specific to the target protein diluted to optimal concentration in NaTT buffer containing 0.3% BSA. Conditions for each antibody are listed in Table 2.6. The next day membranes were washed 6 times in NaTT at 15 minute intervals, shaking gently. Secondary (either rabbit or mouse, depending on primary antibody used) HRP-conjugated antibody was diluted 1:10000 in NaTT with 0.3% BSA, and the membranes were then incubated in dilute secondary antibody for 90 minutes at room temperature. After another 6 washes in NaTT at 15 minute intervals, presence of the protein of interest was detected by chemiluminescence. Membranes were incubated in 6mls of enhanced chemiluminescence (ECL) reagent (1:1 mixture of solution 1 [1M Tris pH8.5, 250mM luminol, 250mM p-cymuric acid and water] and solution 2 [1M Tris pH8.5, 0.19% H₂O₂ and water]) for 3 minutes on a shaker. Membranes were then placed into a cassette, covered with cling film and in the dark room exposed to X-ray film for the required time and developed using an X-OMAT machine.

Protein of interest	Antibody	Species	Blocking conditions	Antibody conditions
IKK α	OP133 Calbiochem	Mouse	4% BSA in NaTT	1:3500 @ 4°C
IKK β	Ab32135 Abcam	Rabbit	4% BSA in NaTT	1:2500 @ 4°C
phospho-p65 S536	3031 Cell signalling	Rabbit	3% BSA in NaTT	1:1000 @ 4°C
p65	sc-8008 Santa Cruz	Rabbit	3% BSA in NaTT	1:10000 @ room temperature
I κ B α	9242 Cell signalling	Rabbit	3% BSA in NaTT	1:7500 @ room temperature
p100/52	05-361 Millipore	Mouse	3% BSA in NaTT	1:3000 @ 4°C
phosho-p100	4810 Cell signalling	Rabbit	3% BSA in NaTT	1:2000 @ 4°C

Table 2.6: Antibodies used for western blot and optimal conditions. Table showing the company, catalogue number and species raised in for each antibody and the optimal conditions for western blotting.

2.3.5 Stripping membrane

Nitrocellulose membranes were reprobed for the subsequent detection of other proteins. Before reprobng, membranes were stripped using a stripping buffer containing 0.05M Tris-HCl and 2% SDS. The membrane was incubated at 60°C in 15ml of stripping buffer with 0.1M (105 μ l) β -mercaptoethanol for 60 minutes on a shaker. The membrane was washed in NaTT buffer 3 times for 15 minutes to remove all stripping buffer. Membranes were then ready for reprobng, following the protocol previously described (section 2.3.4). Lysates were assessed for levels of β tubulin as a loading control (1:10000, ab21058, Abcam).

2.3.6 Quantification of expression levels

Expression levels of proteins observed via western blots were calculated using ImageJ (NIH). Levels were normalised to β tubulin and fold change to control calculated using a one-way ANOVA with Bonferroni correction and Dunnett's test used to compare to control. Changes were considered significant if $P < 0.05$ and highly significant if $P < 0.001$.

2.4 Cell pellets

2.4.1 Preparation of cell pellets

Cells were seeded in a T-75 flask, grown to the correct confluency then quiesced for 24 hours then exposed to TNF α at 20ng/ml for 15 minutes. After the appropriate treatment, flasks were washed with PBS and trypsinised then spun down in 15ml corning tubes. The supernatant was discarded and cells were resuspended in 1ml PBS and transferred to 1.5ml eppendorfs before spinning down again, PBS discarded and cells resuspended in 4% formalin and incubated at room temperature for 15 minutes to fix the cells. Pellets were then formed by centrifugation for 5 minutes, supernatant discarded and all traces of formalin removed by PBS. Heated 1% agarose was then pipetted to the bottom of the tube to lift the pellet, before refrigeration for 30 minutes or until agarose solidified. Pellets in agarose were carefully removed from eppendorfs and placed into a cassette and dehydrated through a series of graded alcohols, before being submersed in xylene twice for 15 minutes and then placed in melted wax for an hour. Pellets were then removed from cassettes and placed into a mould, which was then filled with wax and cooled on cool plate.

2.4.2 Cutting cell pellets

4 μ M thick sections were cut from cooled FFPE blocks using a Leica microtome and placed into a waterbath to smooth out sections which were then attached onto slides and baked overnight at 56°C.

2.4.3 IHC of cell pellets

After cutting onto slides, pellets were used in IHC to assess expression level of the protein of interest. This follows the same protocol as IHC on the TMAs (see section 2.1.3) with the following exceptions:

- Antigen retrieval time was halved to 2 minutes and 30 seconds.
- Antibody concentrations were halved.

2.5 Gene expression profiling

2.5.1 RNA extraction

After treatment, MCF7 cells were trypsinised, media added to neutralise trypsin and two wells of a 6 well plate (of the same treatment) were combined. After centrifugation, Ice cold PBS was used to wash cells and transfer them to RNase free eppendorfs before centrifugation to remove PBS. Cells were then resuspended in 1ml Trizol (15596026, Life Technologies, Paisley, UK) and mixed gently for 10 minutes. 1/5 volume (200µl) of Chloroform (C2432, Sigma) was then added, the mixture vortexed and centrifuged at 12500rpm for 15 minutes. The aqueous top layer was removed (400µl) and added to a fresh RNase free eppendorf with equal volume of Phenol:Chloroform:IAA (AM9732, Ambion) and again vortexed and centrifuged at 12500rpm for 15 minutes. The top layer (300µl) was added to a fresh RNase free eppendorf with equal volume of isopropanol (19576, Sigma), the tube inverted to gently mix and placed in the -80°C freezer for 1 hour. Tubes were then centrifuged for 15 minutes at 12500rpm, the supernatant disposed of and the pellet washed with 75% ethanol in nuclease free water. After centrifugation, all ethanol was removed and tubes left to dry for around 5 minutes before the pellet was resuspended in 15µl of nuclease free water. Concentration and 260/280 ratio of samples were measured using the Nanodrop, and samples stored at -80°C until use.

2.5.2 cDNA synthesis

Before cDNA synthesis, RNA samples were treated with DNase to eliminate any DNA. 2µg of RNA was added with 2µl 10x DNase I buffer (M198A, Promega), 1µl RQ DNase I (M610A, Promega) and 1µl RNase OUT (10777-019, Life Technologies, Paisley, UK), and nuclease free water to a volume of 20µl. After incubation at room temperature for 15 minutes, 1µl of stop solution was added (M199A, Promega) and the mixture heated for 10 minutes at 65°C. Samples were again measured for concentration and 260/280 ratio using the Nanodrop.

For a 100µl cDNA synthesis reaction, 5µl random primers (48190-011, Life Technologies, Paisley, UK), 1µg RNA and nuclease free water was added to a volume of 62µl. This was heated to 65°C for 10 minutes and then 20µl 5x FS buffer, 2.5µl Superscript II reverse transcriptase (18064-014, Life Technologies, Paisley, UK), 10µl dNTPs (N04475, NEB), 3.5µl DMSO and 2.5µl RNase OUT were added to make a final volume of 100µl. This was incubated at 25°C for 10 minutes followed by 50°C for 30 minutes then the transcriptase was inactivated at 85°C for 5 minutes. cDNA was then stored at -20°C until use.

2.5.3 Quantitative Real Time-PCR

In order to compare gene expression between control samples and samples with silenced IKK α or IKK β and to find selective downstream targets, quantitative real time PCR (qPCR) was used. To each well of a 96 well optical fast PCR plate, 40ng cDNA, 10 μ l master mix (4304437, Life Technologies, Paisley ,UK), 5 μ l nuclease free water and 1 μ l of Gene expression assay (Primers for specific genes, see Table 2.7), were added. Blank wells with only the mixture and no cDNA were included to ensure amplification was of the sample cDNA and not contaminants. Plates were then sealed and centrifuged for 3 minutes at 1200rpm and the plate was read on an ABI 7500 real time PCR machine. The programme was as follows: 50°C for 2 minutes, 95°C for 10 minutes then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression was normalised to the *18S* gene and the comparative Ct (cycle threshold) $\Delta\Delta$ Ct method used to quantify relative gene expression using the formula: Fold Change = $2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_t, \text{ target} - C_t, 18S$ and $\Delta(\Delta C_t) = \Delta C_t, \text{ stimulated} - \Delta C_t, \text{ control}$.

Gene	Assay ID	Amplicon length
<i>18S</i>	Hs03928990_g1	61
<i>CHUK</i>	Hs00989507_m1	149
<i>IKBKB</i>	HS00233287_m1	61
<i>CCND1</i>	Hs00765553_m1	57
<i>NCOR2</i>	Hs00196955_m1	75
<i>CXCL10</i>	Hs01124251_g1	135
<i>CRLF1</i>	Hs00191064_m1	74

Table 2.7: Information on Gene expression assays. *The gene, assay ID and amplicon length are detailed.*

2.6 Phenotypic assays

2.6.1 Cell death assay

Cells were assessed for apoptosis level using a Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (11544675001, Roche, USA), both after stimulation and siRNA knockdown. This one step sandwich ELISA quantifies the amount of histone-complexed DNA fragments (mono- and oligonucleosomes) from the cytoplasm of cells, which are enriched in apoptotic cells. Cells were seeded in a 96 well plate at a density of 5×10^3 cells/well in 100 μ L of standard culture medium. Once a confluency of 60-70% was reached siRNA knockdown was performed or cells were quiesced in serum free media for 24 hours followed by stimulation. After treatment cells were pelleted by centrifugation at 200xg for 10 minutes and the supernatant was discarded. The cells were resuspended with 100 μ l of lysis buffer and incubated at room temperature for 30 minutes. The cells were collected by centrifugation at 200x g after lysis, and 20 μ l of the supernatant was transferred to the streptavidin coated microtiter plate. 100 μ l of immunoreagent (containing two monoclonal antibodies: biotin-labelled anti-histone and peroxidase conjugated anti-DNA) was added to the wells and incubated for 2 hours at room temperature. Plates were washed 3x with incubation buffer before 100 μ l of ABTS solution was added to each well and incubated on a shaker at room temperature for 2 hours before 100 μ l of ABTS stop solution was then added. The optical absorbance level was measured using a 96 well microplate reader at 405nm with reference 490nm.

2.6.2 WST-1 viability assay

Cell viability was assessed using the water-soluble tetrazolium salt (WST-1) reagent (05015944001, Roche, Switzerland). This colorimetric assay is based on cleavage of WST-1 to formazan by cellular enzymes. More activity of these mitochondrial dehydrogenases results in an increase in formation of formazan dye, which corresponds to the number of viable cells. The amount of dye can then be measured using a multiwell spectrophotometer. Cells were seeded in 96 well plates at a density 5×10^3 cells/well with 100 μ L of media. Once 60% confluence was reached, cells were silenced for 48 hours or quiesced in serum free media for 24 hours followed by stimulation. The assay was performed by adding 10 μ L of WST-1 reagent to each well and after 2 hours incubation at 37°C, the optical absorbance level was measured using a 96 well microplate reader at 450nm. Each treatment was repeated at least in triplicate on the plate, and experiments repeated to an N=3.

2.6.6 Statistical analysis of WST-1/apoptosis assays

Analysis was performed using SPSS version 18 (IBM, New York, USA). Fold change compared to control was assessed for significance based on p values from a one-way ANOVA. Bonferroni posthoc test was used for multiple comparisons and Dunnett's was used to compare to control. P values were considered significant if $P < 0.05$ and highly significant if $P < 0.001$.

2.6.3 Cell viability via the xCELLigence

The xCELLigence machine (ACEA Biosciences Inc, San Diego, CA, USA) was used to display cell growth/viability in real time. This uses special plates with microelectrodes on the bottom allowing the impedance of each well to be measured. These measurements are continuously sent to the computer, allowing for real time growth curves to be plotted. Measurements are given as "Cell Index" which represents the number and viability of the cells. Cells were seeded in a 96 well *E-plate*TM (012738, ACEA Biosciences, San Diego, CA) at 3×10^3 cells/well with 200 μ l of media in each well and grown for two days to ensure in log phase of growth. Cells were then quiesced in serum free media for 24 hours before appropriate treatment (stimulation or silencing) was applied. After 72 hours graphs showing cell index over time were drawn.

Chapter 3:

Assessment of proliferation, apoptosis and molecular subtypes in breast cancer clinical specimens by immunohistochemistry

3.1 Introduction

Patient tissue is an extremely valuable tool in translational research that allows novel markers to be tested for association with clinico-pathological characteristics such as the size and stage of the tumour, as well as clinical outcomes including breast cancer specific survival and recurrence free interval. Two different cohorts of patients presenting with invasive breast cancer were utilised in this study. The first, the 1800-Bre-TMA was used as a training cohort to identify markers and cut off values, and the ST-Bre-TMA was used as a validation cohort to investigate clinically significant markers in a separate cohort with information available for recurrence on tamoxifen.

Increased proliferation and loss of apoptosis are both hallmarks of cancer [123]. Using MIB1, the monoclonal antibody to detect expression of Ki67 and therefore proliferation, an association between Ki67 and clinical outcome has been reported. Several studies have implicated a poorer disease free and overall survival in patients with tumours that display high Ki67 [158-159]. Lower levels of apoptosis are also likely to result in poorer outcome. Several markers have been employed in tissue studies to determine levels of apoptosis including DNA fragmentation (TUNEL), Bcl2, Bax and caspase-3 [160-161]. In order to further develop the information available in both cohorts, levels of apoptosis and proliferation within the tumours were assessed and tumour subtype established as recommended by the expert panel at the St. Gallen International Breast Cancer Conference [48].

3.2 Clinico-pathological characteristics of the patient cohorts

3.2.1 1800-Bre-TMA

This cohort included 544 breast cancer patients presenting with invasive breast cancer between 1995 and 1998. A database was available with full clinical follow up as well as clinico-pathological data including invasive grade, ER status, HER2 status, necrosis, angiogenesis (measured in a previous study by microvessel density using an anti-CD34 antibody in IHC), lymph node status and any therapy the patient received. The number of patients in each group is detailed in Table 3.1.

The majority of patients (71%) were over 50 years of age, had Grade II (43.5%) or Grade III (36.3%) tumours of 20mm or less (60.1%) with 36.7% having tumours between 21 and 50mm in size. 64.1% of tumours were ER positive and 14.7% were HER2 positive. There was a fairly even split in groups for nodal status (57.4% negative and 42.6% positive), PgR

(54.5% negative and 45.5% positive), necrosis (47.6% absent and 52.4% present) and chemotherapy (59% did not receive and 41% did). The majority of patients (74.4%) received endocrine therapy.

Size, invasive grade, nodal status, ER status, PgR status, HER2 status, levels of angiogenesis and whether the patient received chemotherapy were all significantly associated with clinical outcome, both breast cancer specific survival and recurrence free interval. Age was not significant when either outcome was considered and endocrine therapy did not reach significance with breast cancer specific survival but was significantly associated with recurrence free interval.

Clinico-pathological characteristic	Patient numbers (and %) (Total N = 544)	Clinical outcome significance
Age (≤50/>50 years)	158 (29%) / 386 (71%)	S: P=0.385 R: P=0.094
Size (≤20/21-50/>50 mm)	326 (60.1%) / 199 (36.7%) / 17 (3.1%) m2	S: P<0.001 R: P<0.001
Invasive grade (I/II/III)	109 (20.1%) / 236 (43.5%) / 197 (36.3%) m2	S: P<0.001 R: P<0.001
Nodal status (negative/positive)	309 (57.4%) / 229 (42.6%) m6	S: P<0.001 R: P<0.001
NPI (<3.4/3.4-5.4/>5.4)	169 (31.1%) / 281 (51.7%) / 94 (17.3%)	S: P<0.001 R: P<0.001
ER (ER-/ER+)	194 (35.9%) / 346 (64.1%) m4	S: P=0.002 R: P<0.001
PgR (PgR-/PgR+)	293 (54.5%) / 245 (45.5%) m6	S: P<0.001 R: P<0.001
HER2 (HER2-/HER2+)	446 (85.3%) / 77 (14.7%) m21	S: P<0.001 R: P<0.001
Necrosis (absent/present)	253 (47.6%) / 278 (52.4%) m13	S: P<0.001 R: P<0.001
Angiogenesis (CD34) (≤15/16-23/>23 H score)	175 (35.9%) / 161 (33%) / 152 (31.1%) m56	S: P=0.007 R: P=0.001
Chemotherapy (no/yes)	319 (59%) / 222 (41%) m3	S: P=0.001 R: P<0.001
Radiotherapy (no/yes)	299 (55.3%) / 242 (44.7%) m3	S: P=0.293 R: P=0.742
Endocrine therapy (no/yes)	137 (25.6%) / 398 (74.4%) m9	S: P=0.061 R: P=0.036

Table 3.1: Clinico-pathological characteristics of the 1800-Bre-TMA cohort of breast cancer patients. *The number of patients in each group is detailed as well as significance on clinical outcome. S = breast cancer specific survival, R = recurrence free interval, m = missing.*

3.2.2 ST-Bre-TMA

This cohort included 456 patients presenting with operable breast cancer between 1980 and 1999. The strength of this cohort is that patients were treated with adjuvant tamoxifen and time on tamoxifen was available in this database. Clinico-pathological data including size, invasive grade, PgR status, HER2 status, lymph node status and NPI was also available. The number of patients in each group is detailed in Table 3.2.

Again most patients were over 50 years old with tumours under 50mm in size. 52.5% of tumours were 20mm or less and 42% were 21-50mm. The majority of patients had grade II tumours (48.9%) and were mainly HER2 negative (91.4%). An even division is observed between groups of PgR and nodal status and only 23.7% of patients received adjuvant chemotherapy.

Size, invasive grade, NPI, nodal status and PgR were all significant on both breast cancer specific survival and recurrence free interval, while chemotherapy was only significant on breast cancer specific survival. Age was not associated with clinical outcome, neither was HER2 although very few patients were HER2 positive.

Clinico-pathological characteristic	Patient numbers (and %) (Total N = 456)	Clinical outcome significance
Age (≤50/>50 years)	82 (18.5%) / 361 (81.5%) m13	S: P=0.127 R: P=0.801
Size (≤20/21-50/>50 mm)	220 (52.5%) / 176 (42%) / 23 (5.5%) m37	S: P<0.001 R: P<0.001
Invasive grade (I/II/III)	102 (24.7%) / 202 (48.9%) / 109 (26.4%) m43	S: P<0.001 R: P<0.001
Nodal status (negative/positive)	216 (53.5%) / 188 (46.5%) m52	S: P<0.001 R: P<0.001
NPI (<3.4/3.4-5.4/>5.4)	136 (37.5%) / 165 (45.5%) / 62 (17.1%) m93	S: P<0.001 R: P<0.001
PgR (PgR-/PgR+)	169 (41.5%) / 238 (58.5%) m49	S: P=0.015 R: P=0.002
HER2 (HER2-/HER2+)	395 (91.4%) / 37 (8.6%) m24	S: P=0.126 R: P=0.078
Chemotherapy (no/yes)	338 (76.3%) / 105 (23.7%) m13	S: P=0.002 R: P=0.084
Radiotherapy (no/yes)	317 (69.5%) / 139 (30.5%)	S: P=0.732 R: P=0.378

Table 3.2: Clinico-pathological characteristics of the ST-Bre-TMA cohort of breast cancer patients. *The number of patients in each group is detailed as well as significance on clinical outcome. S = breast cancer specific survival, R = recurrence free interval, m = missing.*

3.3 Ki67 as a marker of proliferation

3.3.1 Ki67 in the 1800-Bre-TMA cohort

Ki67 status was already available in the 1800-Bre-TMA from a previous study by Dr Zahra Mohammed. The percentage of positive cells was calculated and the range of scores is shown in the histogram in Figure 3.1A. When survival analysis was performed using tertiles, and the highest tertile, with values of 15% or more, was associated with a poor clinical outcome and the lower and middle tertiles displayed more favourable outcomes [157].

Studies assessing the prognostic value of Ki67 have used various cut offs, usually ranging from 10-20%. The 15% cut off applied to our cohort, based on tertiles, is reassuringly in the middle of these values and was found to be associated with clinical outcome. Using this 15% cut off Ki67 was associated with breast cancer specific survival ($P=3.3 \times 10^{-5}$, Figure 3.1B). Those with a low Ki67 index had a longer average breast cancer specific survival of 13.1 years compared to 11.2 in those with high Ki67. Similar results were observed with recurrence free interval ($P=0.003$, Figure 3.1C). The 15% cut off was therefore used in this and future studies.

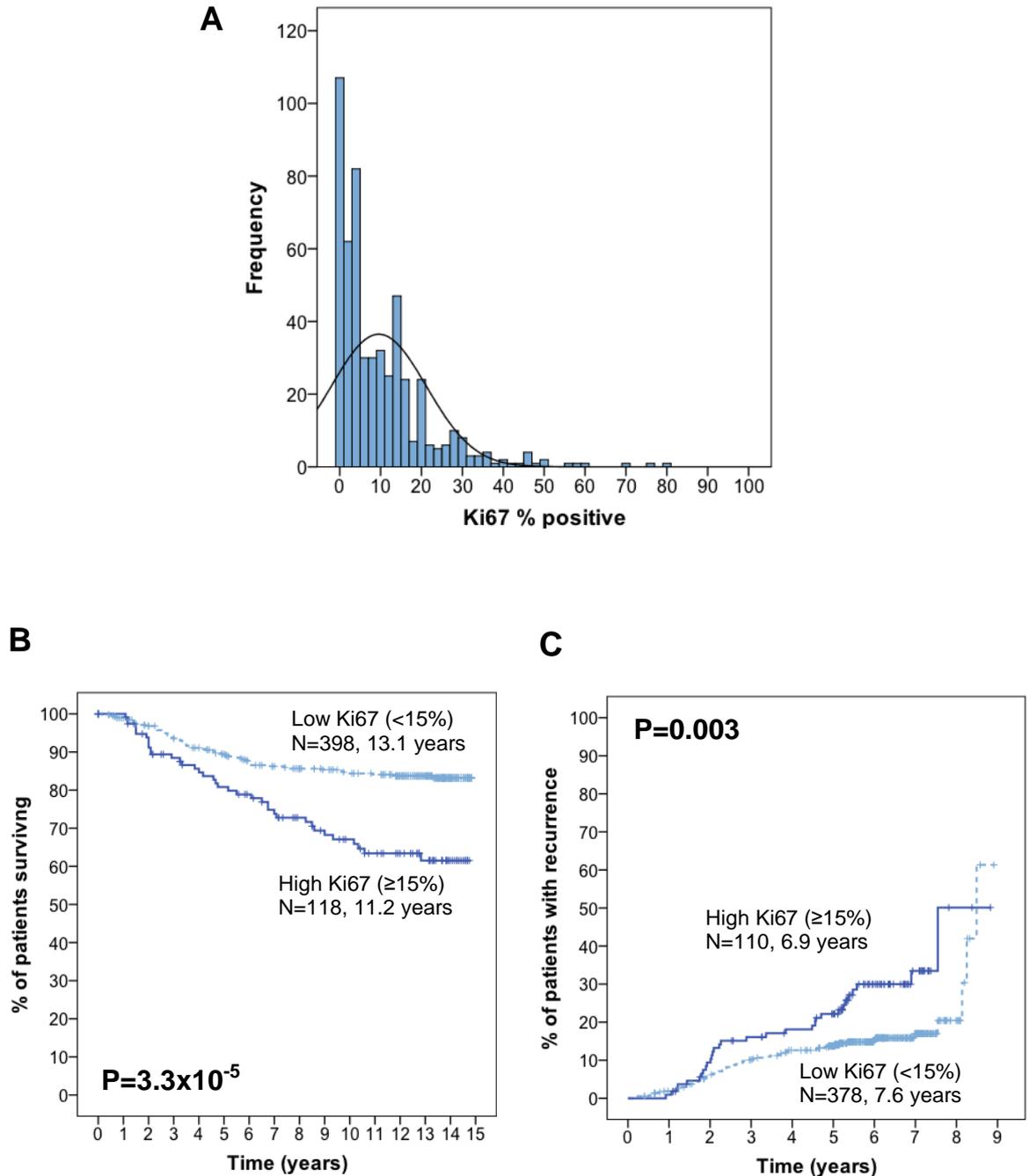


Figure 3.1: Ki67 and outcome in the 1800-Bre-TMA. The range of % positive cells for Ki67, as a marker of proliferation, is shown in the histogram (A). Ki67 was significantly associated with breast cancer specific survival (B) and recurrence free interval (C). Censor lines in B indicate loss to follow up or death due to other cause i.e. not a breast cancer related death. Censor lines in C indicate loss to follow up.

3.3.2 Ki67 in the ST-Bre-TMA cohort

IHC was employed using the MIB1 antibody to assess the levels of Ki67, as a marker of proliferation, in the ST-Bre-TMA cohort. Due to discordance with the results using the automated system and the manually counted level of Ki67, cores were assessed for Ki67 by manually counting the number of positive and negative nuclei and calculating the percentage of positive cells. The 15% cut off established in the previous cohort was applied to the cohort, with those tumours with a Ki67 index of 15% or more being classed as highly proliferative and those with a value of less than 15% being classed as low. Expression of Ki67 is pictured in Figure 3.2. The percentage of positive cells ranged from 0 to 94.7 and of the 375 tumours scored, 264 (70.4%) had a low Ki67 index of less than 15% and 111 (29.6%) displayed high Ki67 with an index of 15% or more. Across the cores from the same patient the average standard deviation of percentage positive cells was 5.7 and the median was 3.2 (IQR = 1.1 – 7.8).

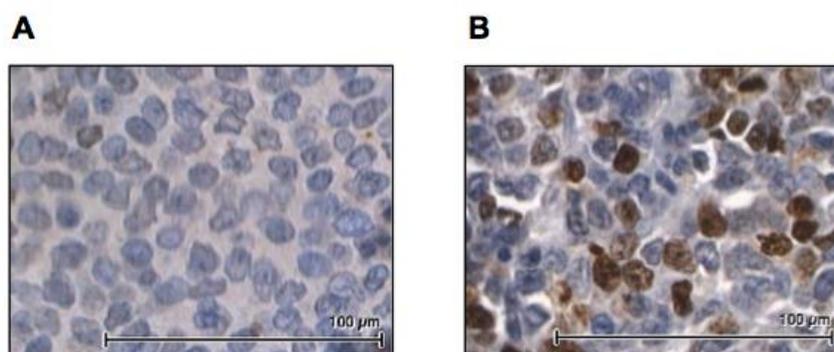


Figure 3.2: Expression of Ki67 in the ST-Bre-TMA cohort. (A) an example of a tumour with no positive nuclei and (B) a tumour displaying high levels of Ki67. Images were taken at 400x magnification, scale bar represents 100µm.

Using the log rank test, high Ki67 was found to be associated with poorer breast cancer specific survival ($P=0.002$, Figure 3.3A), decreased recurrence free interval ($P=1.1 \times 10^{-5}$, Figure 3.3B) and increased recurrence on tamoxifen ($P=1.5 \times 10^{-8}$, Figure 3.3C). Patients with highly proliferative tumours had an average breast cancer specific survival of 12.7 years, 5.2 years less than those with tumours that displayed low Ki67.

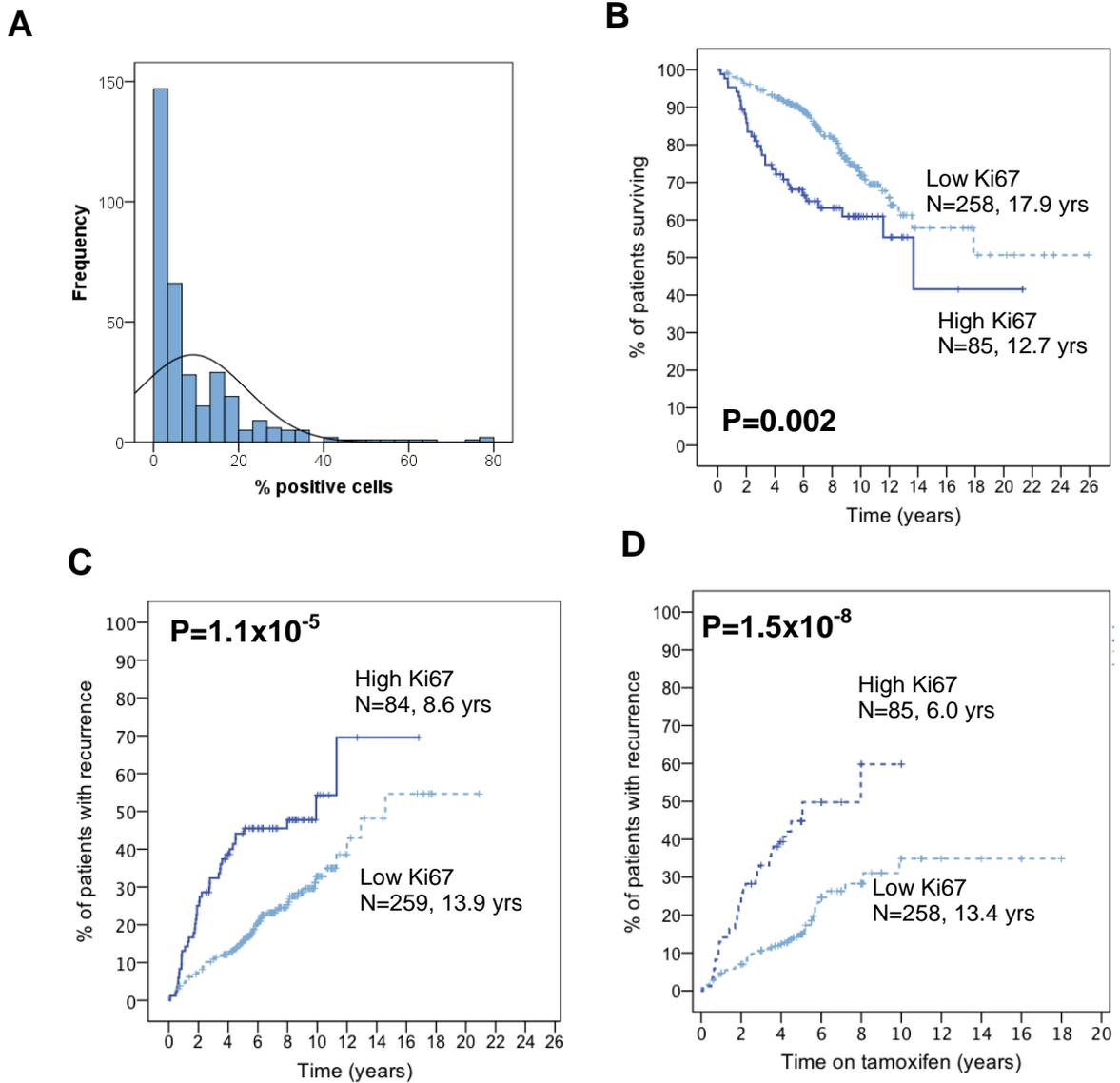


Figure 3.3: Ki67 and clinical outcome in the ST-Bre-TMA cohort. *The range of % positive cells for Ki67, as a marker of proliferation, is shown in the histogram (A). High Ki67 was associated with poor breast cancer specific survival (B), decreased recurrence free interval (C) and an increase in recurrence on tamoxifen (D). Censor lines in panel B indicate loss to follow up or death due to other cause and in panel C and D indicate loss to follow up.*

3.4 Categorising tumours into subtypes using IHC markers

As previously discussed, there are several subtypes of breast cancer, each with distinct characteristics and response to different therapies. Due to lack of fresh tissue, the breast tumours were subtyped based on 4 IHC markers (ER, PgR, HER2 and Ki67) in accordance with the definitions proposed by Cheang *et al* and recommended by the expert panel at St. Gallen [48, 54]

3.4.1 Subtypes in the 1800-Bre-TMA cohort

As the 1800-Bre-TMA cohort includes both ER positive and ER negative patients, and PgR status, HER2 status and Ki67 index were available, it was possible to split the cohort into 4 distinct subtypes as detailed in Table 3.3:

- 1: Luminal A - ER or PgR positive, HER2 negative and low Ki67.
- 2: Luminal B - ER or PgR positive, and HER2 positive and/or high Ki67.
- 3: Triple negative - ER negative, PgR negative and HER2 negative
- 4: HER2 enriched - ER and PgR negative but HER2 positive.

Subtype	IHC markers	Number of patients (%) N=505	Average breast cancer specific survival
Luminal A	ER or PgR positive, HER2 negative, low Ki67 (<15%)	237 (46.9%)	14.0 years
Luminal B	ER or PgR positive, HER2 positive and/or high Ki67 (\geq 15%)	100 (19.8%)	11.7 years
Triple negative	ER, PgR and HER2 negative	122 (24.2%)	11.7 years
HER2 enriched	ER, and PgR negative, HER2 positive	46 (9.1%)	10.3 years

Table 3.3: Subtyping of the 1800-Bre-TMA cohort using 4 IHC markers. *Four IHC markers (ER, PR, HER2 and Ki67) were used to subtype the cohort into four groups. The number of patients in each group is detailed as well as duration of breast cancer specific survival.*

Several patients (39/544) were missing HER2 or Ki67 status and were therefore excluded from analysis. The majority of the 505 breast cancer patients analysed were luminal A (46.9%), 19.8% were luminal B, 24.2% were triple negative and the remaining 9.1% were HER2 enriched. Kaplan-Meier survival graphs were plotted (Figure 3.4) and the log rank test was used to compare low and high Ki67. Patients with luminal A tumours had a significantly longer breast cancer specific survival (14 years, $P=7.2 \times 10^{-9}$) compared to all other subtypes. As multiple comparisons were used in this analysis, using Bonferroni's method a more stringent P value of 0.008 is required since there are 6 possible comparisons. This result remains significant at this more stringent P value. Luminal B patients and triple negative patients both had an average breast cancer specific survival of 11.7 years. The group with the poorest prognosis was the HER2 enriched tumours, in which patients were found to have an average breast cancer specific survival of 10.3 years. This is in line with previous reports examining breast cancer subtypes.

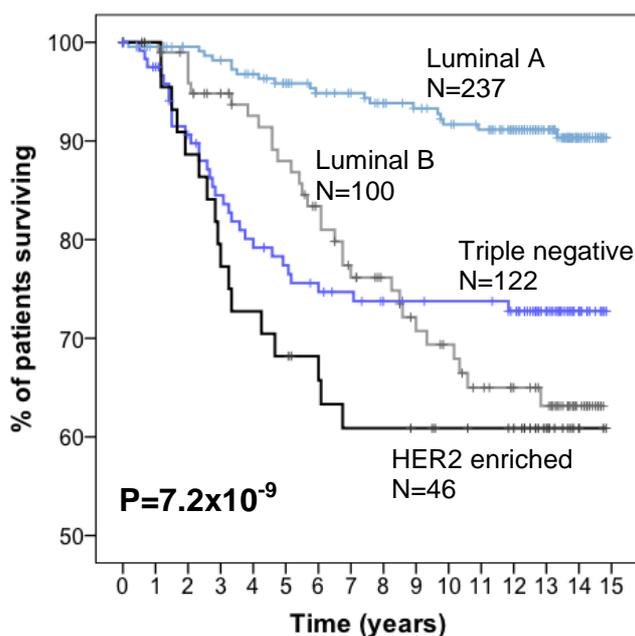


Figure 3.4: Breast cancer subtypes and clinical outcome in the 1800-Bre-TMA cohort. Breast cancer specific survival showed luminal A patients had the longest average survival of 14 years, then luminal B and triple negative (11.7 years), and the worst prognosis was in the HER2 enriched group (10.3 years). Censor lines indicate loss to follow up or death due to other cause.

3.4.2 Subtypes in the ST-Bre-TMA cohort

As this cohort was an ER positive cohort (with the exception of 44 of unknown ER status and 20 ER negative, which were excluded from analysis of subtypes), there were no tumours that fell into the categories of triple negative or HER2 enriched. The cohort was subdivided into luminal A and luminal B using HER2 status and Ki67 index. 79 of the 392 patients were excluded from analysis due to lack of HER2 status or Ki67 index. Of the remaining 313 patients, 223 were categorised as luminal A (71.2%) and 90 as luminal B (35.5%), as detailed in Table 3.4. Luminal A patients had significantly longer average breast cancer specific survival of 16.6 years compared to 14.2 years survival in the luminal B group ($P=0.002$, Figure 3.5).

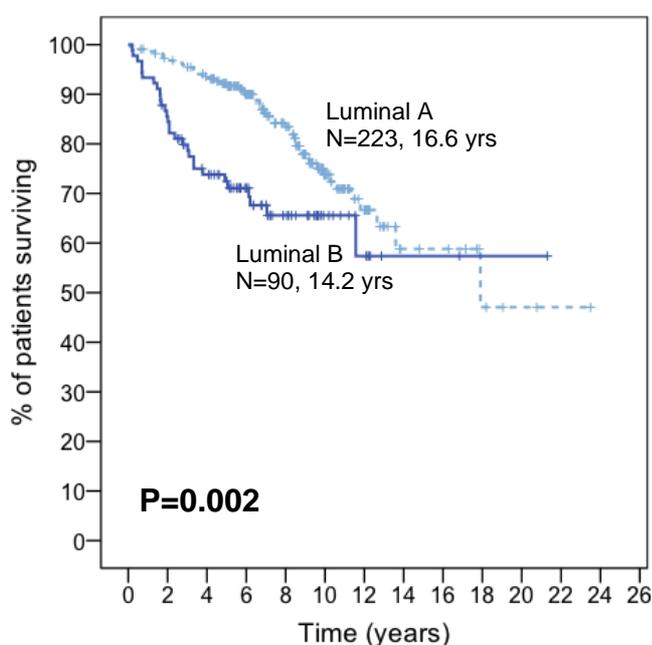


Figure 3.5: Breast cancer subtypes and clinical outcome in the ST-Bre-TMA cohort. Breast cancer specific survival analysis showed luminal A patients had a longer average survival of 16.6 years compared to luminal B patients which had an average survival of 14.2 years ($P=0.002$), Censor lines indicate loss of follow up.

Subtype	IHC markers	Number of patients (%) N = 313	Average breast cancer specific survival
Luminal A	ER or PgR positive, HER2 negative, low Ki67 (<15%)	223 (71.2%)	16.6 years
Luminal B	ER or PgR positive, HER2 positive and/or high Ki67 (≥15%)	90 (28.8%)	14.2 years

Table 3.4: Subtyping of the ST-Bre-TMA cohort into different luminal subtypes using Ki67 and HER2. *The cohort was split into luminal A and luminal B tumours using HER2 status and Ki67 index. The number of patients in each group is detailed as well as duration of breast cancer specific survival.*

3.5 TUNEL as a marker of apoptosis

3.5.1 Apoptosis in the 1800-Bre-TMA cohort

An IHC based approach was also employed to the cohort to detect apoptosis using a commercially available kit to detect DNA fragmentation. Levels of apoptosis were assessed using the number of percentage positive cells, similar to Ki67. Scoring was assessed using the Tissue Image Analysis software on the Slidepath Digital Image Hub. TMAs were dearrayed and matched to the correct position on a map of each TMA allowing separate analysis of each core. The thresholds of a range of size and shape of nuclei and staining colour and intensity were adjusted in the nuclear algorithm. Optimal conditions were established using several cores before the algorithm was applied to the whole cohort. Figure 3.6 shows an example of accepted nuclei and the differentiation between positive and negative nuclei. 10% of cores were scored manually to compare results with the automated system to manual scoring and the ICC was calculated. The ICC was 0.83 indicating good correlation between automated and manual scoring. All cores were checked to ensure the automated score seemed appropriate and no artefacts had been counted and included in analysis.

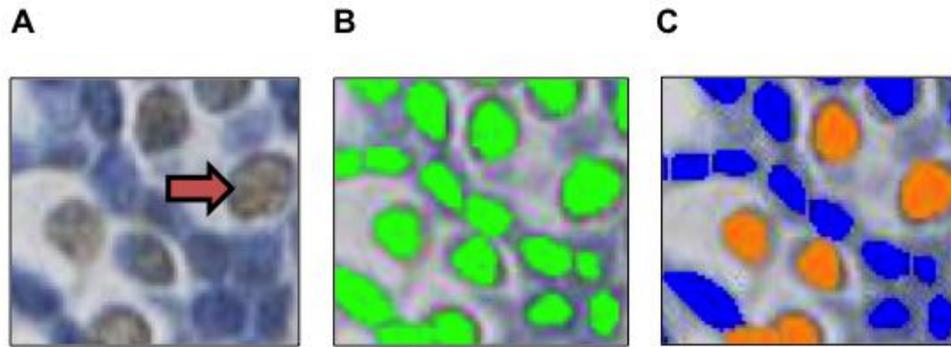


Figure 3.6: Automated scoring of TUNEL using the Slidepath Tissue Image Analysis nuclear algorithm. *The algorithm was optimised for this breast cohort and stain, with thresholds adjusted to include correct size and shaped nuclei and identify positive and negative staining. (A) an example of an area with positive (one is indicated by the arrow) and negative nuclei. (B) Green areas represent accepted nuclei and (C) blue areas indicate the nuclei have been identified as negative and orange as positive.*

Several cores were missing or did not contain tumour, leaving 444 cores available for analysis. The percentage positive values ranged from 0 to 90%, with a median of 0.33%. Average standard deviation of percentage of cells exhibiting staining across the cores from the same patient was 1.25 and median was 0.58 (IQR= 0.0 - 1.0). Figure 3.7 shows an example of staining observed for negative cores and for those with high levels of apoptosis. Using the median as a cut off, tumours were split into those with low levels of apoptosis ($\leq 0.33\%$) and those with high levels of apoptosis ($>0.33\%$). Of the 444 cores available for analysis, 246 (55.4%) were classed as low and 198 (44.6%) as high. Figure 3.8A shows a histogram with the distribution of percentage positive scores for TUNEL.

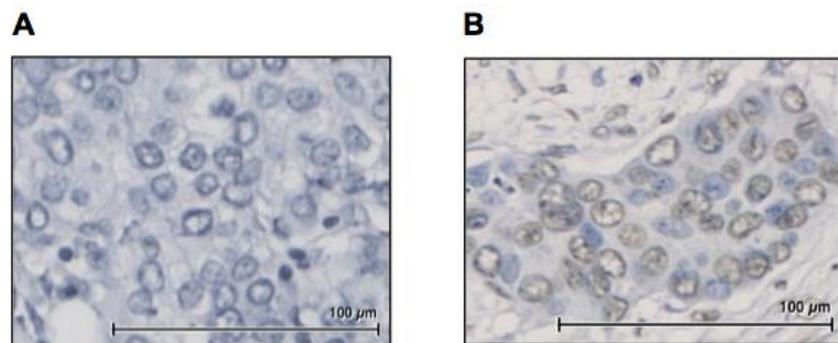


Figure 3.7: Expression of TUNEL in the 1800-Bre-TMA. *(A) an example of a tumour with no positive nuclei and (B) a tumour displaying high levels of apoptosis. Images taken at 400x magnification, scale bar represents 100µm.*

Kaplan-Meier survival curves were drawn for both breast cancer specific and recurrence free interval and high and low expression was compared using the log rank test. Apoptosis was not associated with breast cancer specific survival (Figure 3.8A, $P=0.304$) but was significantly associated with recurrence free interval (Figure 3.8B, $P=0.033$). Patients whose tumours displayed low levels of apoptosis had a shorter recurrence free interval of 7 years compared to 7.8 years in those with high apoptosis. However, using a more stringent P value of 0.01 this is not regarded as significant.

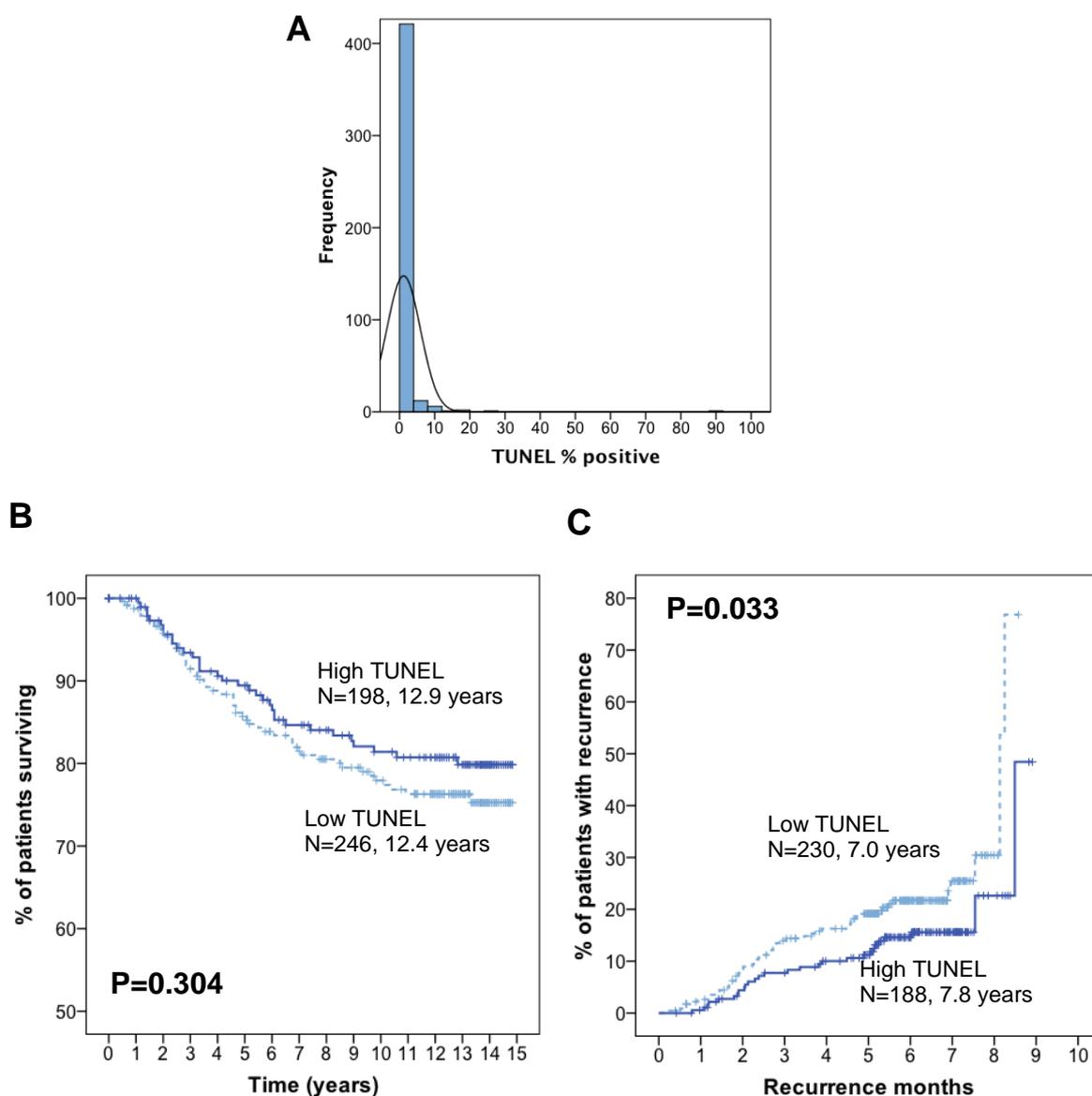


Figure 3.8: Apoptosis and clinical outcome in the 1800-Bre-TMA cohort. (A) Histogram showing distribution of % positive cells for TUNEL. (B) Apoptosis was not associated with breast cancer specific survival ($P=0.304$). (C) Those with high TUNEL had a longer recurrence free interval of 7.8 years compared to 7 years in those with low TUNEL ($P=0.033$). Censor lines in panel A indicate loss to follow up or death due to other cause and in panel B indicate loss to follow up.

3.5.2 Apoptosis in the ST-Bre-TMA cohort

Apoptosis was also assessed in the ST-Bre-TMA by TUNEL. Cores were scored manually due to incorrect percentages reported by the automated Tissue Image Analysis system. The average standard deviation of percentage positive cells in the three cores from one tumour was 16.6 and the median was 11.4 (IQR= 1.2 - 26.5). Examples of tumours with different levels of TUNEL are shown in Figure 3.9. Using the cut off established in the previous cohort, tumours were categorised as having high (>0.33%) and low (\leq 0.33%) levels of apoptosis. Figure 3.10A shows a histogram of percentage positive cells for TUNEL. Many more tumours in this cohort displayed higher levels of tumours, in comparison to the 1800-Bre-TMA where (as shown in the histogram in Figure 3.8A) most tumours displayed no or low levels of apoptosis.

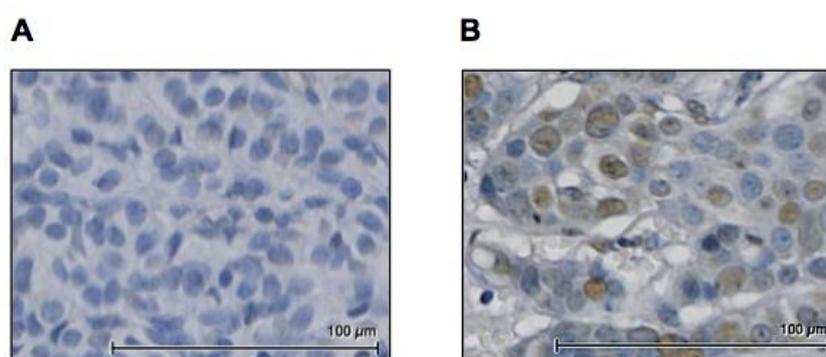


Figure 3.9: Expression of TUNEL in the ST-Bre-TMA. (A) an example of a tumour with no positive nuclei and (B) a tumour displaying high levels of apoptosis. Images taken at 400x magnification, scale bar represents 100 μ m.

Higher levels of apoptosis were associated with a more favourable clinical outcome. Those with high levels of apoptosis had a longer average breast cancer specific survival of 16.1 years compared to 11.8 years for those with low apoptosis ($P=0.034$, Figure 3.10B). Higher apoptosis also resulted in a longer recurrence free interval (9.1 years for low versus 12.8 years for high, $P=0.025$, Figure 3.10C). When recurrence on tamoxifen was considered, those with high levels of apoptosis had a longer time to recurrence of 12.4 years compared to 7.6 years on average for patients with low apoptosis ($P=0.038$, Figure 3.10D). Again, however, the use of a more stringent P value of 0.01 means this association would not be considered significant. It is therefore debatable whether TUNEL, as a marker of apoptosis, is associated with clinical outcome.

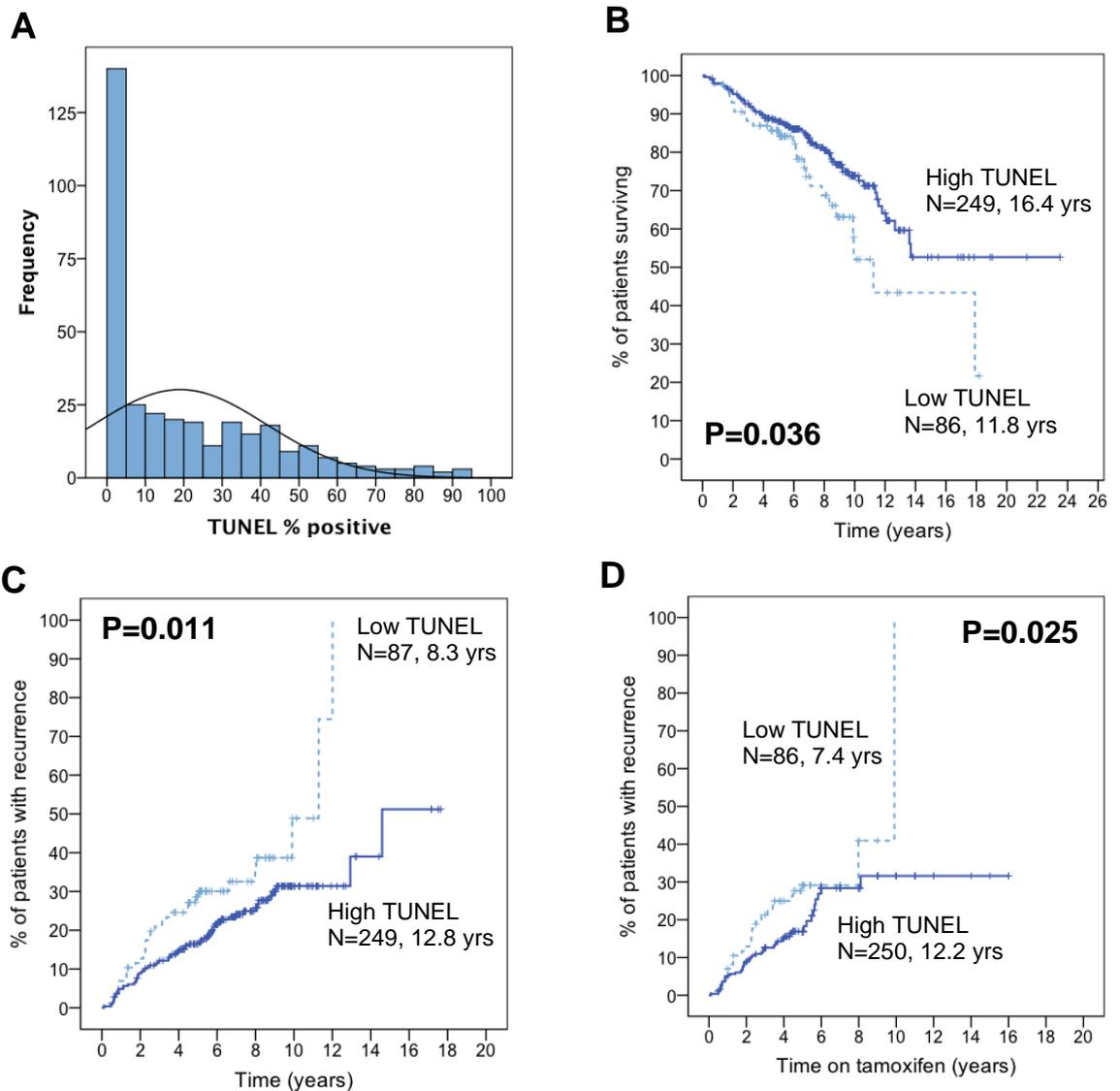


Figure 3.10: Apoptosis and clinical outcome in the ST-Bre-TMA cohort. (A) Histogram of percentage positive cells stained using the TUNEL assay to measure apoptosis. Low apoptosis was associated with poor breast cancer specific survival (B), decreased recurrence free interval (C) and an increase in recurrence on tamoxifen (D). In Figure B censor lines indicate loss of follow up or death due to other cause and in Figures C and D indicate loss to follow up.

Levels of Bcl2 were already available in this cohort as part of a study by Dr Elizabeth Cannings [162]. Chi squared analysis showed expression of TUNEL was significantly associated with expression of Bcl2 ($P=0.034$). TUNEL was found to be a stronger prognostic apoptosis marker, as there was no significant difference between low and high Bcl2 expression on breast cancer specific survival ($P=0.729$), recurrence free interval ($P=0.515$) or recurrence on tamoxifen ($P=0.193$).

3.6 Discussion

IHC is a valuable tool in translational research and also in diagnostic use to assist in the selection of appropriate targeted therapies. There are however many pitfalls as it can be variable due to the quality of the tissue and for routine use in diagnostic laboratories there needs to be standardised protocols for technique and analysis and extensively validated cut offs.

The proliferation rate of a tumour is important in the prognosis of breast cancer and the development of MIB-1, a monoclonal antibody that detects Ki67, has allowed immunohistochemical studies to use this marker in the assessment of tumour proliferation. This antibody was recommended to be considered as the 'gold standard' for Ki67 detection by the International Ki67 in Breast Cancer Working Group [163]. It was also recommended that Ki67 was scored using the percentage of positive cells [163] and it was this method that was applied in this thesis. Several cut offs for Ki67 have been used in studies, usually ranging from 10-20% [159]. A cut off of 15% was previously established in the 1800-Bre-TMA cohort using tertiles and was found to be associated with clinical outcome in both this cohort and the ER positive ST-Bre-TMA cohort. In order to establish a cut off for routine diagnostic use, guidelines on the standardisation of fixation, staining, scoring and analysis are required. This was recognised by the International Ki67 in Breast Cancer Working Group and a consensus on the most appropriate cut off value was not reached as it was recommended further independent studies were required for validation [163]. The tissue available for this thesis used TMAs, which allows for simultaneous assessment of hundreds of patients in a single experiment. Although this is useful for high throughput, variable levels of Ki67 have been reported in whole sections with hotspots occurring at certain areas of tumour [163]. In order to account for tumour heterogeneity, three separate TMAs were constructed to allow analysis of three separate areas of tumour, as identified by a pathologist. The use of TMAs to stain Ki67 is controversial due to heterogeneity observed with certain tumour areas. A validation study was conducted in colorectal cancer comparing TMAs and whole sections for Ki67 and several other markers [164]. Comparing the % positive cells (using a 20% cut off) in TMAs and whole sections, the authors found that there was a 0.79 kappa value between both methods for Ki67 [164]. In a previous study in our laboratory, using a small set of patients with available whole sections, it was found that scores obtained using TMAs and whole sections were comparable (Oral communication, Dr Joanne Edwards). In another study in breast cancer, however, it has been reported that in a cohort of 213 patients whole sections are superior to TMAs for prognostication and correlation with clinic-pathological correlation [165]. This

study also reported poor correlation between each method with a Pearson's correlation coefficient of 0.13417 [165]. It appears the TMAs tended to underestimate the scores, which may be due to missing "hot spots" (areas where Ki67 is particularly high) that are present on the section. Guidelines on the recommended scoring method when hotspots are present yet to be established, with some using hot spots as a focus for scoring, others using them in the general assessment of the section as a whole and others recommending ignoring hot spots. However the International Ki67 in Breast Cancer Working Group have recommended that until proper guidelines are established, that is hot spots are present the assessment of Ki67 should include these and give an overall average score for the whole section [163]. The use of whole sections therefore may be more appropriate for assessment of Ki67, however unfortunately only TMAs were available for this study. Further investigations are needed with much larger datasets to assess the level of correlation between each method. In both cohorts Ki67 was demonstrated to be a useful marker, which correlated with breast cancer specific survival, recurrence free interval and recurrence on tamoxifen and allows stratification of patients. However for use clinically strict adherence to guidelines on fixation, staining and scoring would have to be followed in order to make it reproducible across diagnostic laboratories. Additional assessment is also needed to compare the use of TMAs for Ki67.

Apoptosis, the loss of which is another hallmark of cancer, was also investigated in both patient cohorts. Various markers have been used in studies such as caspases, Bax, Bcl2 and DNA fragmentation using the TUNEL method. This study used a commercially available kit to measure apoptosis levels via the TUNEL method. It was hypothesised that a higher level of apoptosis in breast tumours would result in a better clinical outcome in patients. TUNEL was associated with recurrence free interval in the first cohort with those patients with lower TUNEL expression having a quicker time to recurrence. However levels of TUNEL were not associated with disease specific survival, although a similar trend is observed. The association with clinical outcome was validated in the second cohort, using the previously established cut off, with low levels of TUNEL associating with decreased breast cancer specific and recurrence free interval and a quicker time to recurrence on tamoxifen. To compare different markers of apoptosis and their significance on clinical outcome, expression of TUNEL was assessed as well as Bcl2, which was available from a previous study in the ST-Bre-TMA cohort. Bcl2 is anti-apoptotic, blocking pro-caspases, and therefore may be expected to correlate with low levels of TUNEL. Levels of Bcl2 were significantly associated with TUNEL when chi squared test was performed. Apoptosis detection via the TUNEL assay was found to be a stronger prognostic marker

than Bcl2, which was not found to be significantly associated with breast cancer specific survival, recurrence free interval or endocrine resistance.

As this study used TMAs with three representative cores from each tumour and scores were calculated as the average across the three cores, the standard deviation was also calculated across these cores as a measure of heterogeneity and the median and interquartile range have also been calculated. This is particularly important for Ki67 and apoptosis, where the markers may exhibit variable expression due to the tumour microenvironment. Cores were generally taken from the centre of the tumour and although some heterogeneity was observed, overall there was fairly good agreement in the expression of markers examined in this study, shown by the standard deviation values. There was heterogeneity observed with Ki67 with the maximum standard deviation of 38.4, this may be due to the hotspots observed with Ki67 staining. The use of three cores in the TMAs is designed to account for heterogeneity and result in a representative score for the whole tumour. A much larger deviation is likely to have been observed had the TMA included cores from the invasive edge as well as the centre of the tumour and this would also allow comparison of expression of various markers at different areas.

Although an association with disease recurrence was observed, TUNEL is not warranted for inclusion in routine analysis at present until the most appropriate marker is established and guidelines on its use developed. Additionally, the findings of this study contradict others, which have found that a high apoptotic index is associated with poorer survival, although these used different methods of apoptosis detection [163, 166-168]. Another study of clinical outcome and apoptosis used DNA fragmentation as a marker of apoptosis and also investigated Bcl2 expression on clinical outcome [169]. High Bcl2 was associated with a favourable outcome but was not associated with apoptosis. Apoptosis was not significantly associated with clinical outcome in this study [169]. There is therefore controversy surrounding apoptosis and clinical outcome in breast cancer, however these results support the hypothesis that low levels of apoptosis are associated with poorer outcome. The use of markers to quantify levels of apoptosis are however useful in the research setting when investigating other markers as it allows markers to be correlated with different processes involved in the development and progression of breast cancer. Furthermore, detection of apoptosis may be a useful marker to show response to therapy [168]. Consensus and extensive validation is required on the use of markers and cut offs before assessment of apoptosis can be considered for adoption clinically.

In order for assessment of markers of proliferation and apoptosis to be implemented in routine diagnostic laboratories, as well as the development of standard protocols and guidelines on assessment being required, a method of automatically assessing expression is necessary. Analysis can be very tedious as it is not possible to accurately estimate the percentage of cells that are positive and manual counting is a very arduous task. The introduction of image analysis software offers the possibility of automated assessment of IHC slides, reducing the workload of the pathologist. However use of such software remains to be demonstrated to be reproducible and highly accurate. As part of a previous study Ki67 was counted both visually and automatically using the Slidepath Image Analysis system and an excellent correlation was observed between the two methods [157]. A stronger association was observed, however, with Ki67 levels and clinical outcome when counting was performed visually rather than with the automated system [157]. Other studies investigating the use of automated assessment of Ki67 index in breast cancer have also reported good correlation between manual and automated scoring [170-171]. Although good correlation is observed between the two scoring methods, more investigation and optimisation is required to ensure automation is able to generate reliable clinical measurements before it is adopted in the routine clinical pathology laboratory.

In this study apoptosis, measured using a TUNEL assay, was scored in the 1800-Bre-TMA cohort using the automated system and although several artefacts had to be corrected, there was generally good correlation between manual and automated counting. There was however discordance with percentages obtained for apoptosis and Ki67 percentages in the ST-Bre-TMA cohort, meaning all cores had to be scored visually. This again highlights that further work is required before the use of automated systems is appropriate. The time to fixation has been reported to have little effect on visual analysis but is important when using automated image analysis as it impacts on the integrity of the nucleus [163]. Antigen retrieval is required and the use of a standard protocol for this is recommended [163]. The counterstain is another aspect that can affect the results obtained from automated scoring. If the stain is too weak the software does not detect all the nuclei, and it is important all nuclei are counted as it can drastically alter the percentage obtained if the number of negative nuclei are underestimated [163]. Equally, when counterstaining is too strong, it is often difficult for the software to differentiate between negative dark blue nuclei and brown positive nuclei. Every step of the process of IHC and scoring therefore needs to be standardised to improve reliability of automated scoring. Additionally, the problems with the automatic scoring highlights a limitation of this part of the study due to the use of two different analysis methods, visual and automatic, for each cohort in the assessment of

apoptosis, making comparison of results from each cohort for apoptosis problematic and therefore caution should be applied in interpretation of these results. In order to be used for validation and to apply the same cut offs the same method of analysis is required and ideally the 1800-Bre-TMA cohort should be scored visually due to the problems in optimisation of the automated scoring system in the ST-Bre-TMA. These problems may be due to slightly different fixation methods or strength of counterstaining which, as previously discussed, can affect the results of automatic scoring. The use of Ki67 is arguably of greater importance in this study due to the inclusion of Ki67 in stratification by molecular subtype. For this reason Ki67 values used in each cohort for Kaplan-Meier graphs and log-rank tests used visual scoring for both cohorts and these are therefore comparable.

The use of proliferation requires additional validation and guidelines on its use, but an expert panel at the St. Gallen International Breast Cancer Conference supported the use of Ki67 staining to routinely split the luminal subtypes once guidelines are available [48]. Using the 15% cut off for Ki67, the clinical cohorts were stratified into four subgroups: luminal A (ER+ and/or PgR+, HER2-, low Ki67), luminal B (ER+ and/or PgR+ and HER2+ and/or high Ki67), HER2 enriched (ER-, PgR-, HER2+) and triple negative (ER-, PgR-, HER2-). Percentages were in line with those reported elsewhere, with luminal A tumours being the most frequent in both cohorts. Additionally, as expected patients with luminal A tumours displayed a more favourable outcome compared with other subtypes. Levels of Ki67 and HER2 therefore appropriately stratified luminal tumours into luminal A and B. As discussed in Chapter 1, the different subtypes of breast cancer have been reported to respond differently to various therapies, which should be considered when deciding on the most appropriate treatment and therefore the availability of this information is beneficial to clinicians. Ki67 therefore has been demonstrated to be an extremely useful marker overall, which once proper guidelines are in place to standardise would provide valuable clinical information. Performing Ki67 assessment in both cohorts in this chapter allowed the division of both cohorts into molecular subtypes, allowing stratification of patients in future chapters.

In conclusion, this chapter has shown that Ki67, as a marker of proliferation, is able to split the cohorts into groups with different prognosis and in combination with ER, PgR and HER2 appeared to be able to stratify tumours into the four main breast cancer subtypes. Apoptosis, in this case measured using a TUNEL assay, appears to be prognostic, but this does not appear to be as useful as proliferation. Additionally a standardised marker is required before any guidelines on protocol and cut offs could be produced. This study also demonstrated that extensive optimisation of image analysis is needed and at present manually scoring these markers is a superior method than using computer software. Furthermore, Ki67 along with HER2 status appears to appropriately subdivide the ER positive tumours into luminal A and B and markers that allow stratification may become increasingly important as more is learned about the different subtypes.

Chapter 4:

Expression of members of the NF- κ B pathways in breast cancer clinical specimens

4.1 Introduction

The NF- κ B pathways have been implicated in the progression of breast cancer. Studies with large numbers of patients exploring the role of different members are however lacking. We aimed to investigate the expression of key family members of both the canonical and non-canonical NF- κ B pathways in breast cancer patient tissue and assess the clinical significance of each member. Before use in valuable patient tissue, each antibody was validated to confirm it specifically targeted the protein of interest. Antibodies were then optimised for IHC in breast tissue before protein expression of each marker was assessed in the 1800-Bre-TMA cohort.

4.2 Antibody validation of members of the canonical pathway

Antibodies are one of the most commonly used tools in research, particularly in translational tissue based research. In order to produce reliable results, antibodies must be demonstrated to be specific and immunostaining reproducible. Selection of appropriate antibodies and verification of specificity are extremely important steps before use in patient tissue. All antibodies used in this study were confirmed as specific.

4.2.1 Validation of anti-IKK β antibody

The antibody against IKK β was previously validated in our laboratory by Mr L MacKenzie to ensure specificity using several methods. First, a single band of the predicted size of 87 kDa was observed by western blotting (Figure 4.1A). In paraffin embedded cell pellets of LNCaP prostate cancer cells, a decrease in expression of IKK β was shown in IKK β silenced cells compared to control, but expression was retained in cells treated with IKK α or non-targeting siRNA (Figure 4.1B). Blocking peptide experiments using IHC on archival prostate tissue showed loss of expression when the antibody was pre-incubated with excess IKK β protein before use on tissue, but not when pre-incubated with IKK α or p65 protein (Figure 4.1C).

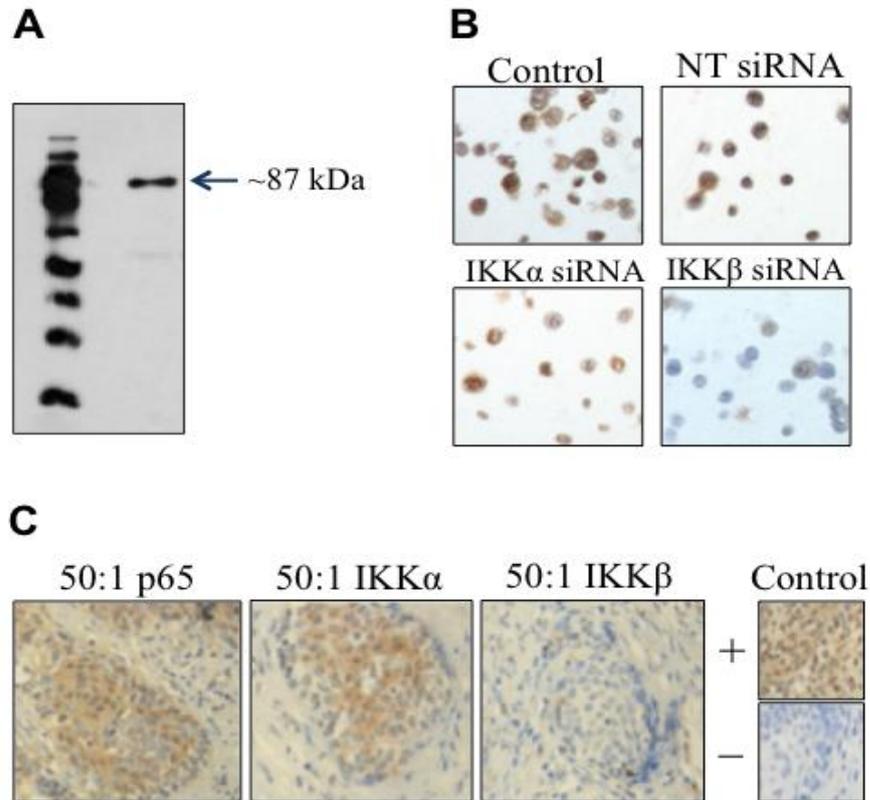


Figure 4.1: Validation of the anti-IKK β antibody. (A) Western blotting showed a clear band of appropriate size (87 kDa) in extracts from LNCaP prostate cancer cells. (B) Silenced LNCaP cell pellets showed a decrease in expression of IKK β in cells where IKK β was silenced, but expression was retained in IKK α silenced cells and non-targeting (NT) control cells. (C) Blocking peptide experiments in prostate cancer tissue showed an excess of IKK β , but not IKK α or p65, reduced expression of IKK β .

4.2.2 Validation of antibodies detecting the p65 subunit

Antibodies for p65, phosphorylated p65 were confirmed as specific as part of this study. Dr Pamela McCall demonstrated the p65 NLS antibody to be specific as part of a previous study. A single band of correct size (65 kDa) was shown on a western blot of MCF7 cell lysates for p65 (Figure 4.2A) and phospho-p65 (Figure 4.2C). In IHC of cell pellets of MCF7 breast cancer cells, nuclear translocation was observed when exposed to 20ng/ml of TNF α . Blue (negative) nuclei are observed in the untreated control cells and translocation to the nucleus after TNF α treatment resulted in brown nuclei due to p65 (Figure 4.2B) and phospho-p65 (Figure 4.2D) expression. Western blotting of LNCaP cell extracts showed a single band of around 65 kDa (Figure 4.2E), and nuclear translocation and an increase in expression of p65 NLS was observed when IHC was performed on cell pellets of LNCaP cells treated with TNF α compared to untreated cells (Figure 4.2D).

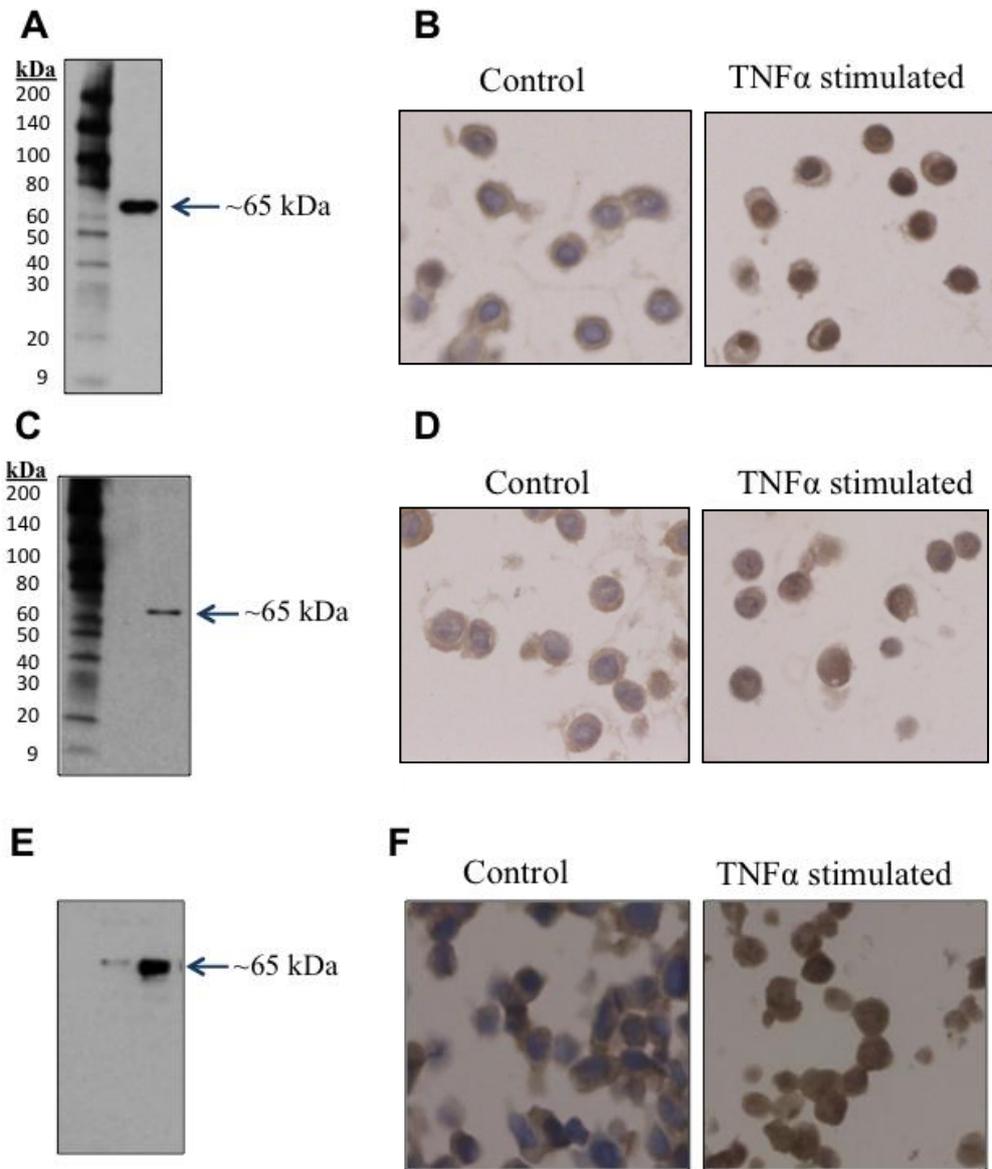


Figure 4.2: Validation of the anti-p65, anti-phospho-p65 and anti-p65-NLS antibodies. (A) Western blot for anti-p65 showing a single band of appropriate size (65 kDa) in lysates of MCF7 breast cancer cells. (B) IHC was employed to show nuclear translocation of p65 in TNF α stimulated MCF7 cell pellets. Blue nuclei are observed in the untreated cells and brown are observed in the stimulated cells due to nuclear expression of p65. (C) Western blot using the phospho-p65 antibody showed a single band of appropriate size (65 kDa) in MCF7 cells. (D) IHC on TNF α treated MCF7 cell pellets showed nuclear translocation of phospho-p65. (E) Western blot for anti-p65-NLS showing a single band of 65 kDa. (F) Cell pellets showing increase in p65 NLS and nuclear translocation in TNF α treated LNCaP cells.

4.3 Expression and clinical outcome of members of the canonical pathway

4.3.1 Expression of IKK β and clinical outcome

IKK β expression was analysed in the 1800-Bre-TMA cohort. 144 patients had cores which were missing or contained no tumour leaving 400 patients available for analysis. The histoscores for cytoplasmic expression of IKK β ranged from 0 to 275 with a median of 120 (with an interquartile range (IQR) of 95.4 - 150), and for the nuclear compartment ranged from 0 to 230 with a median of 96.7 (IQR = 65 - 135). An example of high and low expression of IKK β is displayed in Figure 4.3A. The cohort was subdivided into those with low and high expression using the median as a cut off. Tumours with a cytoplasmic histoscore over 120 were classed as having high cytoplasmic IKK β and tumours with a nuclear histoscore of more than 96.7 assigned to the high nuclear expression group. The average standard deviation in histoscore across the cores from the same patient for cytoplasmic expression was 32.2 and the median was 26.5 (IQR = 14.1 - 45.5). For nuclear expression the average standard deviation was 38.4 with a median of 32.1 (IQR = 20.8 - 50.8). An example of immunostaining of IKK β is displayed in Figure 4.3, with low expression shown in 4.3A and high in 4.3B. Also included in Figure 4.3 are histograms displaying distribution of cytoplasmic (Figure 4.3C) and nuclear (Figure 4.3D) histoscores. Bland altman plots demonstrated no bias between observers and the ICC was 0.89 for cytoplasmic expression and 0.81 for nuclear expression showing good correlation between observers.

To determine whether IKK β expression was significantly associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of IKK β were plotted and low and high expression was compared using the log rank test. No association was observed with recurrence free interval for cytoplasmic (P=0.213, hazard ratio (HR) = 1.3 (0.8 - 2.1), Figure 4.3E) or nuclear (P=0.769, HR = 0.9 (0.6 - 1.5), Figure 4.3F) expression. Chi squared analysis was performed to assess the association with clinic-pathological factors of the cohort including age, size, grade, nodal status, NPI, ER status, PgR status, HER2 status, Ki67 index, apoptosis, necrosis, angiogenesis and necrosis. Expression of IKK β , in the cytoplasm or nucleus, was not associated with any clinico-pathological factors of the cohort.

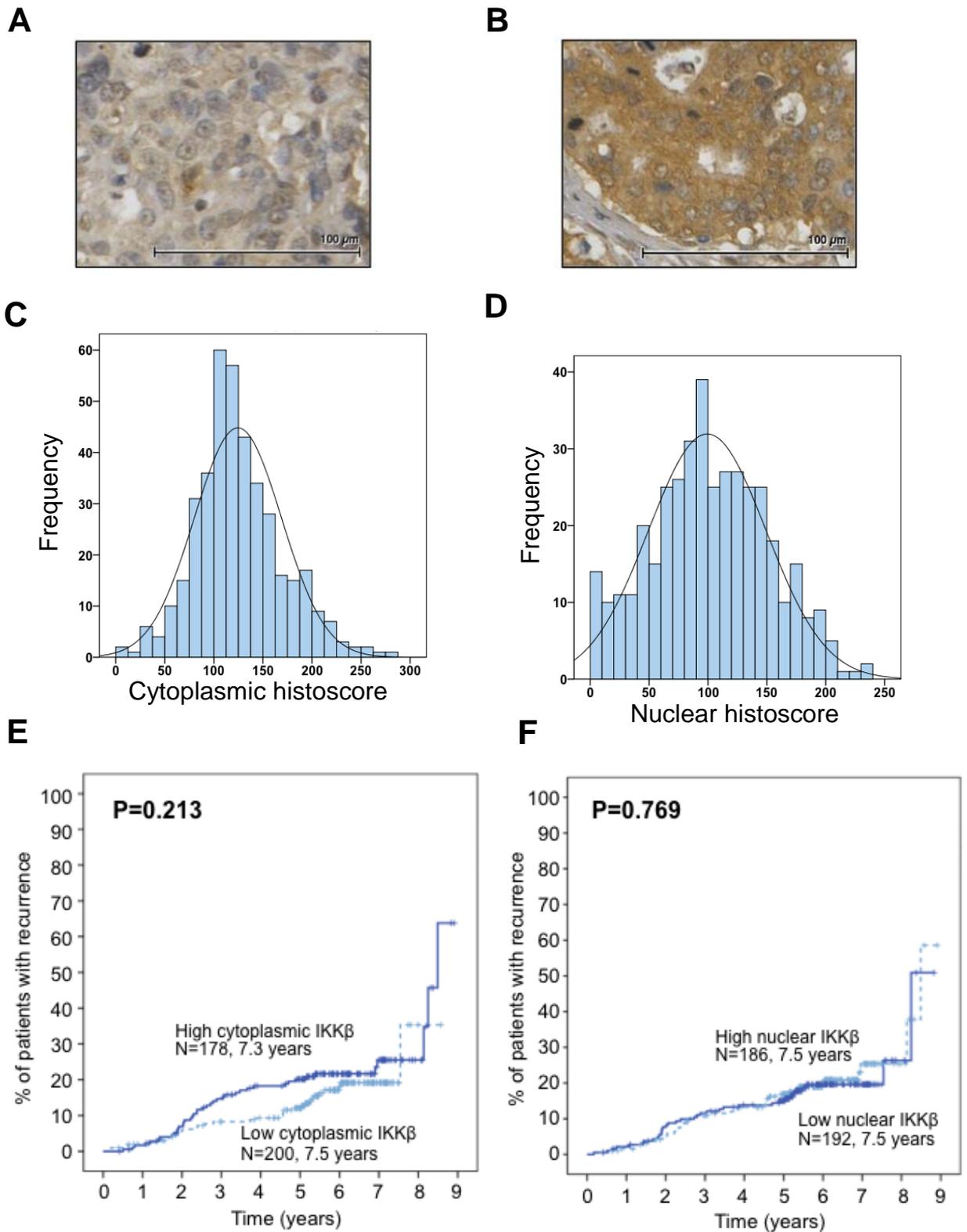


Figure 4.3: Expression of IKK β and clinical outcome in the 1800-Bre-TMA. An example of low (A) expression and of high (B) expression of IKK β in breast cancer tissue is shown (images taken at 400x magnification, scale bar represents 100 μ m) as well as histograms with normal distribution of cytoplasmic (C) and nuclear (D) histoscores. Expression of IKK β in the cytoplasm (E) or nucleus (F) was not associated with recurrence free interval. Censor lines indicate loss to follow up.

4.3.2 Expression of p65 and clinical outcome

Expression of p65, both cytoplasmic and nuclear, was assessed in 438 patients (80.5%) in the 1800-Bre-TMA cohort, with 19.5% (N=106) missing or containing no tumour. Of those tumours assessed, 94.1% (412) showed cytoplasmic expression with a histoscore range of 0 to 170. The median histoscore was 60 (IQR = 30 - 86.7). Only 13.7% (N=60) showed nuclear expression with a range of 0 to 66.7 and a median histoscore of 0 (IQR = 0 - 0). Across the cores from the same patient the average standard deviation in histoscore for cytoplasmic expression was 23.8 and the median was 20.8 (IQR = 10.0 - 35.5), and for nuclear expression the average was 1.5 with a median of 0.0 (IQR = 0 - 0). An example of immunostaining showing low and high p65 expression is shown in Figure 4.4. This figure also displays histograms of cytoplasmic (Figure 4.4C) and nuclear (Figure 4.4D) histoscores. Bland altman plots again demonstrated no bias between observers. Correlation between observers scoring was excellent for cytoplasmic expression with an ICC of 0.91. Good correlation was also evident for nuclear expression with an ICC of 0.8.

To determine whether p65 expression was significantly associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of p65 were plotted and low and high expression were compared using the log rank test. No association was observed with recurrence free interval for cytoplasmic (P=0.904, HR = 0.9 (0.7 - 1.6), Figure 4.4C) or nuclear (P=0.464, HR = 1.3 (0.7 - 2.3), Figure 4.4D) expression of p65. When chi squared analysis was performed to assess the association with clinic-pathological factors of the cohort expression of p65, in the cytoplasm was associated with tumour size (P=0.020) and also moderately with Ki67 (P=0.047) and in the nucleus was associated with nodal status (P=0.013) and necrosis (P=0.017).

Correlation between members of the pathway was measured using Pearson correlation coefficient. Expression levels of p65 and IKK β in the cytoplasm were found to weakly correlate (Figure 4.5). The correlation coefficient between cytoplasmic expression of p65 and IKK β was 0.299 (P=2.84x10⁻⁹). Although this P value is highly significant, the correlation coefficient (which in this analysis is of greater importance) is low showing only weak correlation between cytoplasmic expression of p65 and IKK β .

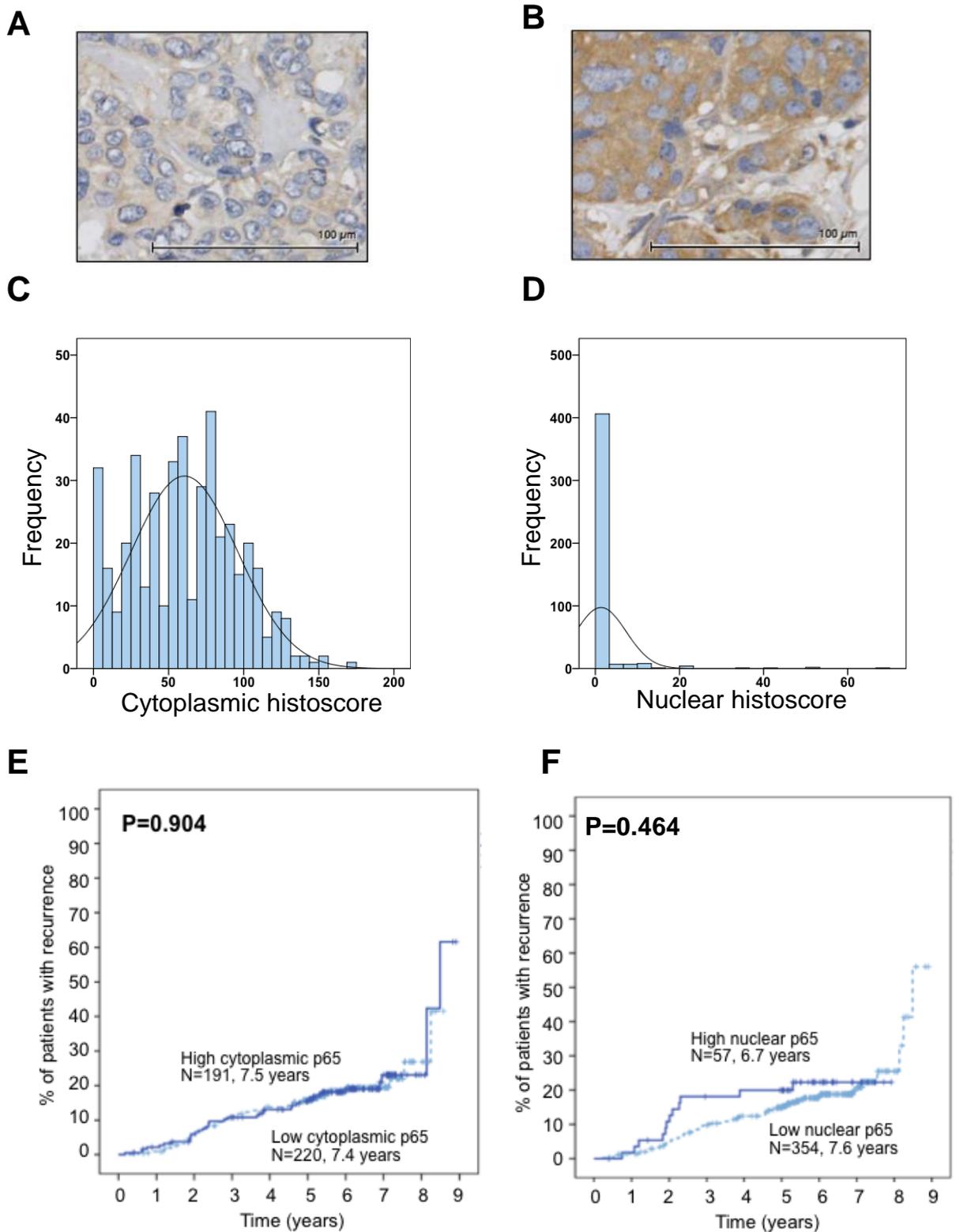


Figure 4.4: Expression of p65 and clinical outcome in the 1800-Bre-TMA. An example of low (A) expression and of high (B) expression of p65 in breast cancer tissue is shown (images taken at 400x magnification, scale bar represents 100 μ m) as well as histograms with normal distribution of cytoplasmic (C) and nuclear (D) histoscores. Expression of p65 in the cytoplasm (E) or nucleus (F) was not associated with recurrence free interval. Censor lines indicate loss to follow up.

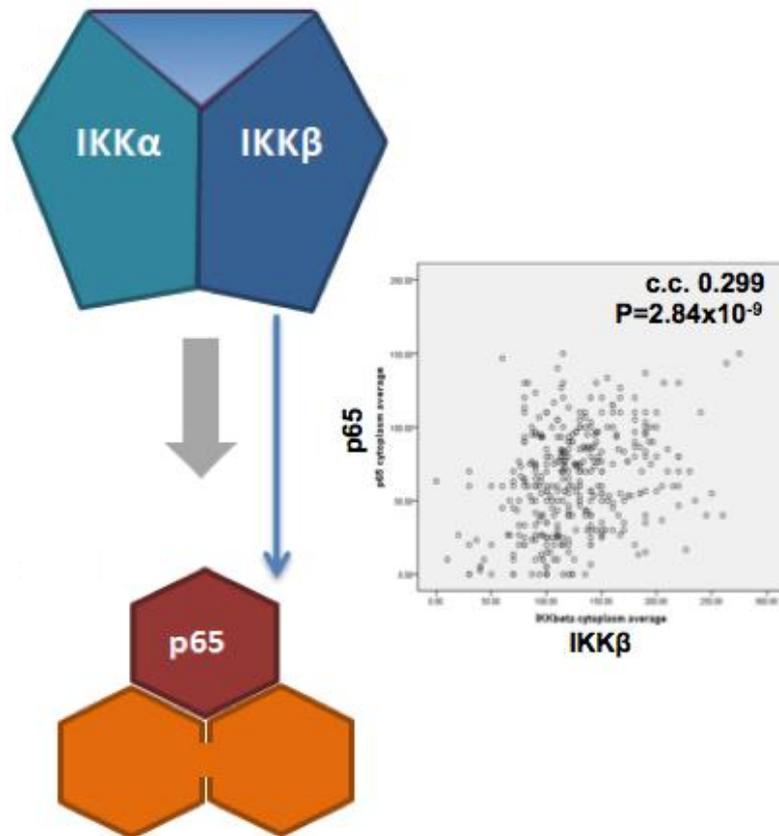


Figure 4.5: Correlation in expression between members of the canonical pathway. *Cytoplasmic expression of p65 and IKKβ were found to weakly correlate. Although a highly significant P value was obtained, the correlation coefficient was only 0.299 and therefore only a weak correlation was observed. C.c = Pearson correlation coefficient.*

4.3.3 Phosphorylation of p65 and clinical outcome

Expression of p65 phosphorylated at Serine residue 536 (phospho-p65) was assessed in 76.1% of patients (N=414). Of these, 98.3% (N=405) showed cytoplasmic expression with a range in expression of 0 to 220 and a median histoscore of 80 (IQR = 46.7 - 110). 81.6% (N=338) showed nuclear expression of phospho-p65 S536 ranging from 0 to 160 histoscore units, with a median histoscore of 14.2 (IQR = 5 - 30). The canonical NF- κ B pathway is therefore found to be active in the majority of breast cancers. The average standard deviation in cytoplasmic histoscore across the cores from the same patient was 38.4 and the median was 35.4 (IQR = 15.3 - 56.6). Across the cores the average standard deviation in nuclear histoscore was 14.8 with a median of 7.1 (IQR = 2.9 - 21.2). Figure 4.6A shows immunostaining of a tumour with low phospho-p65 expression and Figure 4.6B shows an example of high expression. Histograms in Figure 4.6 show distribution of histoscores for p65 expression in the cytoplasm (Figure 4.6C) and nucleus (Figure 4.6D). Scoring of cytoplasmic expression showed excellent correlation between the two observers with an ICC of 0.91 for cytoplasmic expression and nuclear scores had good correlation with an ICC of 0.84. Additionally, bland altman plots showed no bias between observers.

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of p65 were plotted and low and high expression compared using the log rank test. Cytoplasmic expression of phospho-p65 was not associated with recurrence free interval (P=0.904, HR = 1.0 (0.7 - 1.7), Figure 4.6C). Nuclear expression of phospho-p65 was however associated with decreased recurrence free interval (P=0.005, HR = 2.0 (1.2 - 3.1), Figure 4.6D). Patients with tumours with high levels of phospho-p65 in the nuclear compartment had a shorter recurrence free interval of 7 years on average compared to 7.9 years for those with low expression. This was independent in cox regression multivariate analysis when combined with tumour size, invasive grade, nodal status, NPI, ER status, PgR status, HER2 status, Ki67, Necrosis and angiogenesis (P=0.01, HR = 1.9 (1.2 - 3.2)). When chi squared analysis was performed to explore the association of phosphorylation of p65 in the cytoplasm with factors of the cohort, it was found to associate with tumour size (P=0.010) and weakly with Klintrup (P=0.044).

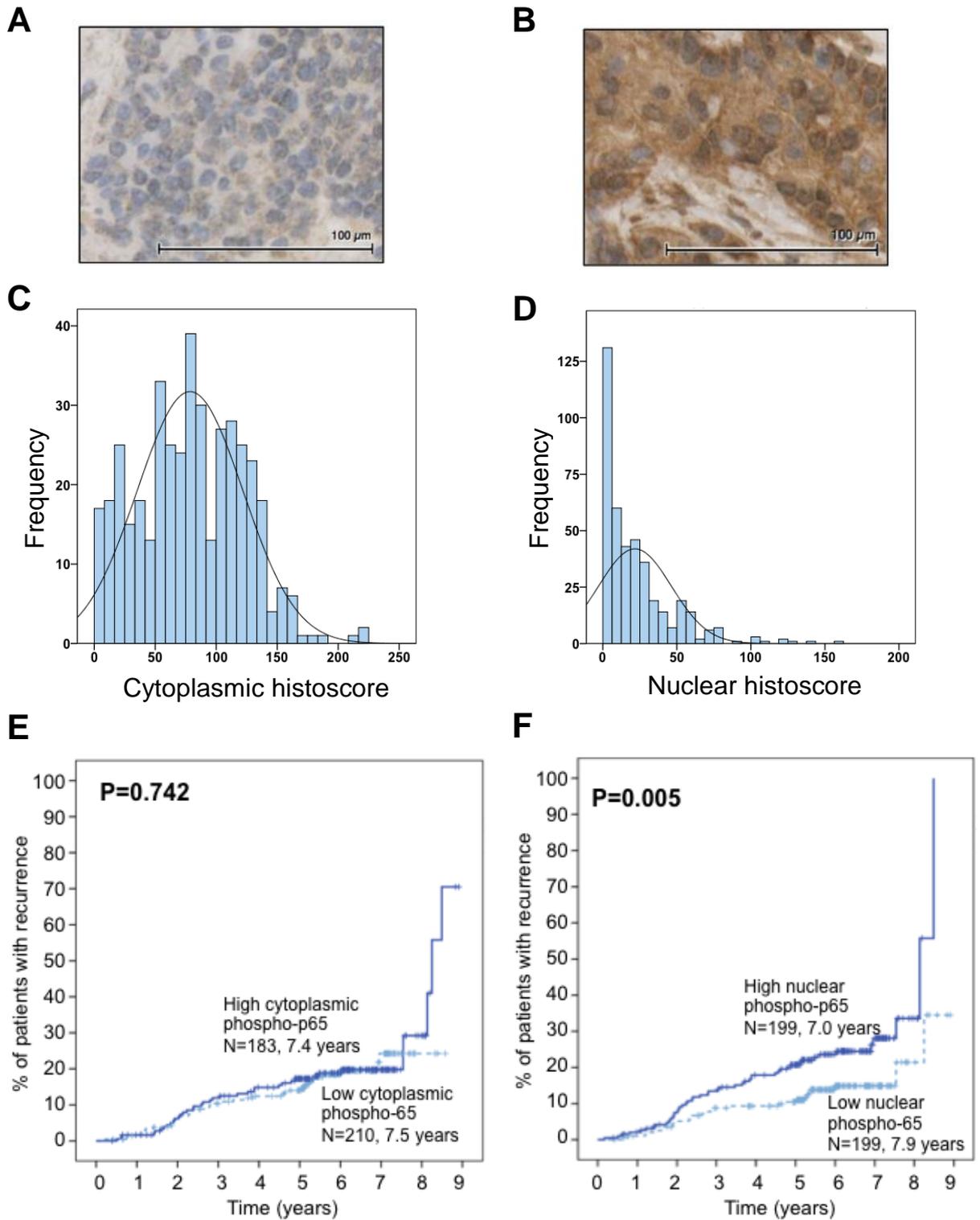


Figure 4.6: Expression of phospho-65 and clinical outcome in the 1800-Bre-TMA. (A) Example of low expression and (B) of high expression of p65 phosphorylated at S536 in breast cancer tissue. Images were taken at 400x magnification, scale bar represents 100 μ m. (C) Histogram showing normal distribution of cytoplasmic and (D) nuclear histoscores (E) Expression of phospho-p65 in the cytoplasm was not associated with recurrence free interval. (F) High nuclear phospho-p65 was associated with quicker recurrence. Censor lines indicate loss to follow up.

Chi-squared analysis was performed to establish whether expression of nuclear phospho-p65 was associated with any clinico-pathological characteristics of the cohort. These factors and their association with nuclear phospho-p65 are detailed in Table 4.1. As part of a previous study by Dr Zahra Mohammed, microvessel density was assessed using IHC with an anti-CD34 antibody. Expression of phospho-p65 in the nuclear compartment was found only to associate with angiogenesis, as measured by this microvessel density (P=0.041).

Clinico-pathological characteristic	Association with nuclear phospho-p65
Age	P=0.663
Size	P=0.478
Grade	P=0.377
Nodal Status	P=0.741
NPI	P=0.304
ER status	P=0.269
PgR status	P=0.764
HER2	P=0.798
Ki67	P=0.169
Apoptosis (TUNEL)	P=0.342
Necrosis	P=0.219
Klintrup	P=0.215
Angiogenesis (CD34)	P=0.041

Table 4.1: Association of nuclear phospho-p65 with clinico-pathological characteristics of the 1800-Bre-TMA cohort. *Chi-squared analysis explored the relationship of nuclear expression of phospho-p65 with various characteristics of the cohort. Nuclear expression of phospho-p65 was found to associate with angiogenesis measured by CD34.*

4.3.4 Expression of phosphorylated p65 versus p65 NLS and clinical outcome

Due to problems with antibodies targeting phosphorylated protein, we explored the prognostic significance of p65 NLS as an alternative marker of the canonical pathway. Figure 4.7A and 4.7B show an example of immunostaining of breast tumours with low and high expression and Figure 4.7C shows the distribution of nuclear histoscores. Nuclear expression of p65 NLS ranged from 0 to 300 with a median histoscore of 163.3 (IQR = 106.7 - 215). Interobserver correlation between the two scorers was good with an ICC of 0.86 and bland altman plots showed there was no bias evident between observers. Kaplan-Meier survival analysis shows that nuclear expression of p65 NLS was not associated with recurrence free interval ($P=0.379$, HR = 1.2 (0.8 - 2.0), Figure 4.7D), and phospho-p65 was therefore found to be a stronger prognostic marker of recurrence in breast cancer.

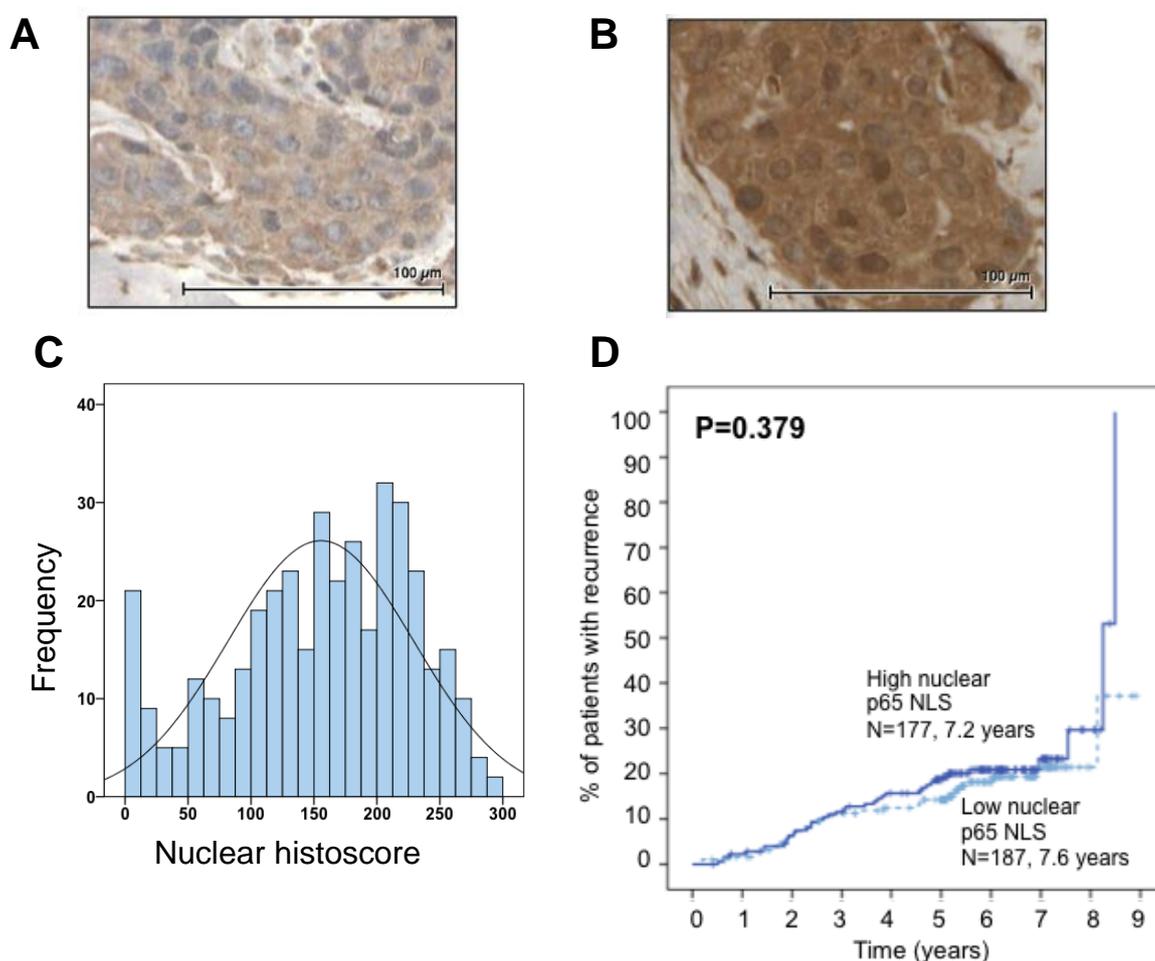


Figure 4.7: Comparison of p65 NLS and phospho-p65 nuclear expression on clinical outcome in the 1800-Bre-TMA. (A) Example of low expression and (B) of high expression of p65 NLS in breast cancer tissue (images taken at 400x magnification, scale bar represents 100µm). (C) Histogram showing distribution of histoscores. Nuclear expression of p65 NLS was not associated with recurrence free interval. (D) Kaplan-Meier survival analysis showing % of patients with recurrence over time (years) for high nuclear p65 NLS (N=177, 7.2 years) and low nuclear p65 NLS (N=187, 7.6 years). P=0.379.

4.3.5 Expression of phospho-p65 in different subgroups of breast cancer and clinical outcome

In order to determine whether nuclear expression of phospho-p65 was associated with recurrence in a particular subgroup, the cohort was divided using ER status. The observation of higher nuclear phospho-p65 associating with quicker recurrence was accentuated in ER positive tumours ($P=0.002$, HR = 3.1 (1.5 - 6.4), 7.10 years vs. 8.34 years, Figure 4.8B), and negated in ER negative tumours ($P=0.670$, HR = 1.1 (0.6 - 2.2), Figure 4.8A). In patients who were ER positive and received endocrine therapy this was even more significant ($P=2.67 \times 10^{-4}$, HR = 4.2 (1.8 - 9.9), Figure 4.8C)

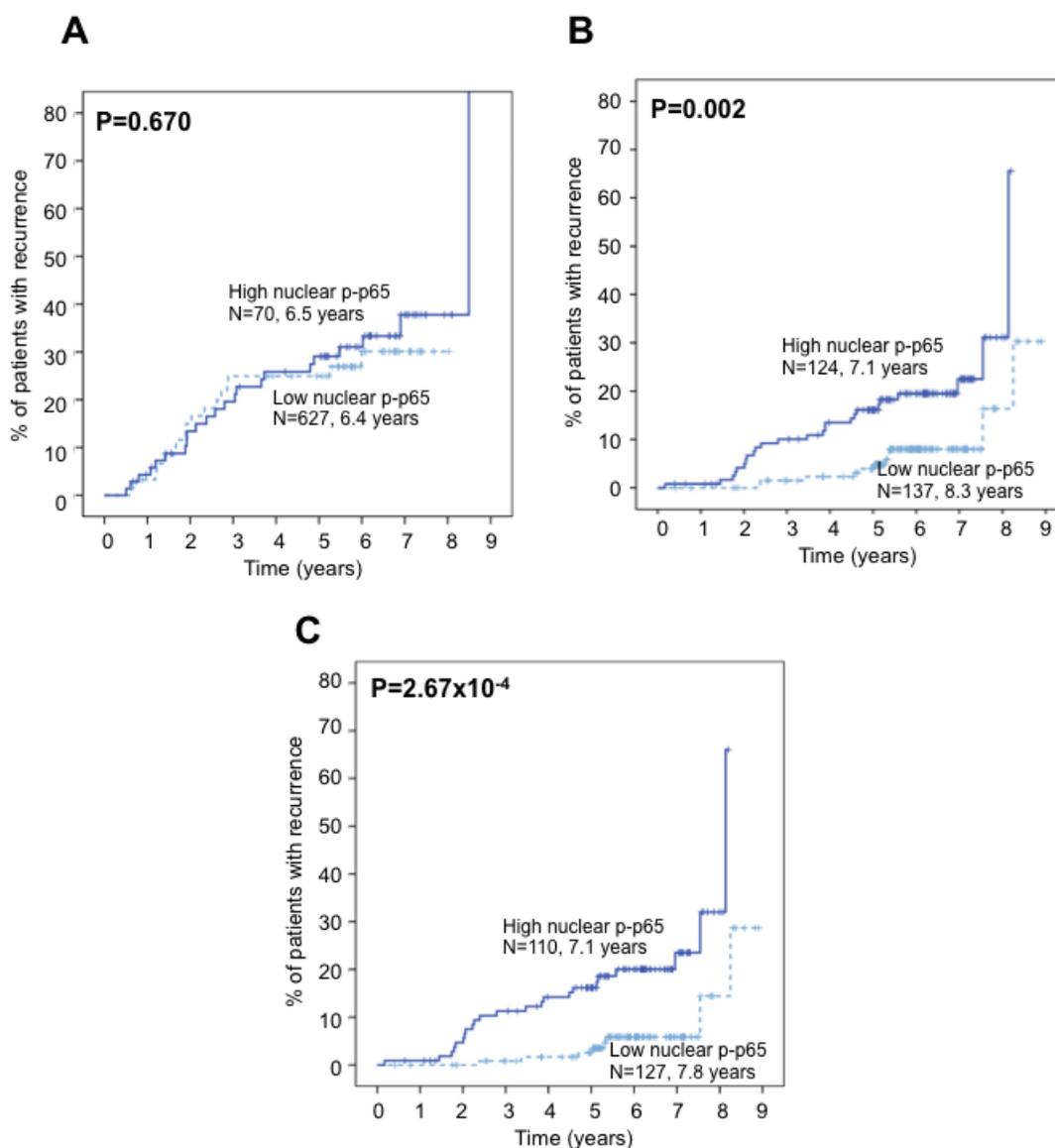


Figure 4.8: Nuclear expression of phospho-p65 is associated with recurrence free interval in ER positive patients. *The relationship between nuclear p-p65 S536 and recurrence free interval is negated in ER negative tumours (A), but accentuated in ER positive (B) and even further in ER positive patients who received endocrine therapy (C).*

In patients on endocrine therapy, when split further into subtype this was negated in luminal A tumours ($P=0.083$, HR = 3.1 (0.8 - 11.6), Figure 4.9A) but significance increased in luminal B tumours ($P=2.54 \times 10^{-5}$, HR = 7.2 (2.4 - 22.0), 5.5 years vs. 8.2 years, Figure 4.9B). There was no association between phospho-p65 nuclear expression and recurrence free interval in the triple negative ($P=0.926$, HR = 1.0 (0.4 - 2.5), Figure 4.9C) or HER2 enriched subtypes ($P=0.708$, HR = 1.2 (0.5 - 3.1), Figure 4.9D).

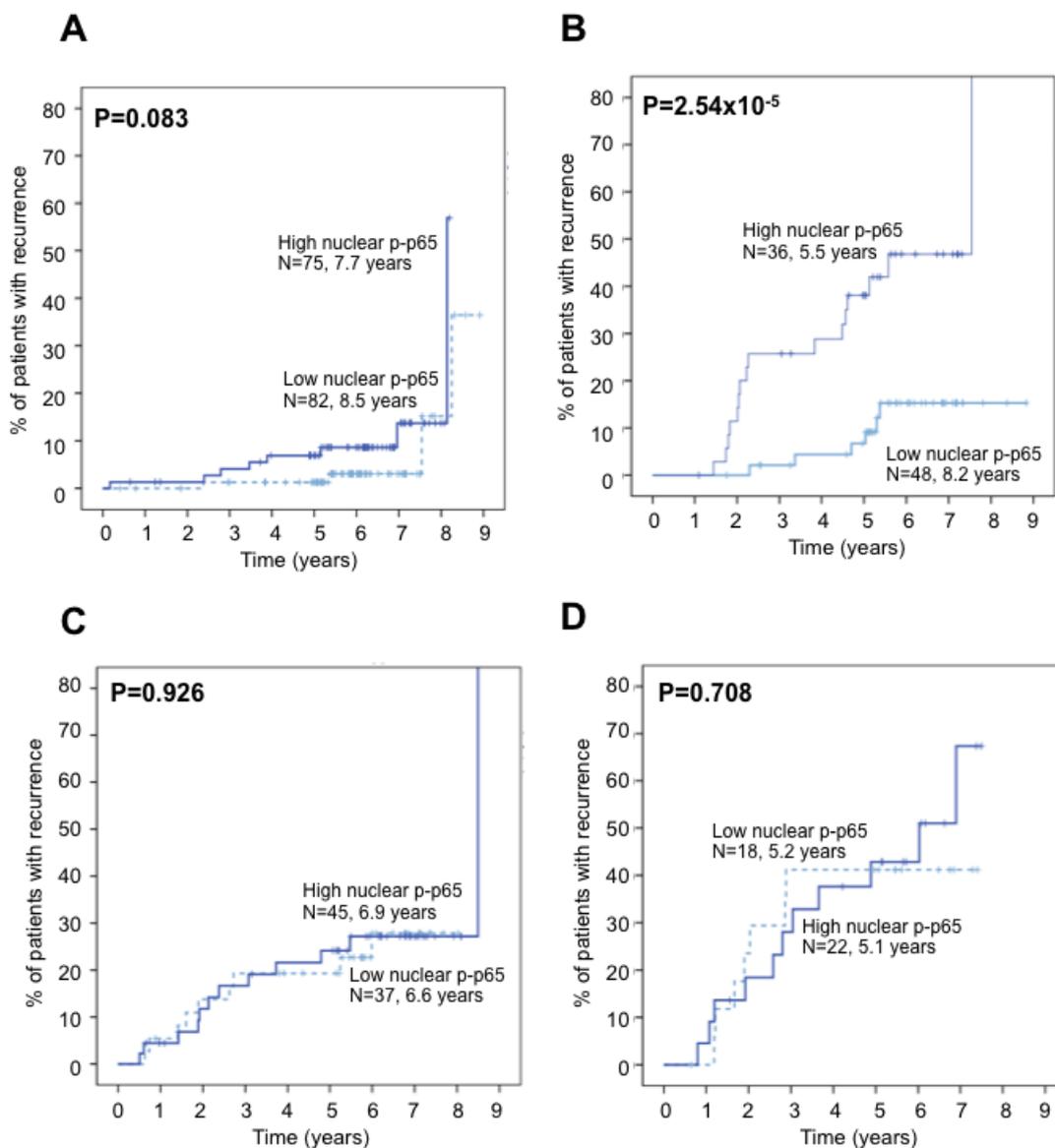


Figure 4.9: Nuclear expression of phospho-p65 and recurrence free interval in different subtypes of breast cancer. *In patients on endocrine therapy, luminal A tumours were not associated with recurrence (A), but in luminal B tumours (B) high expression of phospho-p65 in the nucleus was significantly associated with increased recurrence. The relationship between nuclear phospho-p65 S536 and recurrence free interval is negated in triple negative (C) and HER2 enriched (D) subtypes.*

These patients were further divided into the different types of luminal B tumours (HER2+ or high Ki67), this relationship was only significant in highly proliferative tumours ($P=2.74 \times 10^{-4}$, HR = 7.6 (2.1 - 27.1), Figure 4.10A), and not in those with HER2 positive tumours ($P=0.073$, HR = 5.9 (0.6 - 53.5), Figure 4.10B), although very small numbers were available for analysis in this group and a similar trend is observed.

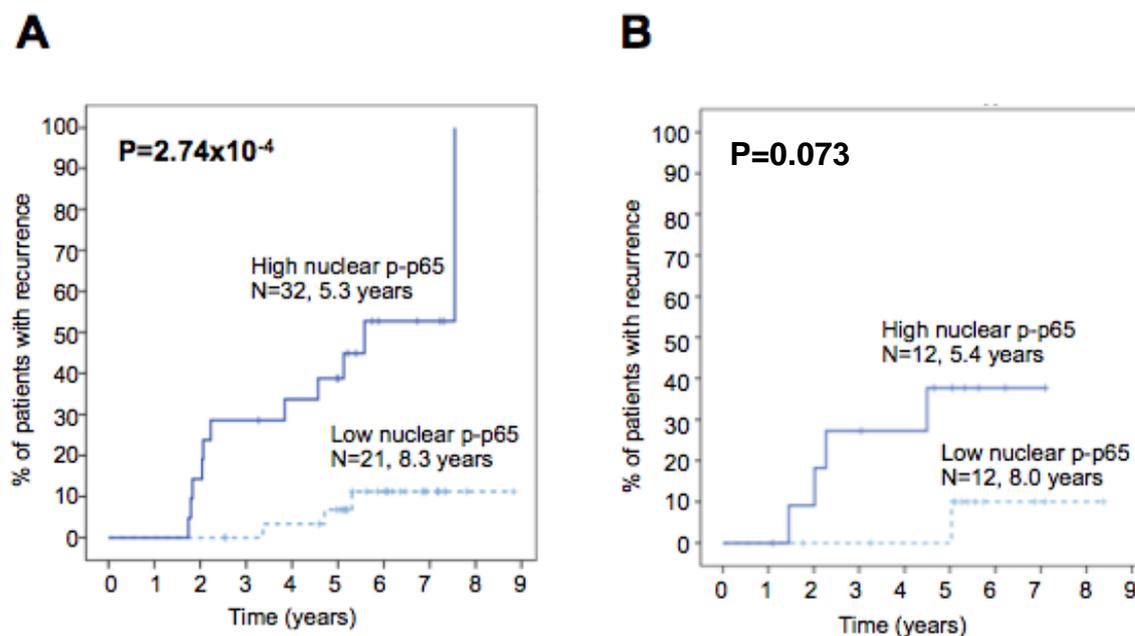


Figure 4.10: Nuclear expression of phospho-p65 and recurrence free interval in luminal B patients. Patients on endocrine therapy with luminal B tumours were further subdivided into those with HER2- high Ki67 tumours and HER2+ tumours. Nuclear expression of phospho-p65 was significantly associated with recurrence free interval in patients with luminal B HER2- high Ki67 tumours (A) but not in those with HER2 amplification (B), although only small numbers were available for analysis.

When recurrence was considered only in the first five years in patients receiving endocrine therapy (tamoxifen), and therefore likely to be recurrence on tamoxifen, there was a significant difference between high and low expression ($P=0.015$, HR = 5.3 (1.2 to 24.1), Figure 4.11). However, using a more stringent P value of 0.01 this is not regarded as significant, however there are only a low number of patients in each group and in a larger cohort this may reach significance using this P value.

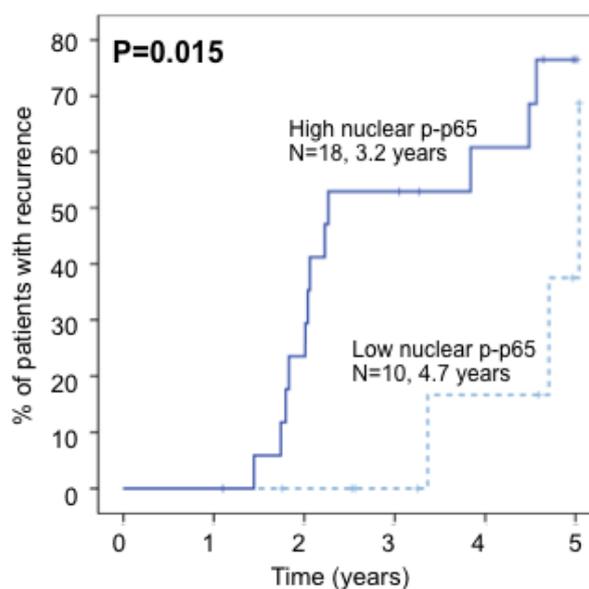


Figure 4.11: Nuclear expression of phospho-p65 and recurrence free interval in the first 5 years of tamoxifen treatment. *In patients on endocrine therapy with luminal B tumours, when recurrence was considered in the first five years and therefore likely to be recurrence on tamoxifen, a significant relationship was observed between phospho-p65 nuclear expression and recurrence free interval.*

In conclusion, phosphorylation of p65, a marker of activation of the canonical NF- κ B pathway, was associated with shorter recurrence free interval in ER positive breast cancer patients, particularly those with luminal B tumours. Of the family members tested in this study, phosphorylation of p65 appears to be the best biomarker of the canonical pathway in breast cancer clinical specimens.

4.4 Antibody validation of members of the non-canonical pathway

4.4.1 Validation of anti-NIK antibody

Western blotting and IHC on cell pellets were carried out using the anti-NIK antibody to confirm specificity as part of a previous study in our laboratory. A single band of correct size (125 kDa) was observed on a western blot (Figure 4.12A). In cell pellets of HUVEC cells infected with β -galactosidase (β -gal) or NIK adenoviruses, IHC using the anti-NIK antibody showed an increase in cells infected with NIK adenovirus (Figure 4.12B).

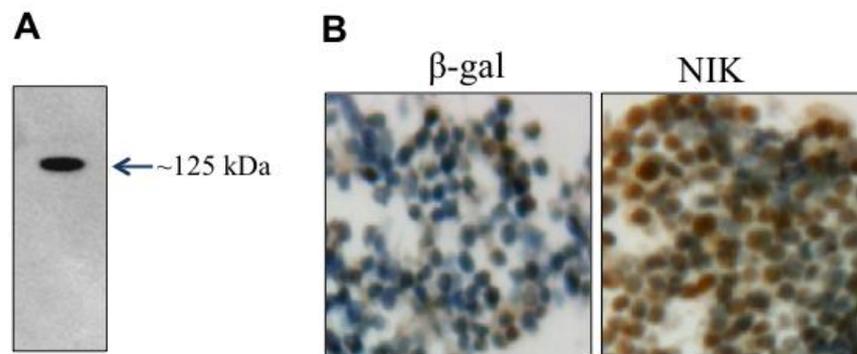


Figure 4.12: Validation of the anti-NIK antibody. (A) Western blot showing a single band of appropriate size (125 kDa). (B) IHC was employed to cell pellets of HUVEC cells infected with β -galactosidase (β -gal) or NIK adenoviruses and showed an increase in staining in the NIK adenovirus infected cells.

4.4.2 Validation of anti-RelB antibody

A previous study in our laboratory confirmed specificity of the anti-RelB antibody. Western blotting showed a single band of appropriate size observed (70 kDa, Figure 4.13A). Additionally, IHC on cell pellets treated with 20ng/ml lymphotoxin for 24 hours showed a marked increase in nuclear localisation of RelB compared with untreated controls (Figure 4.13B)

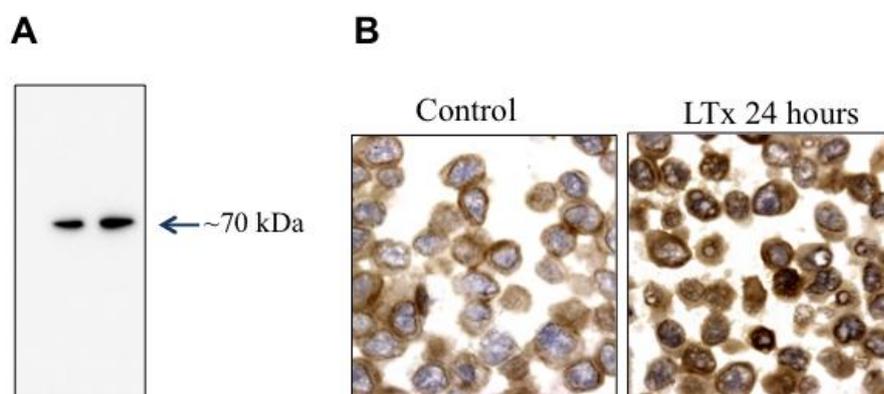


Figure 4.13: Validation of the anti-RelB antibody. (A) Western blot showing a single band of appropriate size (70 kDa). (B) Paraffin embedded cell pellets of LNCaP cells treated with lymphotoxin showed an increase in RelB nuclear expression compared with control cells.

4.4.3 Validation of anti-IKK α antibody

Western blotting using the anti-IKK α antibody showed a single band of the predicted molecular weight (85 kDa). LNCaP cells were pre-treated with 200nM siRNA against either IKK α or IKK β to check specificity for IKK α . A reduction in expression was observed in IKK α -silenced but not IKK β -silenced cells (Figure 4.14A). Additionally, IKK α -silenced and IKK β -silenced LNCaP cells pellets were made and immunostaining for IKK α showed a reduction in IKK α -silenced cells, but not in IKK β -silenced or control cells (Figure 4.14B).

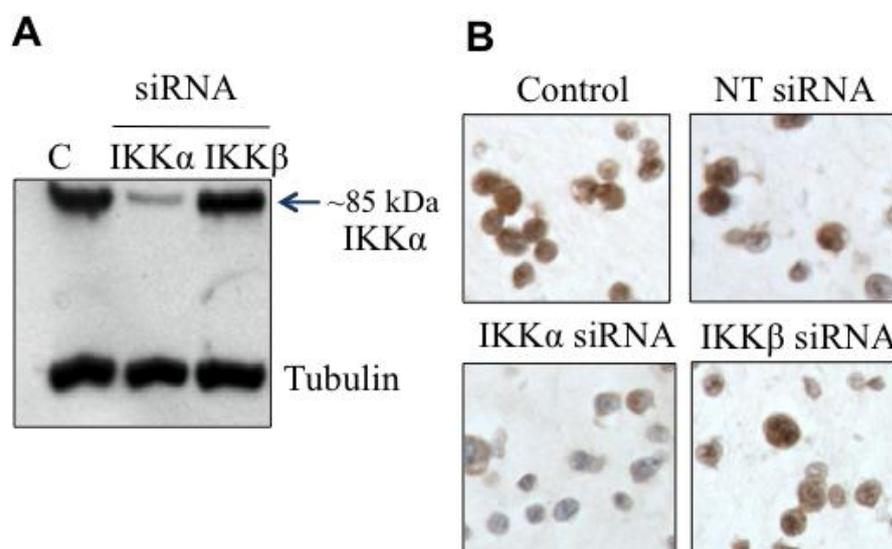


Figure 4.14: Validation of the anti-IKK α antibody. (A) Western blot showing a single band of appropriate size (85 kDa). A reduction in expression was observed in lysates from IKK α -silenced but not IKK β -silenced LNCaP cells (B) Paraffin embedded cell pellets of LNCaP cells treated with siRNA showed a decrease in expression in IKK α -silenced but not IKK β -silenced, compared to control cells.

4.5 Expression and clinical outcome of members of the non-canonical pathway

4.5.1 Expression of NIK and clinical outcome

Expression of NIK, the kinase upstream of IKK α in the non-canonical pathway, was analysed in the 1800-Bre-TMA cohort. 119 patients had cores that were missing or contained no tumour leaving 429 patients available for analysis. The histoscores for cytoplasmic expression of NIK ranged from 0 to 240 with a median of 95 (IQR = 70 - 113.3), and for the nuclear compartment ranged from 0 to 300 with a median of 191.7 (IQR = 159.2 - 226.7). The cohort was subdivided into those with low and high expression using the median as a cut off. Across the cores from the same patient the average standard deviation in cytoplasmic histoscore was 29.1 and the median was 26.5 (IQR = 14.1 - 42.4). For nuclear expression the average standard deviation was 32.8 with a median of 28.2 (IQR = 15.3 - 43.6). An example of immunostaining of NIK is displayed in Figure 4.3, with low expression shown in 4.15A and high in 4.15B. Bland altman plots demonstrated no bias between observers and an ICC of 0.86 for cytoplasmic expression and 0.81 for nuclear expression showed good correlation between observers.

To determine whether NIK expression was significantly associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression were plotted and low and high expression was compared using the log rank test. No association was observed with recurrence free interval for cytoplasmic (P=0.287, HR = 0.8 (0.5 - 1.2), Figure 4.15C) or nuclear expression of NIK (P=0.914, HR = 1.0 (0.6 - 1.5), Figure 4.15D). When chi squared analysis was performed to test the association of expression of NIK and clinic-pathological factors of the cohort, expression in the cytoplasm was found to be associated with Ki67 (P=0.012) and apoptosis (P=0.006) and in the nucleus showed a weak association with nodal status (P=0.047).

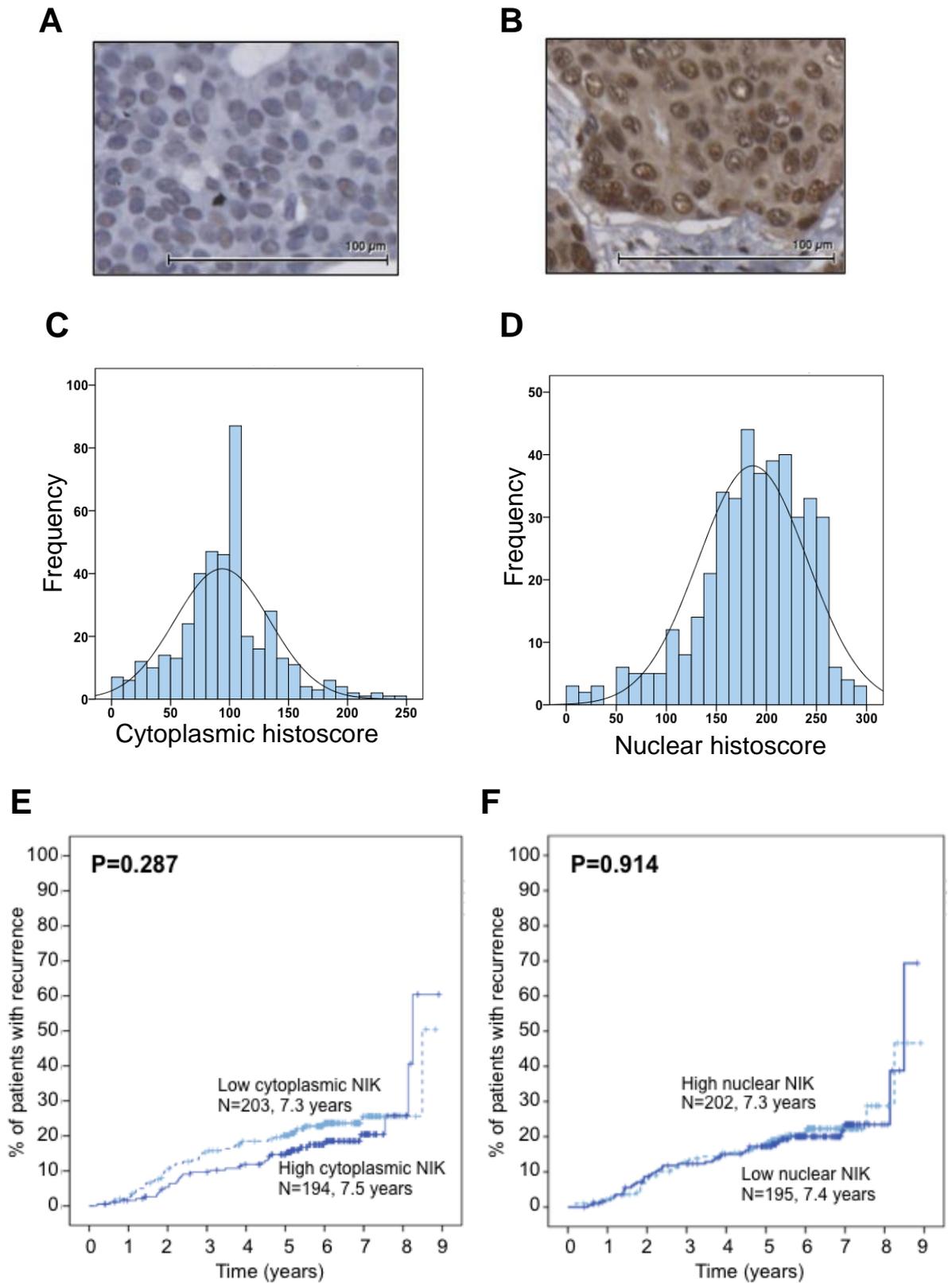


Figure 4.15: Expression of NIK and clinical outcome in the 1800-Bre-TMA. (A) Example of low expression and (B) of high expression of NIK in breast cancer tissue (images taken at 400x magnification, scale bar represents 100 μm). Histograms show distribution of histoscores for cytoplasmic (C) and nuclear NIK (D). Expression of NIK in the cytoplasm (E) or nucleus (F) was not associated with recurrence free interval. Censor lines indicate loss of follow up.

4.5.2 Expression of RelB and clinical outcome

In order to investigate activation of the non-canonical pathway, RelB expression was assessed in the 1800-Bre-TMA cohort. 20.4% (N=111) of patients had cores that were missing or containing no tumour leaving 433 (79.6%) available for analysis. Of those tumours assessed, 99.3% (430) showed cytoplasmic expression with a range of 0 to 290 and a median histoscore of 110 (IQR = 86.7 - 145). Only 32.7% (N=146) showed nuclear expression with range of 0 to 226.7 and a median histoscore of 0 (IQR = 0 - 6.7). A low amount and very low intensity of nuclear staining observed for RelB suggested lack of activation of the non-canonical pathway in the majority of breast tumours. The average standard deviation in histoscore across the cores from the same patient for cytoplasmic expression was 31.5 and the median was 26.5 (IQR= 14.1 - 43.6). For nuclear expression the average standard deviation was 9.7 and the median was 0 (IQR = 0 - 11.5).

An example of immunostaining showing low and high RelB expression is shown in Figure 4.16A and 4.16B and histograms in Figure 4.16 display distribution of histoscores for cytoplasmic (Figure 4.16C) and nuclear (Figure 4.16D) expression of RelB. Excellent correlation was seen between observers scoring with an ICCC of 0.9 for cytoplasmic expression. An ICCC of 0.82 for nuclear expression showed good correlation and bland altman plots also showed no bias between observers. RelB was not associated with recurrence free interval for cytoplasmic (P=0.139, HR = 0.7 (0.5 - 1.1), Figure 4.16E) or nuclear (P=0.418, HR = 0.8 (0.5 - 1.3), Figure 4.16F) expression. When chi squared analysis was performed, expression of RelB in the cytoplasm was found to show a weak association with both size (P=0.039) and angiogenesis (P=0.038), but no associations were observed with expression in the nucleus.

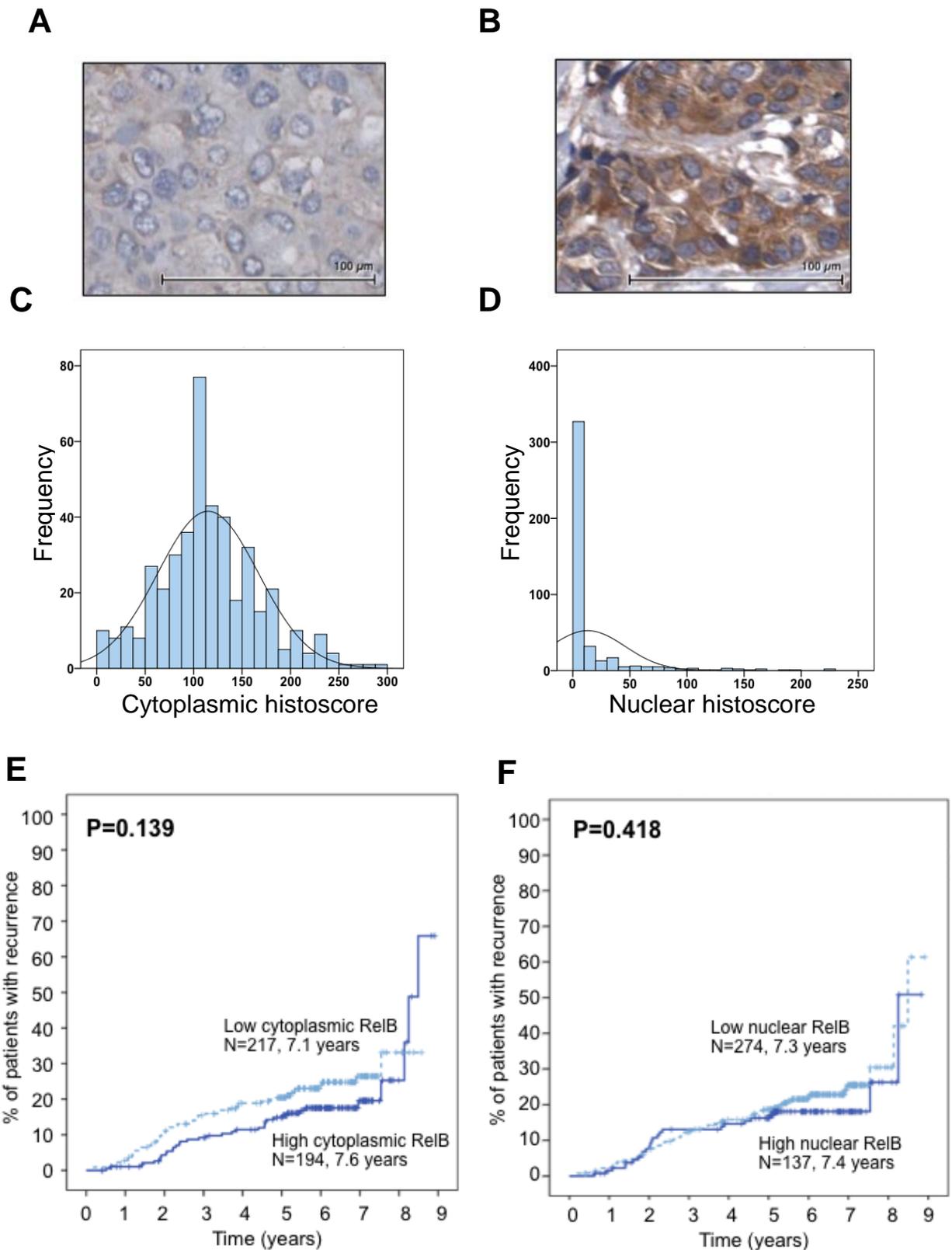


Figure 4.16: Expression of RelB and clinical outcome in the 1800-Bre-TMA. (A) Example of low expression and (B) of high expression of RelB in breast cancer tissue (images taken at 400x magnification, scale bar represents 100 μ m). Histograms show distribution of histoscores for cytoplasmic (C) and nuclear RelB (D). Expression of RelB in the cytoplasm (E) or nucleus (F) was not associated with recurrence free interval. Censor lines indicate loss to follow up.

4.5.3 Expression of IKK α and clinical outcome

Expression of IKK α was assessed in 73.3% of patients (N=399). Of these, 97.2% (N=388) showed cytoplasmic expression with a range of 0 to 260 and a median histoscore of 100 (IQR = 66.7 - 140). 94% (N=375) showed nuclear expression of IKK α with a range of 0 to 280 and a median histoscore of 130 (IQR = 75 - 180). Across the cores from the same patient the average standard deviation in cytoplasmic histoscore was 47.5 and the median was 43.6 (IQR = 21.2 - 68.1). For nuclear expression the average standard deviation was 58.3 and the median was 52.4 (IQR = 28.3 - 81.5).

Figure 4.17A shows immunostaining of a tumour with low IKK α expression and Figure 4.17B shows an example of high expression. Histograms in Figure 4.17 show distribution of histoscores for cytoplasmic (Figure 4.17C) and nuclear (Figure 4.17D) expression of RelB. Scoring between the two observers was found to have excellent correlation for both cytoplasmic and nuclear expression both with ICC values of 0.95.

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of IKK α were plotted, and low and high expression was compared using the log rank test. Nuclear expression of IKK α was not associated with recurrence free interval (P=0.502, HR = 0.9 (0.5 - 1.4), Figure 4.17F). Cytoplasmic expression of IKK α was however associated with decreased recurrence free interval (P=0.014, HR = 1.8 (1.1 - 2.9), Figure 4.17E). However, using a more stringent P value of 0.01 this is not regarded as significant and on multivariate analysis when combined with with tumour size, invasive grade, nodal status, NPI, ER status, PgR status, HER2 status, Ki67, Necrosis and angiogenesis this was not independently prognostic (P=0.524 HR = 1.2 (0.7 - 2.0)). Chi squared analysis showed nuclear expression of IKK α was associated with necrosis (P=0.020) and Klintrup (P=0.014).

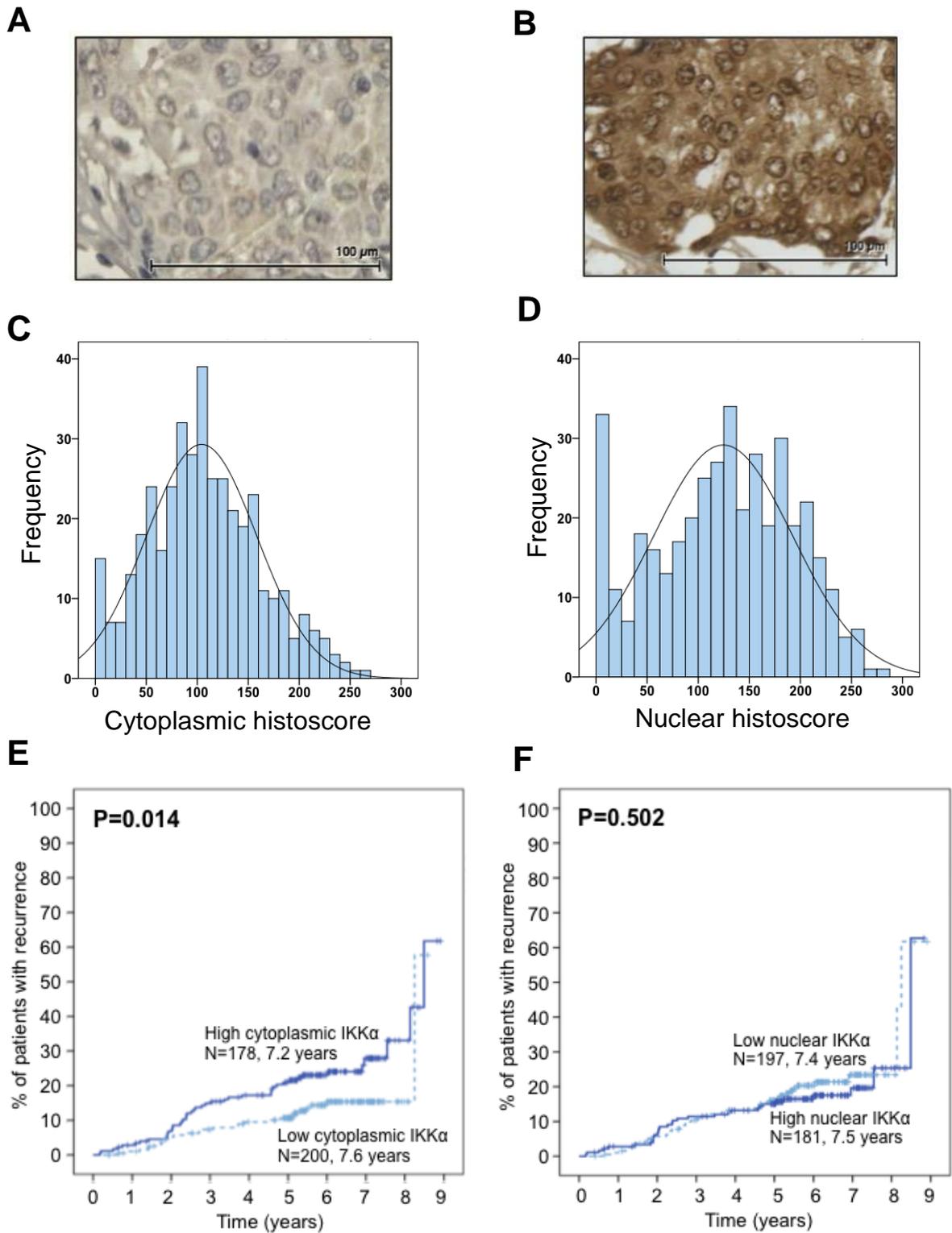


Figure 4.17: Expression of IKK α and clinical outcome in the 1800-Bre-TMA. (A) Example of low expression and (B) of high expression of IKK α in breast cancer tissue (images taken at 400x magnification, scale bar represents 100 μ m). Histograms show distribution of histoscores for (C) cytoplasmic (D) and nuclear IKK α . (E) High cytoplasmic IKK α was associated with quicker recurrence. (F) Expression of IKK α in the nucleus was not associated with recurrence free interval. Censor lines indicate loss to follow up.

Chi-squared analysis was performed to determine whether expression of IKK α was associated with any clinico-pathological characteristics of the cohort. These factors and their association with nuclear IKK α are detailed in Table 4.2. Expression of IKK α in the cytoplasm was associated with size (P=0.028), grade (P=0.018), hormone status (ER P=0.002 and PgR P<0.001), HER2 status (P=0.019), Klintrup (P=0.029), apoptosis measured by TUNEL (P=0.024) and necrosis (P=0.019).

Clinico-pathological characteristic	Association with cytoplasmic IKK α
Age	P=0.642
Size	P=0.028
Grade	P=0.018
Nodal Status	P=0.914
NPI	P=0.019
ER status	P=0.002
PgR status	P<0.001
HER2	P=0.019
Ki67	P=0.614
Apoptosis (TUNEL)	P=0.024
Necrosis	P=0.019
Klintrup	P=0.029
Angiogenesis (CD34)	P=0.131

Table 4.2: Association of cytoplasmic IKK α with clinico-pathological characteristics of the 1800-Bre-TMA cohort. *Chi-squared analysis explored the relationship of nuclear expression of IKK α with various characteristics of the cohort. Cytoplasmic expression of IKK α was associated with size, grade, NPI, hormone receptor status, HER2 status, apoptosis measured by TUNEL and necrosis.*

Correlation between members of the pathway was measured using Pearson correlation coefficient. Correlations in expression of NIK, RelB and IKK α in the cytoplasm are shown in Figure 4.18. Although highly significant P values were obtained, the correlation coefficient is of much greater importance and more consideration should be placed on this and therefore none of these markers show a strong association. The correlation coefficient between NIK and IKK α cytoplasmic expression was very weak with a value of 0.161 (P=0.002) and there was a weak correlation between IKK α and RelB with a correlation coefficient of 0.228 (P=1.12x10⁻⁵). Histoscores of NIK and RelB cytoplasmic expression also showed a weak association with a correlation coefficient of 0.316 (P=6.13x10⁻¹¹). Additionally, the correlation coefficient between cytoplasmic expression of IKK α and IKK β , which form heterodimers in the canonical pathway, was moderate with a value of 0.392 (P=1.4x10⁻¹⁵).

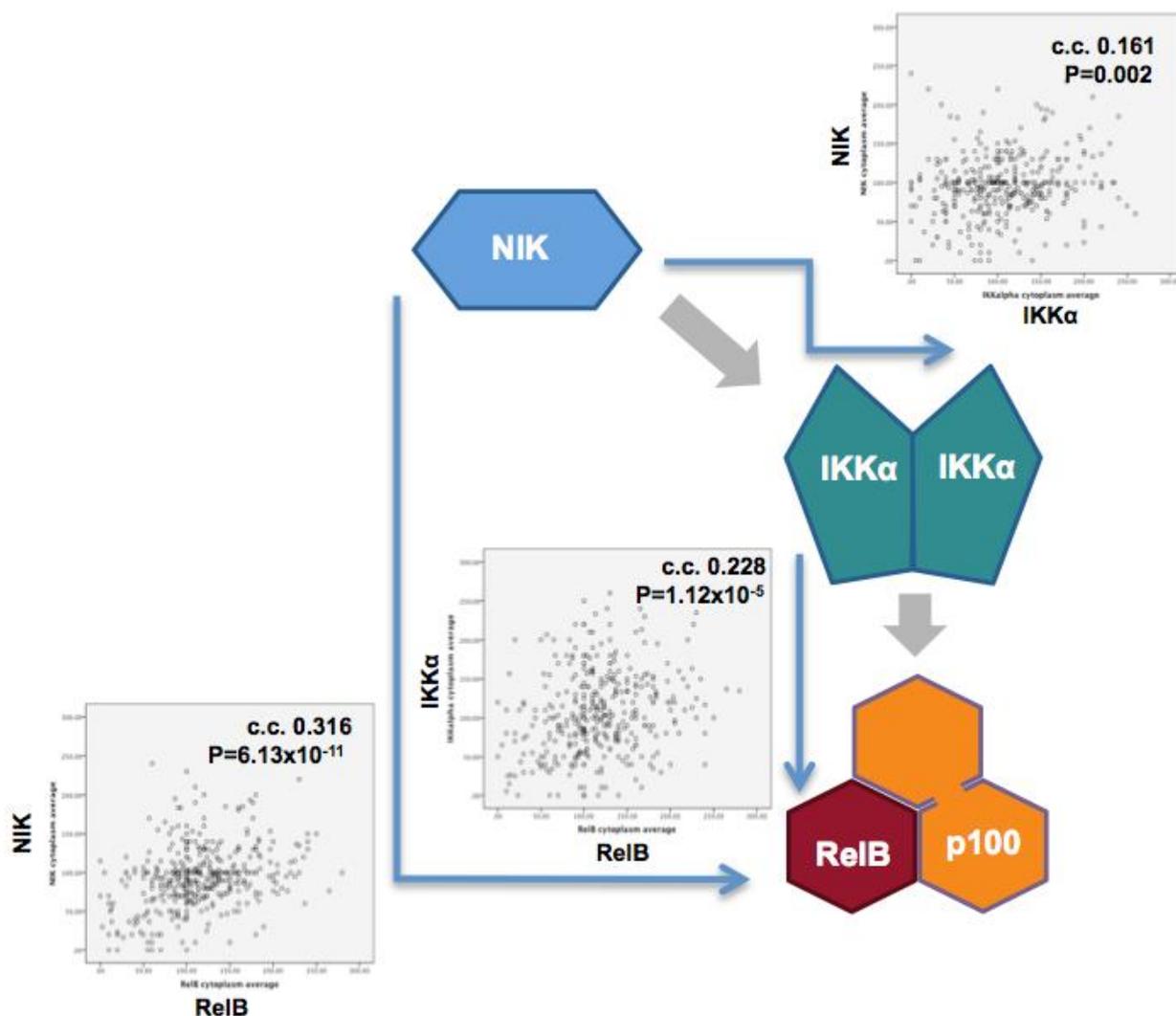


Figure 4.18: Correlation in expression between members of the non-canonical pathway. Correlation between cytoplasmic expression of NIK, RelB and IKK α is shown, these family members showed weak correlation in expression. C.c = Pearson correlation coefficient.

The association between shorter recurrence free interval and high cytoplasmic expression of IKK α was negated in ER negative tumours (P=0.998, HR = 1.0 (0.5 - 1.9), Figure 4.19A) and accentuated in ER positive tumours (P=0.008, HR = 2.5 (1.3 - 5.1), 7.6 years vs. 8.0 years, Figure 4.19B). When this was considered in ER positive patients on endocrine therapy, the relationship between higher cytoplasmic IKK α and quicker time to recurrence remained significant (P=0.043, HR = 2.1 (1.0 - 4.2), Figure 4.19C). However, using a more stringent significance value of 1% (P<0.01), this is not regarded as significant.

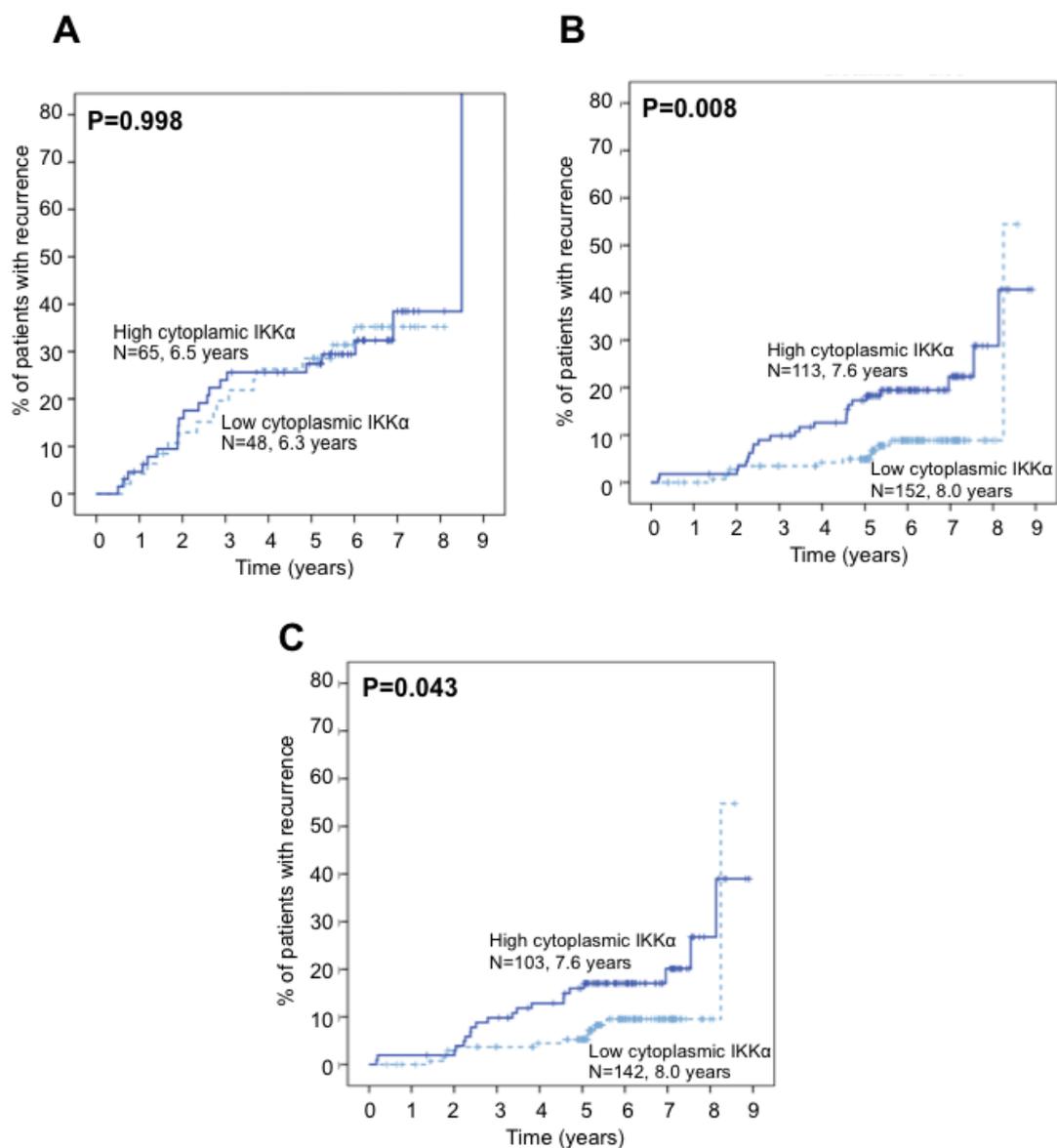


Figure 4.19: Cytoplasmic expression of IKK α is associated with recurrence free interval in ER positive patients. *The relationship between cytoplasmic IKK α and recurrence free interval was negated in ER negative tumours (A), but accentuated in ER positive (B). Significance was retained in ER patients on endocrine therapy (C).*

When split into subtype this association was negated in all subtypes except luminal A. In patients on endocrine therapy with luminal A tumours, those with high cytoplasmic expression of IKK α was associated with a quicker recurrence than those with low cytoplasmic IKK α (P=0.028, HR = 3.9 (1.1 - 14.6), 7.9 years vs. 8.3 years, Figure 4.20A). Using a more stringent significance value of P<0.01, this would not be regarded as significant. The use of a larger cohort would be useful to investigate this further.

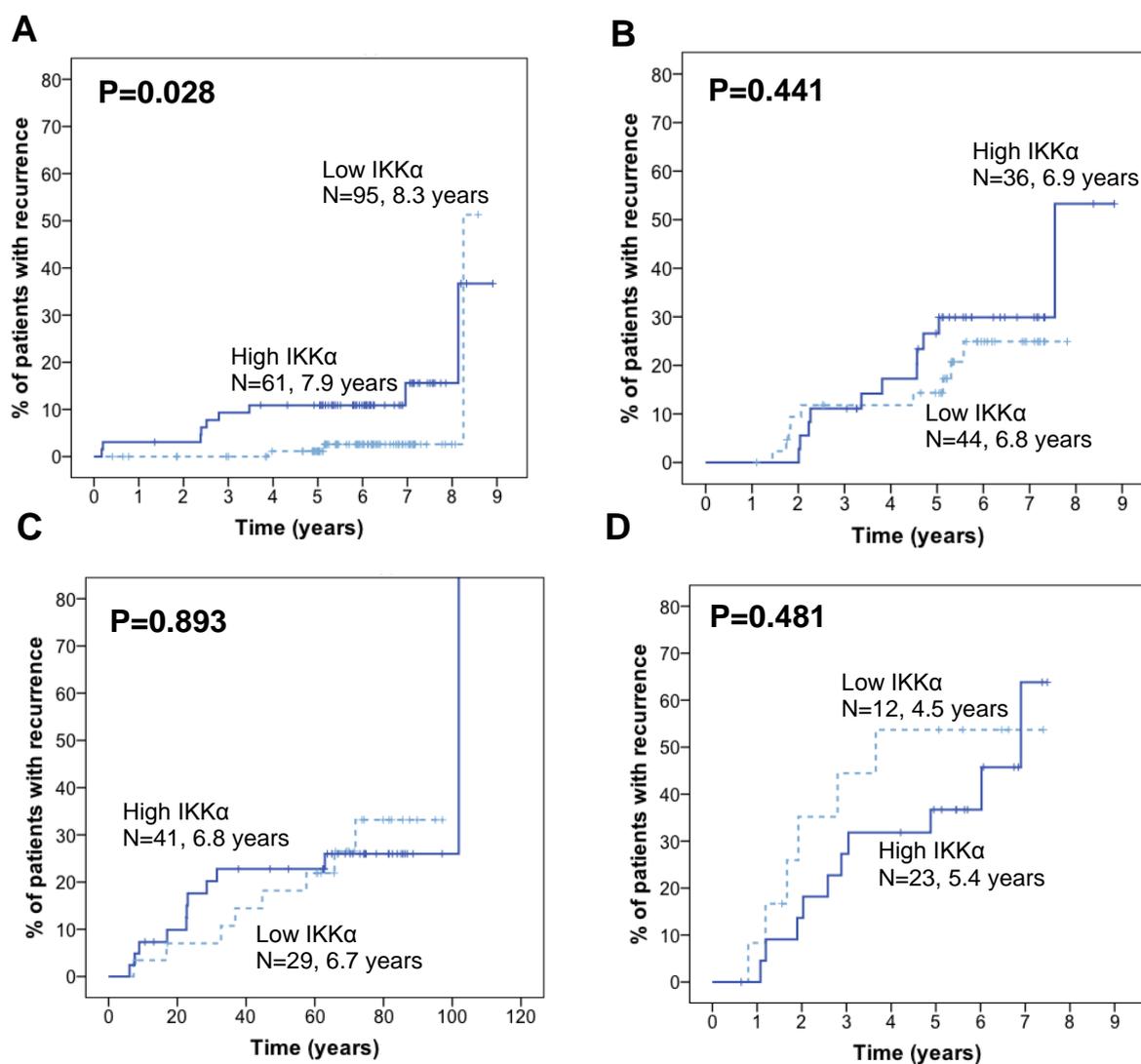


Figure 4.20: Cytoplasmic expression of IKK α and recurrence free interval in different breast cancer subtypes. When split into the different subtypes, the relationship between cytoplasmic IKK α and recurrence free interval in patients who received endocrine therapy was retained in luminal A tumours (A) but was negated in the luminal B tumours (B). IKK α was not associated with recurrence in triple negative breast cancer (C) or the HER2 enriched subtype (D).

Patients on endocrine therapy with luminal B tumours did not have a significant difference in recurrence free interval between low and high cytoplasmic IKK α (P=0.441, HR = 1.5 (0.6 - 3.4), Figure 4.20B). IKK α was not associated with recurrence in triple negative breast cancer (P=0.893, HR = 0.9 (0.4 - 2.4), Figure 4.20C) or the HER2 enriched subtype (P=0.481, HR = 0.7 (0.3 - 1.9), Figure 4.20D).

This relationship between high cytoplasmic IKK α and recurrence in patients with luminal A tumours was also observed when recurrence was considered in the first 5 years (and therefore likely recurrence on tamoxifen). Those with higher cytoplasmic IKK α had a shorter time to recurrence on tamoxifen than those with low cytoplasmic IKK α (P=4.4x10⁻⁵, HR = 22.0 (2.7 - 180.7), 3.2 years vs. 5.0 years, Figure 4.21). This was independent in cox regression multivariate analysis when combined with tumour size, invasive grade, nodal status, NPI, ER status, PgR status, HER2 status, Ki67, necrosis and angiogenesis (P=0.003, HR = 24.9 (2.9 - 212.7)). There are however very small numbers available for analysis and caution is therefore required when interpreting these results.

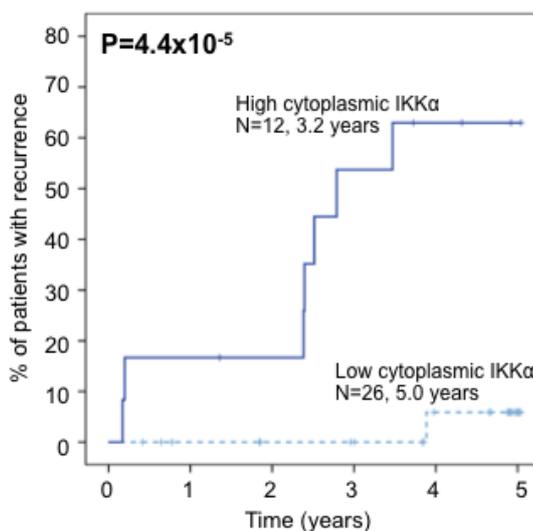


Figure 4.21: Cytoplasmic expression of IKK α is associated with recurrence on tamoxifen in luminal A patients. *Patients with luminal A tumours that have higher expression of cytoplasmic IKK α have a quicker time to recurrence on tamoxifen than those with low expression. Censor lines indicate loss to follow up.*

In conclusion, IKK α , a kinase involved in activation of the non-canonical NF- κ B pathway, was associated with shorter recurrence free interval in ER positive breast cancer patients. IKK α appears to be the best biomarker of the non-canonical pathway in breast cancer clinical specimens.

4.6 Discussion

For many years an association between inflammation and cancer has been suspected and evidence has emerged in the last decade demonstrating the importance of this link. NF- κ B regulates genes involved in many processes including inflammation, proliferation and apoptosis and has a critical role in both innate and adaptive immunity. In many cancers NF- κ B is constitutively active and the central regulators of this pathway, the kinases IKKs, are likely candidates for the link between the two processes [172].

Tissue biomarkers have the potential to improve the diagnosis and prognostication of cancer, including breast cancer where many are already used routinely including ER and HER2. It may also benefit the selection of therapy using stratified medicine, allowing maximum benefit to the patient and avoiding unnecessary use of therapies that will have little effect for many patients. Before use in patient tissue all antibodies were optimised and validated. Although antibodies detecting p65 and phosphorylation of p65 were validated in both breast and prostate cancer cells, all other antibodies were validated in only prostate cancer cells. This possibly could lead to problems with further use in other tissue types as certain antibodies may have tissue specific immune-reactivity and therefore ideally all antibodies should be confirmed as specific in each tumour type. In order to validate results obtained in the breast cancer patient cohort, antibodies used to detect expression of members of the NF- κ B pathways should be confirmed as specific in breast cancer cells and tissue, particularly IKK α . IHC was employed to breast cancer tissue to detect phosphorylated status of the p65 subunit at serine residue 536, which is important for transcriptional activation of the NF- κ B dimer [102], and therefore signifies activation of the canonical pathway. In a cohort of 544 breast cancer patients it was found that higher levels of phospho-p65 in the nucleus was associated with a shorter recurrence free interval. However, there are often difficulties with phosphorylated antibodies, limiting their possibility for use in diagnostic laboratories where a high sensitivity and reproducibility is required. One study compared the phosphorylation status of Akt and Erk1/2 in tissue taken immediately after surgery and fixed, tissue taken after X-ray of the tumour then fixed, and in tissue from specimens fixed in the histopathology department using routine fixation procedures [173]. No difference was observed between tissue that was taken immediately after surgery and fixed and tissue that was taken and fixed after X-ray of the tumour. There was however a significant discordance in results obtained in specimens subject to routine fixation, with many tumours now displaying lack of phosphorylation when many had shown high phosphorylation [173]. Fixation procedures are therefore important in the use of phospho-specific antibodies and limit their use as larger tumours take longer to formalin fix and therefore reduction in phospho-staining is likely. The

NLS of p65 has been used in prostate cancer tissue and high expression was demonstrated to be associated with shorter time to disease recurrence and shorter cancer specific survival [135]. The use of p65 NLS as an alternative marker of activated p65 in breast cancer tissue was therefore explored. No significant association with recurrence free interval and p65 NLS nuclear expression was observed however, and phospho-p65 nuclear expression was therefore found to be of more prognostic significance. A different marker of activation of the canonical pathway is therefore required. Other studies have utilised degradation, as well as phosphorylation, of I κ B α . Levels of I κ B α were higher in prostate cancer tissue compared to adjacent normal tissue and in prostate cancer cells the addition of an IKK inhibitor diminished the ability of TNF α to degrade I κ B α [174]. Further investigation into selective markers of the canonical NF- κ B pathway in breast cancer is required and downstream markers will be explored.

Expression of NF- κ B has been reported to be elevated in breast cancer cell lines and in rat mammary tumours when compared to normal tissue [138]. A difference has been observed between ER negative and ER positive cells. Furthermore, NF- κ B expression has been demonstrated to correlate with oestrogen independence, with high levels reported in ER-negative tumours [142]. It may therefore be expected that it would be beneficial to block NF- κ B activity in ER negative patients, however the results from the current study suggest a role in ER positive disease and that these patients would benefit most from inhibitors of the pathway. This is in agreement with a study in ER positive tumours that found higher DNA binding activity of p50 was associated with shorter disease free survival [139].

The non-canonical NF- κ B pathway has also been implicated in breast cancer development and progression. In mice that transgenically overexpress p100/52, a delay in mammary gland development is observed and these mice developed multiple tumours [141]. Additionally, although during mammary gland development p100/52 levels were low, during tumourigenesis expression of p100/52 was elevated [141]. There are few studies exploring the non-canonical NF- κ B pathway in breast cancer patients. Comparison between breast cancer tissue and adjacent normal tissue has shown expression of the p50 and p52 subunits is significantly higher in cancer tissue with an increase in p52 in the nucleus [140]. IHC was employed to assess levels of NIK, RelB and IKK α to investigate the impact of their expression on clinical outcome in breast cancer patients. Patients with tumours that expressed high levels of IKK α in the cytoplasm had shorter recurrence free interval. However expression of NIK or RelB, in the nucleus or cytoplasm, was not associated with recurrence. Unfortunately effective markers of activation of the non-canonical NF- κ B pathway for use in

tissue studies are lacking. In our cohort only around a third of tumours displayed RelB expression even at low levels in the nuclear compartment. The median histoscore was 0 with an interquartile range of just 0 - 6.7. This low amount and very low intensity of nuclear staining observed for RelB suggested lack of activation of the non-canonical pathway. A possible alternative explanation is that the protein is present in the nucleus in many tumours however a confirmation change upon nuclear translocation may have resulted in the antigen that the antibody binds to is masked resulting in a lack of detection by the antibody. Expression of p100/52 has also been reported as a marker of the non-canonical pathway in clinical tissue [140, 175], however although nuclear expression suggests activation, antibodies are unable to differentiate between p100 and p52 and therefore a more appropriate and specific marker is required. Induction of the non-canonical NF- κ B pathway results in phosphorylation of p100, however disappointingly the only commercially available phospho-p100 antibody shows many non-specific bands rendering it inadequate for use in patient tissue. Phosphorylation of the IKKs has also been used as a marker of NF- κ B activation, however available antibodies do not distinguish between IKK α or IKK β phosphorylation [174]. Additionally, any phospho-p100 or phospho-IKK antibody again would be subject to the variability observed for phospho-specific antibodies. Establishment of an appropriate marker is therefore required and will be explored as part of this study.

There are several subtypes of cancer, all with different molecular profiles and varied responses to chemotherapy and other therapies [176]. This is reflected in the results from this study, as there appears to be diverging roles for NF- κ B in the different luminal subtypes. High activation of the canonical NF- κ B pathway, measured by nuclear expression of p65 phosphorylated at S536, was found to associate with angiogenesis and the luminal B subtype. The non-canonical NF- κ B pathway, more specifically cytoplasmic IKK α , was associated with cell death (apoptosis and necrosis) and hormone receptor status. High expression of IKK α was found to associate with shorter recurrence free interval. As phospho-p65 and IKK α were associated with recurrence in different subtypes, luminal B and A respectively, this suggests that the increased recurrence observed with IKK α expression is a result of its role in the non-canonical NF- κ B pathway. Another study recently investigated which signalling pathways are dominant in each subtype and defined additional protein subgroups [52]. The number of different subgroups of breast cancer continue to grow and a better understanding of the signalling pathways involved in each is likely be important when considering treatment options for patients.

In this study, where there was a range in expression the median was used as the cut off for high and low expression and if a high proportion of tumours were negative (as was the case with RelB and p65 nuclear expression) groups were split using presence and absence of staining into positive and negative. Software has been developed that allows users to enter their range of scores and this selects the optimal cut off based on this distribution, such as X-Tile [177] and Cutoff Finder [178]. The use of such software may have been more appropriate for selection of optimal cut off values and should be considered in future work.

The results from this chapter suggest that the NF- κ B pathways may be involved in promoting recurrence in patients with ER positive tumours and may be involved with development of endocrine resistance. However, each pathway seems to be involved in different subtypes with the canonical pathway associating with recurrence in the luminal B subtype and the non-canonical with the luminal A subtype. Further studies in an independent cohort combined with mechanistic studies are required to further investigate the role of the pathways in the progression of breast cancer. These results do however highlight ER positive tumours may have different signalling pathways driving disease progression and therefore patients may benefit from different therapeutic strategies

Chapter 5:

Expression of phospho-p65 and IKK α in an independent cohort of ER positive breast cancers

5.1 Introduction

It was previously demonstrated that high expression of phosphorylated p65 in the nuclear compartment and high cytoplasmic IKK α were associated with reduced recurrence free interval. These findings require validation in an independent cohort, and due to the observation that both were associated with recurrence in ER positive breast cancer a cohort consisting of ER positive patients was selected. Luminal subtypes had been established using HER2 and Ki67 to subdivide luminal A and luminal B tumours, as described in chapter 3. An additional strength of this cohort, other than providing a platform to validate previous findings, is that information was available on how long each individual patient received tamoxifen therapy, therefore allowing the markers to be tested for recurrence on tamoxifen.

5.2 Expression of phosphorylated p65 in the ST-Bre-TMA

This cohort included 456 patients, 20 of which were found to have ER negative tumours and 44 were of unknown ER status. These patients were therefore excluded from analysis in this chapter, leaving 392 patients available for investigation. Immunohistochemistry was employed to TMAs using an antibody to detect phosphorylation of the p65 subunit at serine residue 536. As with the previous cohort, three representative cores were available for each tumour in order to account for tumour heterogeneity. Expression was scored using the weighted histoscore method to quantify intensity and percentage positive staining [156].

5.2.1 Nuclear expression of phospho-p65 in the ST-Bre-TMA

Expression of phospho-p65 was assessed in 78% of patients (322/392). Of these patients, 98% (316/322), had tumours that displayed expression of phospho-p65 in the nucleus. The median histoscore in these tumours was 110 with an interquartile range of 80 to 140. The median from the previous cohort was 15, however only 12 patients had a histoscore of 15 or less meaning the previously established cut off could not be applied for analysis due to insufficient numbers. The median histoscore of 110 observed in this cohort was therefore used to subdivide patients into those with low and high expression of nuclear phospho-p65. 186 patients were found to have low expression of phosphorylated p65 in the nuclear compartment and 173 patients were classified as having high expression. An example of immunostaining showing low and high phospho-p65 expression is shown in Figure 5.1A and 5.1B. Across the cores from the same patient the average standard deviation of the histoscores was 34.2 and the median was 30.6 (IQR= 17.3 - 46.5).

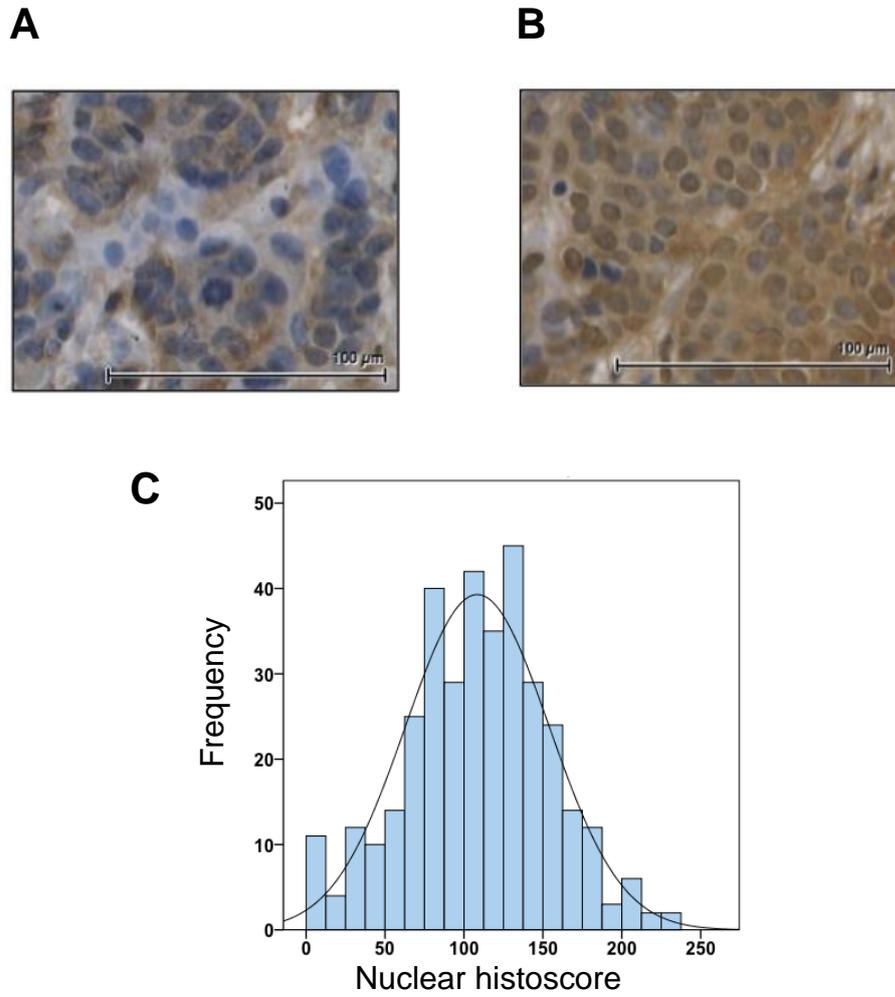


Figure 5.1: Nuclear expression of phospho-p65 in the ST-Bre-TMA. (A) An example of low nuclear expression and (B) of high nuclear expression of phospho-p65 in breast cancer tissue is shown (images taken at 400x magnification, scale bar represents 100µm). (C) Histogram displaying frequency of nuclear phospho-p65 histoscores.

5.2.2 Nuclear expression of phospho-p65 in the ST-Bre-TMA and clinical outcome

To examine whether nuclear expression of phospho-p65 was significantly associated clinical outcome in the ST-Bre-TMA cohort, Kaplan-Meier survival curves were plotted and low and high expression compared using the log rank test. No association was observed with recurrence free interval ($P=0.177$, HR = 1.3 (0.9 - 2.0), 11.5 years vs. 12.6 years, Figure 5.2A) or recurrence on tamoxifen ($P=0.137$, HR = 1.4 (0.9 - 2.3), 8.9 years vs. 11.2 years, Figure 5.2B), although this trended towards high expression having slightly poorer outcome. Nuclear expression of phospho-p65 was, however, associated with poorer breast cancer specific survival ($P=0.041$, HR = 1.6 (1.0 - 2.5), 14.1 vs. 15 years, Figure 5.2C).

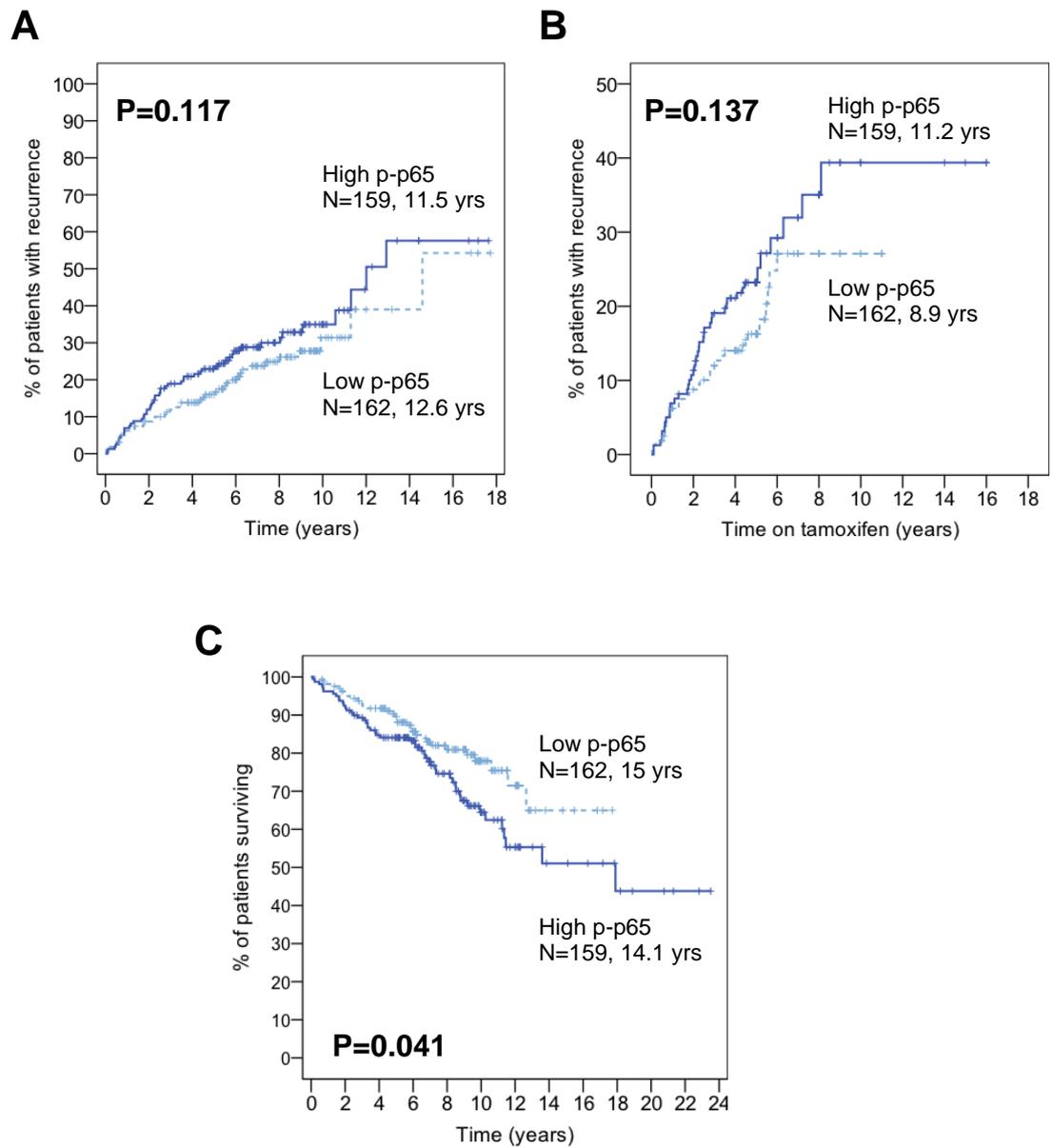


Figure 5.2: Nuclear expression of phospho-p65 and clinical outcome in the ST-Bre-TMA. (A) Expression of phospho-p65 in the nucleus was not associated with recurrence free interval or (B) recurrence on tamoxifen. (C) High nuclear phospho-p65 was associated with poorer breast cancer specific survival. Censor lines in (A) and (B) indicate loss to follow up and in (C) indicate loss to follow up or death due to other cause.

5.2.3 Association of phospho-p65 nuclear expression with clinico-pathological characteristics of the ST-Bre-TMA

Chi-squared analysis was performed to establish whether expression of nuclear phospho-p65 was associated with any clinico-pathological characteristics of the cohort. These factors and their association with nuclear phospho-p65 are detailed in Table 5.1. Unlike the previous cohort a measure of angiogenesis, klntrup and necrosis was not available to correlate with. From the available parameters expression of phospho-p65 in the nuclear compartment was not found to associate with any of the clinico-pathological factors. This is consistent with the previous study, in which only CD34 as a marker of angiogenesis was found to correlate with expression of phospho-p65.

Clinico-pathological characteristic	Association with nuclear phospho-p65
Age	P=0.482
Size	P=0.494
Grade	P=0.056
Nodal Status	P=0.206
NPI	P=0.756
PgR status	P=0.873
HER2	P=0.430
Ki67	P=0.414
Apoptosis (TUNEL)	P=0.532

Table 5.1: Association of nuclear phospho-p65 with clinico-pathological characteristics of the ST-Bre-TMA cohort. *Chi-squared analysis explored the relationship of nuclear expression of phospho-p65 with various characteristics of the cohort.*

5.2.4 Nuclear expression of phospho-p65 in the ST-Bre-TMA and clinical outcome in different luminal subtypes

When this was subdivided into luminal subtypes, phospho-p65 nuclear expression was not significantly associated with breast cancer specific survival in either luminal subtype, although a relationship was observed in luminal B tumours, consistent with the previous cohort, however this did not reach significance ($P=0.087$, Figure 5.3B). This relationship was not observed in luminal A tumours, again consistent with previous clinical data ($P=0.629$, Figure 5.3A). When tumours were subdivided by luminal subtype and recurrence free interval was analysed, phospho-p65 was not significantly associated with recurrence free interval in either subtype (luminal A, $P=0.842$, Figure 5.4A; luminal B $P=0.160$, Figure 5.4B). Again, when recurrence on tamoxifen was considered, high phospho-p65 was not significantly associated with quicker time to recurrence in luminal B tumours although a relationship was observed ($P=0.141$, Figure 5.4D). No relationship was observed between phospho-p65 and recurrence on tamoxifen in luminal A tumours ($P=0.962$, Figure 5.4C).

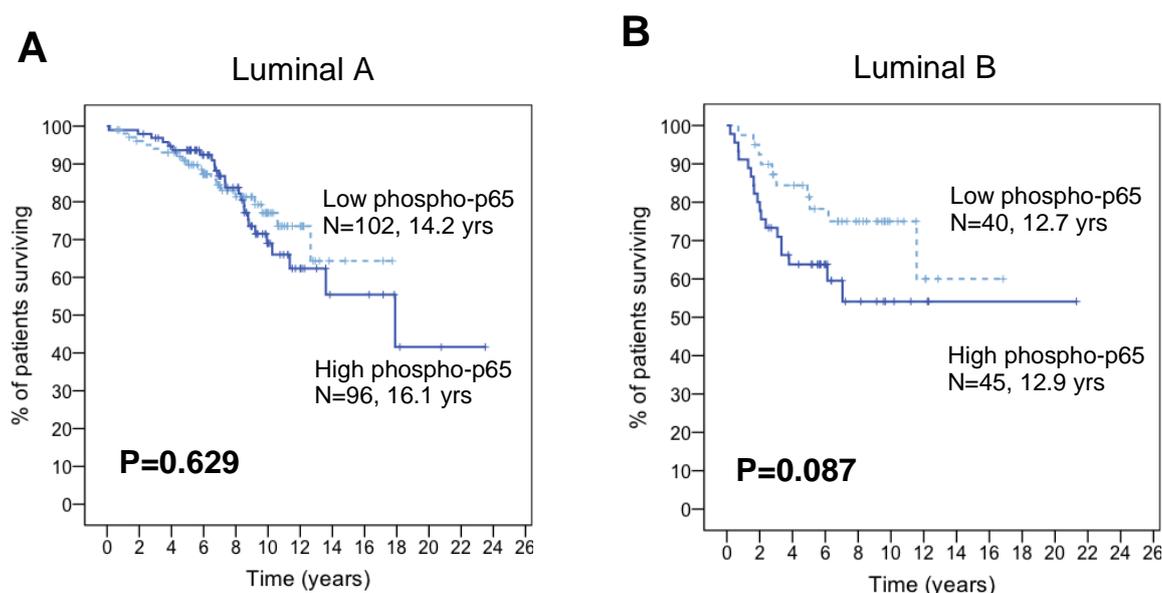


Figure 5.3: Nuclear expression of phospho-p65 and breast cancer specific survival in luminal subtypes in the ST-Bre-TMA. Expression of phospho-p65 in the nucleus in patients with luminal A tumours (A) and luminal B tumours (B) and breast cancer specific survival. *Censor lines indicate loss to follow up or death due to other cause.*

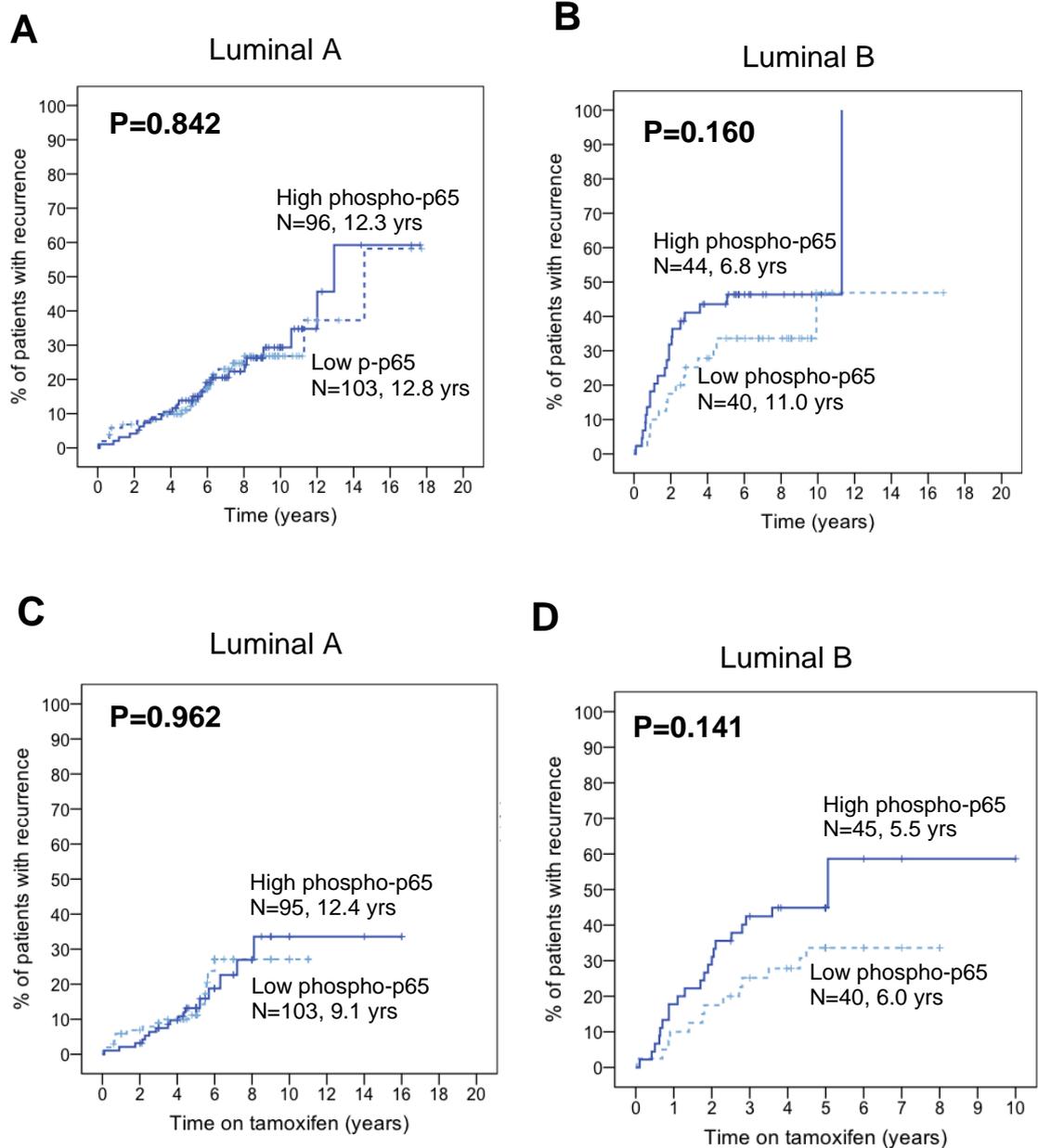


Figure 5.4: Nuclear expression of phospho-p65 and clinical outcome in luminal subtypes in the ST-Bre-TMA. Expression of phospho-p65 in the nucleus in patients with luminal B tumours was associated with recurrence free interval (A), but not recurrence on tamoxifen (C) or breast cancer specific survival (E). No relationship was observed with recurrence free interval (B), recurrence on tamoxifen (D) and breast cancer specific survival (F) in patients with luminal B tumours. Censor lines in (A-D) indicate loss to follow up and in (E) and (F) indicate loss to follow up or death due to other cause.

5.3 Expression of IKK α in the ST-Bre-TMA

Immunohistochemistry was employed to TMAs to assess levels of IKK α . Evidence from the literature and our previous clinical cohort suggest IKK α is associated with ER and it was therefore hypothesised that expression of IKK α would be involved in the development of endocrine resistance.

5.3.1 Cytoplasmic expression of IKK α in the ST-Bre-TMA

Expression of IKK α was assessed in 78.8% (309/392) of the ER positive patients included in analysis and of these 99% (308/309) displayed cytoplasmic expression. Histoscores in these tumours ranged from 0 to 190 with a median histoscore of 95 (IQR = 73.3 - 116.7). The cut off from the previous cohort, 100 histoscore units, was applied to stratify patients into those with high IKK α expression and those with low IKK α expression in the cytoplasm. 181 patients were found to have low cytoplasmic IKK α and 128 patients were classified as having high expression. Figure 5.2 shows an example of immunostaining showing low (Figure 5.5A) and high (Figure 5.5B) IKK α expression. The average standard deviation in histoscore across the cores from the same patient was 29.3 and the median was 26.5 (IQR= 14.1 - 37.9).

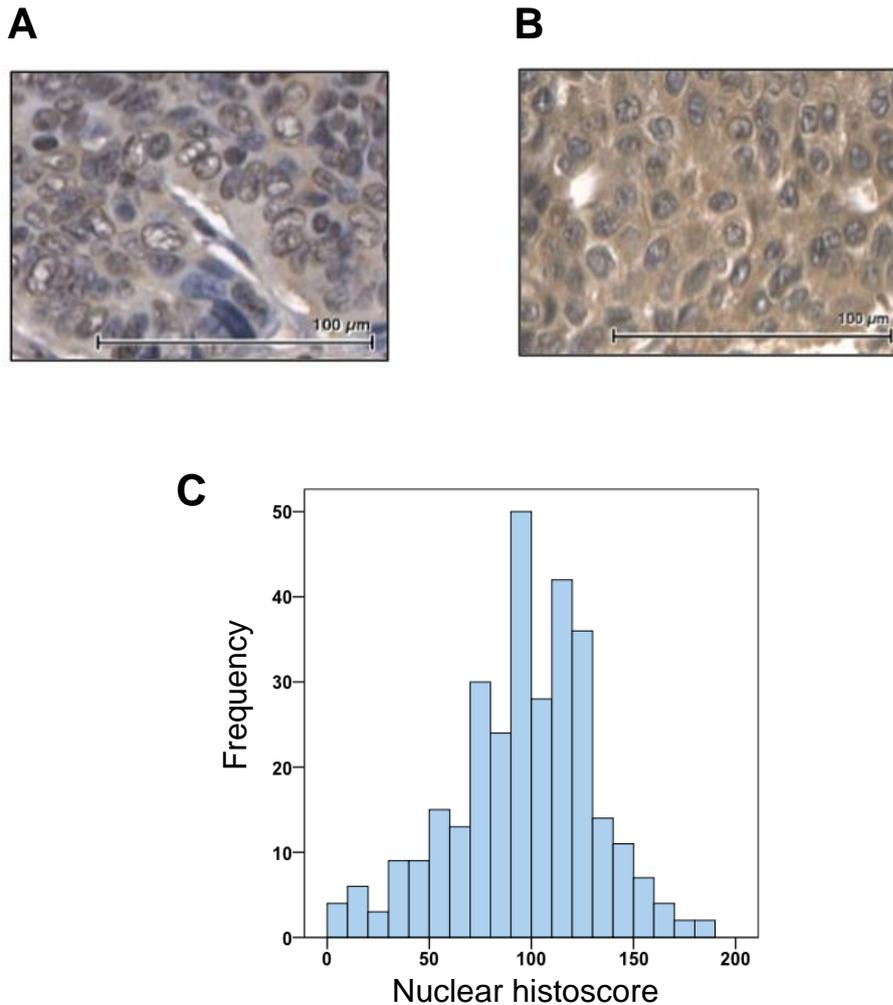


Figure 5.5: Cytoplasmic expression of IKK α in the ST-Bre-TMA. (A) An example of low nuclear expression and (B) of high nuclear expression of IKK α in breast cancer tissue is shown (images taken at 400x magnification, scale bar represents 100 μ m). (C) Histogram displaying frequency of cytoplasmic IKK α histoscores.

5.3.2 Cytoplasmic expression of IKK α in the ST-Bre-TMA and clinical outcome

In order to investigate whether expression of IKK α was significantly associated with clinical outcome in the ST-Bre-TMA cohort, Kaplan-Meier survival curves were plotted and low and high expression compared using the log rank test. Cytoplasmic IKK α was significantly associated with reduced recurrence free interval (P=0.024, HR = 1.6 (1.1 - 2.4), 11.3 years vs. 15 years, Figure 5.6A), quicker recurrence on tamoxifen (P=0.013, HR = 1.8 (1.1 - 2.9), 10.3 years vs. 12.6 years, Figure 5.6B), and shorter recurrence free interval (P=0.016, HR = 1.7 (1.1 - 2.7), 15.4 vs. 16 years, Figure 5.6C).

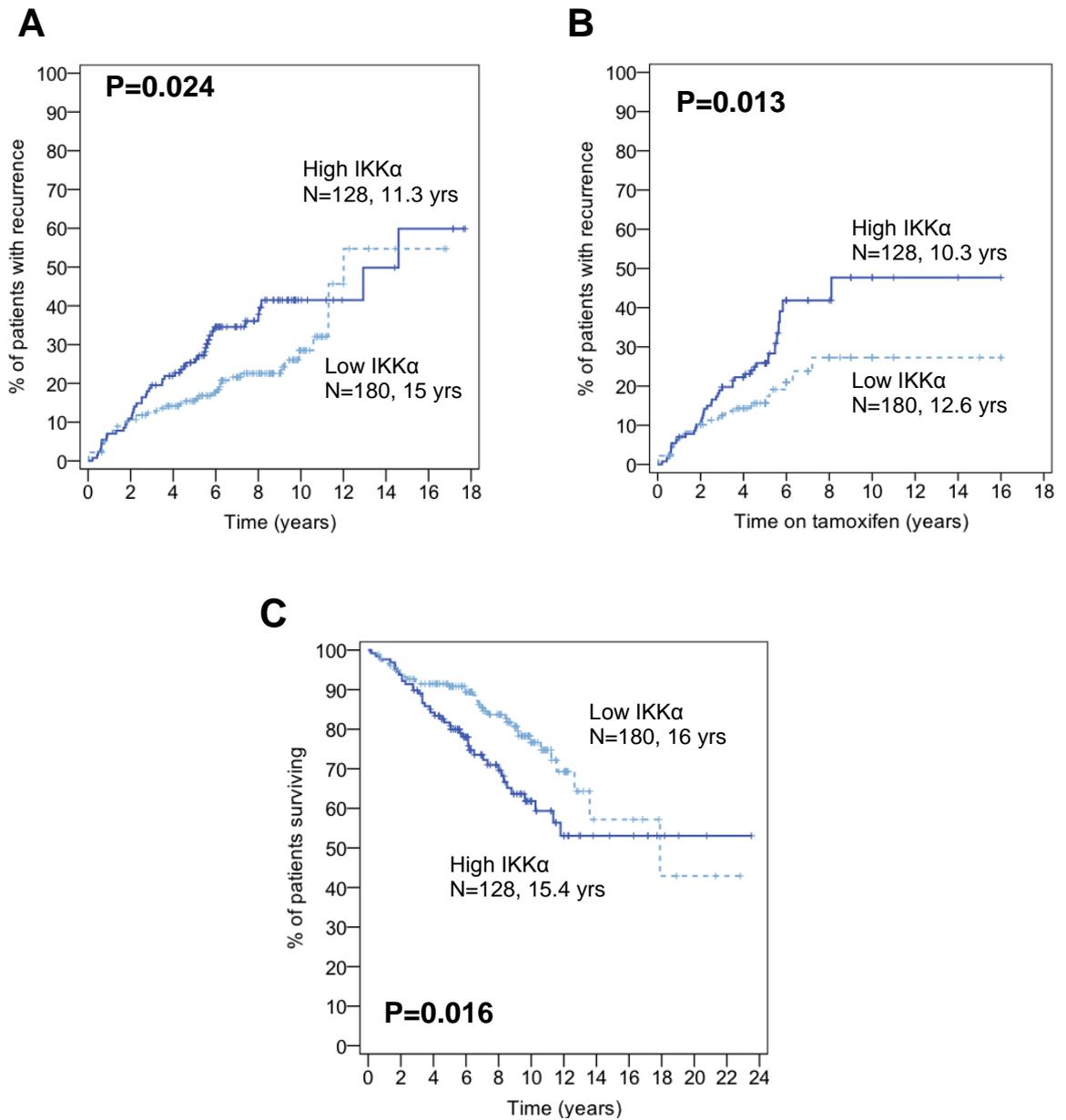


Figure 5.6: Cytoplasmic expression of IKK α and clinical outcome in the ST-Bre-TMA. (A) Expression of IKK α in the cytoplasm was associated with shorter recurrence free interval, (B) quicker time to recurrence on tamoxifen and (C) decreased breast cancer specific survival. Censor lines in (A) and (B) indicate loss to follow up and in (C) indicate loss to follow up or death due to other cause

5.3.3 Association of IKK α cytoplasmic expression with clinico-pathological characteristics of the ST-Bre-TMA

In order to establish whether expression of cytoplasmic IKK α was associated with any clinico-pathological characteristics of the cohort, chi-squared analysis was performed. These factors and their association with cytoplasmic IKK α are detailed in Table 5.2. As previously observed expression of IKK α in the cytoplasm was associated with invasive grade, nodal status and also NPI, again we were unable to determine if correlated with klintrup, as this wasn't available for this cohort. In the previous cohort IKK α was associated with cell death, however necrosis information was unavailable in this cohort and TUNEL (as a marker of apoptosis) was not found to correlate, although there were small amounts of tumours with low TUNEL for analysis.

Clinico-pathological characteristic	Association with cytoplasmic IKK α
Age	P=0.546
Size	P=0.272
Grade	P=0.036
Nodal Status	P=0.043
NPI	P=0.011
PgR status	P=0.593
HER2	P=0.738
Ki67	P=0.212
Apoptosis (TUNEL)	P=0.427

Table 5.2: Association of cytoplasmic IKK α with clinico-pathological characteristics of the ST-Bre-TMA cohort. *Chi-squared analysis explored the relationship of cytoplasmic expression of IKK α with various characteristics of the cohort.*

5.3.4 Cytoplasmic expression of IKK α in the ST-Bre-TMA and clinical outcome in different luminal subtypes

When this was subdivided into luminal subtypes, in luminal A tumours cytoplasmic IKK α nuclear expression was not significantly associated with survival ($P=0.214$, Figure 5.7A), recurrence free interval ($P=0.137$, Figure 5.8A) or recurrence on tamoxifen ($P=0.110$, Figure 5.8C). In patients with luminal B tumours high IKK α was associated with poorer breast cancer specific free survival ($P=0.018$, Figure 5.7B), decreased recurrence free interval ($P=0.031$, Figure 5.8B) and quicker recurrence on tamoxifen ($P=0.029$, Figure 5.8D). This is not consistent with the previous cohort where a relationship with recurrence free interval was observed in patients with luminal A tumours, and further testing in a larger cohort is required.

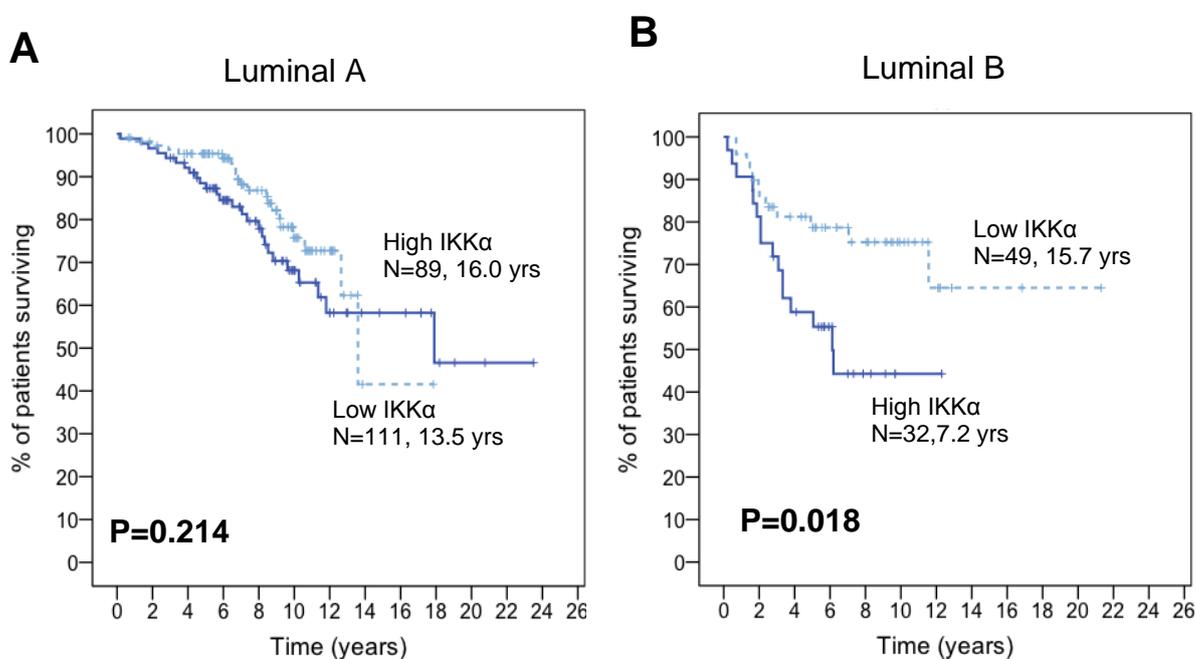


Figure 5.7: Cytoplasmic expression of IKK α and breast cancer specific survival in luminal subtypes in the ST-Bre-TMA. Expression of IKK α in the cytoplasm in patients with luminal A tumours was not associated with breast cancer specific survival (A). In patients with luminal B tumours (B), high IKK α was associated with reduced breast cancer specific survival. Censor lines indicate loss to follow up or death due to other cause.

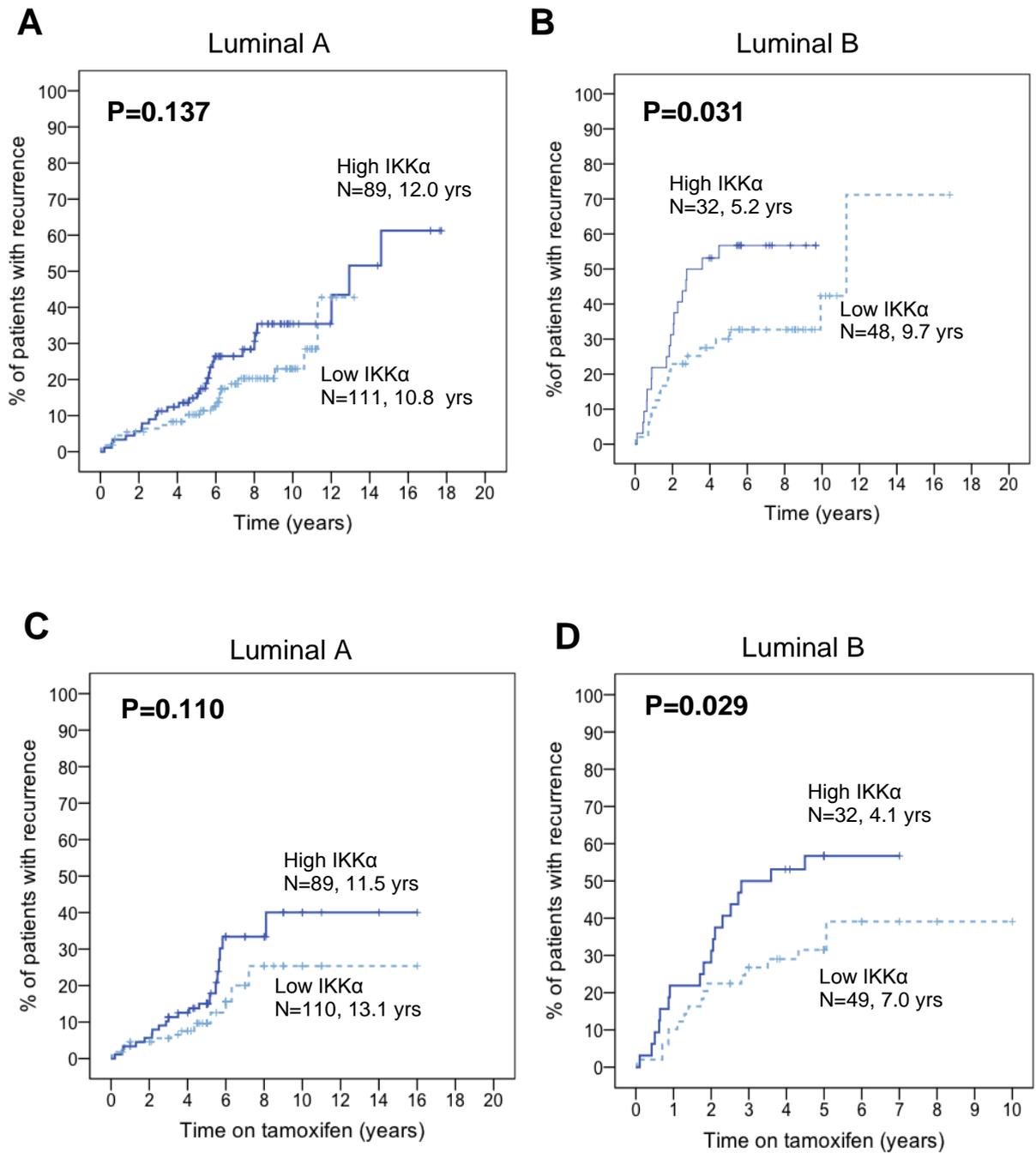


Figure 5.8: Cytoplasmic expression of IKK α and clinical outcome in luminal subtypes in the ST-Bre-TMA. Expression of IKK α in the cytoplasm in patients with luminal A tumours was not associated with recurrence free interval (A) or recurrence on tamoxifen (C). In patients with luminal B tumours however, high IKK α was associated with shorter recurrence free interval (B) and quicker time to recurrence on tamoxifen (D). Censor lines indicate loss to follow up.

5.4 Discussion

It was previously observed that nuclear expression of phospho-p65 and cytoplasmic expression of IKK α were both associated with recurrence in ER positive breast cancer and these markers were therefore tested in an independent cohort of ER positive patients.

When levels of phosphorylated p65 at serine residue 536 were assessed, these were much higher overall than the previous cohorts. The median histoscore of nuclear phospho-p65 was 110 with an interquartile range of 80 to 140. The cut off established in the previous cohort was much lower at 15 histoscore units, however only 12 patients in the ST-Bre-TMA had a histoscore of 15 or less meaning the previously established cut off could not be applied for analysis due to insufficient numbers. This highlights the variability observed with levels of phosphoproteins, in agreement with the study by Pinhel *et al* previously described [173]. There are several studies that have investigated the stability of phosphoproteins in tissue studies by examining the effect of time to fixation and levels of phosphoprotein observed. Expression of Akt phosphorylated at Ser 473 was lost in gastroesophageal tumour resections but present in biopsies [179]. Additionally, western blotting showed a greater decrease in levels of phospho-Akt over time with a half life of 20 minutes, in comparison to total Akt, which had a half life of 180 minutes [179]. Due to the often transient nature of the process, and the results from Baker *et al* and Pinhel *et al*, it may be expected that phosphorylation may be lost with delays in fixation; however increase in phosphorylation has also been reported. Jones *et al* studied the effect of a delay in fixation on markers associated with Src tyrosine kinase activity by assessing levels of phosphorylation of focal adhesion kinase and Paxillin, both downstream markers of Src [180]. No difference was observed in bladder tumours however, in breast cancer specimens, an increase in phosphorylation of Paxillin was observed over time [180]. Furthermore, another study reported an increase in certain proteins and decreases in others and reasoned that this is dependent on the function of the protein [181]. This collection of research again demonstrates the importance of time to fixation and the need for standardisation of procedures involved in every stage of tissue studies, particularly when using phosphoprotein antibodies.

High levels of phospho-p65 in the nucleus were not significantly associated with breast cancer recurrence or recurrence on tamoxifen, inferior breast cancer specific survival was however observed. A more reliable marker of activation of the canonical pathway is therefore required. Elevated IKK α in the cytoplasm was again associated with briefer recurrence free interval and also decreased breast cancer specific survival, validating that IKK α is a marker of poor prognosis in ER positive breast cancer. However, when subdivided into luminal A and

luminal B tumours, unlike the previous cohort in which IKK α was associated with recurrence in the luminal A tumours, in this cohort IKK α was associated with breast cancer specific survival, recurrence free interval and recurrence on tamoxifen only in luminal B tumours. The results from chapter 4 and chapter 5 therefore confirm that IKK α is involved with ER positive disease but question whether its role is dominant in the luminal A or luminal B subtype, suggesting that further validation is required in a larger cohort. The results do however again suggest that different pathways are active in different breast tumours and emphasises the need to consider breast cancer a collection of distinct diseases, which should be treated accordingly.

Validation in a similar, but independent, cohort with patients from the same geographical location as the discovery cohort is considered internal validation [182]. Although this provides valuable evidence of the reproducibility of findings from the initial cohort, external validation is therefore still required. Using an independent cohort of patients from outwith the Greater Glasgow and Clyde area would provide substantiation that these markers are associated with breast cancer progression in other areas and strengthen evidence for use in a clinical setting. Larger numbers of patients are also required to ensure each subgroup is of sufficient statistical power. However, rather than to propose that these be used as biomarkers, the primary objective of investigation in a second cohort was instead to provide validation to strengthen the implication of IKK α involvement in endocrine resistance and that inhibitors of IKK α may be beneficial to patients with ER positive breast cancer.

Although there are therapies currently available for ER positive breast cancer, many patients develop resistance to endocrine therapy and a better understanding of mechanisms driving endocrine resistance is required [84]. The results from both cohorts suggest a role for NF- κ B, in particular IKK α , in endocrine resistance, with patients who have luminal (ER positive) tumours that have high IKK α showing quicker time to recurrence on tamoxifen. It has also been found that tamoxifen resistant MCF7/HER2 and BT474 cells have enhanced NF- κ B activity compared to MCF7 tamoxifen sensitive cells [183]. This along with the findings in the clinical cohort indicates that inhibitors of components of the NF- κ B pathways, particularly IKK α , may therefore be beneficial to patient with ER positive tumours following development of endocrine resistance or in combination with endocrine therapy. Mechanistic studies examining the effect of preventing activation of the NF- κ B pathways are required examining the most efficient method of suppression, such as inhibition of IKK α and IKK β , and the impact this has on cellular viability.

In 2005 guidelines were published for tissue studies examining prognostic markers in tumours. These guidelines, known as REporting recommendations for tumor MARKer prognostic studies (REMARK), were established with the aim of improving the reporting of these studies by providing clear and complete reporting of data, allowing other researchers to interpret how useful the results are and the context of the findings [153]. As discussed on P54, these guidelines were adhered to in this chapter and the previous two chapters including clinical tissue. The only exception was that a power calculation was not used to justify sample size as tissue was used based on availability, however there was around 100 events for each end point in both cohorts and therefore these sample numbers are sufficient for a study of this kind, although validation of findings in a much larger cohort would be extremely beneficial.

This chapter has therefore shown that IKK α was associated with recurrence free interval in a second cohort and also with recurrence on tamoxifen, however in contrast to the first cohort IKK α was only associated with clinical outcome in patients with luminal B tumours. Validation in a larger cohort from a different geographical location is therefore necessary before any conclusions can be drawn. The variability in phospho-specific antibodies was highlighted by the much higher levels of phosphorylation of p65 in this cohort. This has therefore demonstrated that selective markers of both the canonical and the non-canonical pathway are required.

Chapter 6:

Expression of components of the NF- κ B pathways in breast cancer cell lines

6.1 Introduction

The NF- κ B pathways have been reported to be elevated in cancer cell lines, and it was observed in the clinical cohorts that high levels of expression of certain key members of the pathways are associated with poor clinical outcome. Further investigation was therefore carried out into expression of the canonical and non-canonical NF- κ B pathways in breast cancer cell lines. Due to the differences observed in ER positive and negative tumours, both ER positive MCF7 cells and ER negative MDA-MB-231 cells were utilised as model cell lines to reflect the different ER subgroups of breast cancer. Expression of several family members, from both the canonical and non-canonical pathway, was assessed in both cell lines after exposure to ligands in order to compare the differences in NF- κ B signaling in ER positive and ER negative breast cancer. Additionally, as difficulties with phosphorylated antibodies were highlighted in the tissue study using phosphorylation of p65 as a marker of activation of the canonical pathway and as there is no specific marker for activation of the non-canonical pathway, siRNA was employed to silence the kinases in each pathway and observe downstream effects using qPCR.

6.2 Activation of the canonical NF- κ B pathway in breast cancer cell lines

To establish that induction of the canonical NF- κ B pathway could be observed in a cellular setting, key markers of pathway activation including the phosphorylation of the p65 subunit and degradation of I κ B α were measured. Western blotting was performed using protein extracts prepared from ER positive MCF7 cells and ER negative MDA-MB-231 cells.

Both TNF α and IL-1 β have been reported to drive activation of the canonical NF- κ B pathway, and these ligands were selected to investigate NF- κ B activation in the breast cancer cell lines. A pilot experiment was performed to confirm which ligand could be utilised in future experiments. First cells were rendered quiescent by incubation in serum free media for 24 hours and cells were then exposed to either TNF α or IL-1 β , both at 20ng/ml, for various incubation periods as stated below. Proteins were then extracted from cells, separated by SDS-PAGE and transferred to a nitrocellulose membrane before visualisation (as described in Section 2.3). Expression of members of the canonical NF- κ B pathway was then investigated including I κ B α , p65 and its phosphorylation status at serine 536, IKK β and IKK α . The expression of these proteins at various time points following TNF α or IL-1 β stimulation are shown in Figure 6.1. In order to compare the amount of protein in each sample and allow proper assessment of the blotting, β -tubulin expression, a housekeeping gene independent of NF- κ B, was used as a loading control.

6.2.1 Activation of the canonical NF- κ B pathway in MCF7 and MDA-MB-231 cells

A preliminary experiment (N=1) was constructed to identify activation of the pathway following exposure to different ligands. This experiment was only performed once to guide us on future use of ligands and quantification of the blots was therefore not performed in this instance. In ER positive MCF7 cells, both degradation of I κ B α and induction of phosphorylation of p65 were observed with TNF α and IL-1 β exposure (Figure 6.1A). Compared to untreated cells, TNF α exposure resulted in a time dependent increase in p65 phosphorylation, which remained elevated until 120 minutes. However response to IL-1 β was more transient, returning to basal levels by approximately 120 minutes. Both agonists also induced a parallel stimulation of I κ B α degradation with a time dependent decrease in I κ B α from 15 minutes exposure and lowest levels at 30 minutes. After 60 minutes exposure to TNF α , expression of I κ B α began to increase again. In ER negative MDA-MB-231 cells, although some degradation of I κ B α was evident and slight increase is observed in p65 phosphorylation in response to TNF α , phosphorylation is high at all time points including untreated control cells, showing constitutive activation of the canonical pathway in these cells (Figure 6.1B). This made further induction of the pathway difficult to observe and neither ligand was able to induce the pathway to the same extent as that observed in the ER positive cells. Small levels of induction were, however, observed in response to TNF α , therefore TNF α was chosen as the ligand of choice for future experiments examining the canonical NF- κ B pathway. Expression of p65, IKK α and IKK β did not change over the time course examined.

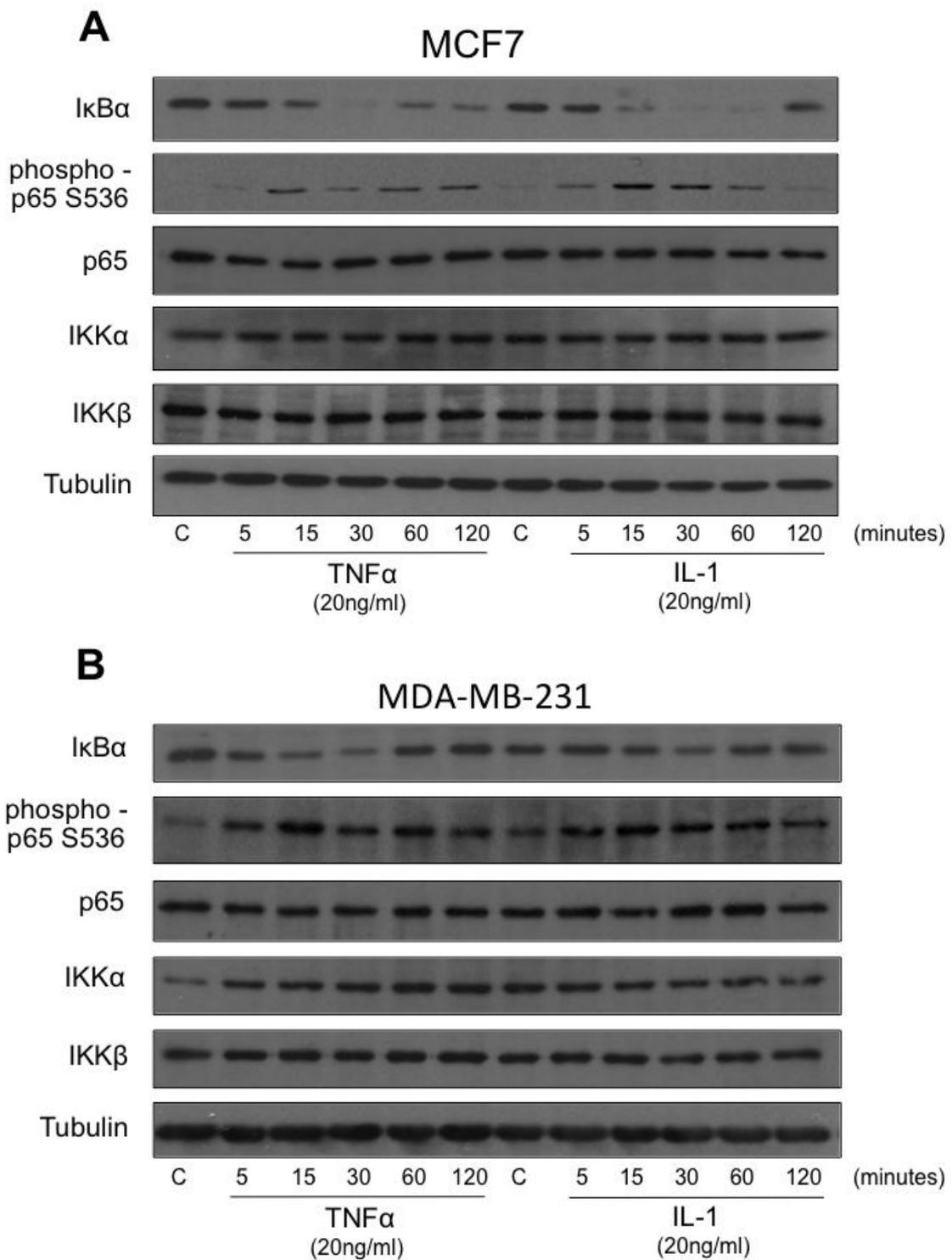


Figure 6.1: Expression of members of the canonical NF- κ B pathway in MCF7 and MDA-MB-231 breast cancer cell lines at following TNF α or IL-1 β exposure. MCF7 (A) and MDA-MB-231 (B) cells were exposed to 20ng/ml of TNF α or IL-1 β for various time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of I κ B α , p65 and its phosphorylation status at serine 536, IKK β and IKK α (N=1). β -tubulin was used as a loading control.

6.2.2 TNF α exposure and expression of components of the canonical NF- κ B pathway in MCF7 cells

TNF α was selected for stimulation of MCF7 and MDA-MB-231 cells in 3 separate experiments to allow quantification of expression levels of I κ B α and phosphorylated p65. Again, cells were exposed to 20ng/ml TNF α for various time periods before protein was extracted, separated by SDS-PAGE and expression of members of the pathway visualised. Images were then quantified using ImageJ. Protein expression was normalised to the β -tubulin loading control and expression compared to untreated cells. Differences in expression were calculated using fold change to control, and graphs of I κ B α and phospho-p65 expression were drawn. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels.

TNF α induced I κ B α degradation and phosphorylation of p65 in a time dependent manner in ER positive MCF7 cells (Figure 6.2). Degradation of I κ B α occurred at 15 minutes incubation with TNF α , with an average reduction in expression to 55% of control levels (P=0.005). The minimal level was observed at 30 minutes stimulation, with only 17% of that of the levels in untreated cells (P<0.001). Expression of I κ B α returned at 60 minutes. Phosphorylation of p65 occurred earlier with around a 2.7 average fold increase at 5 minutes TNF α exposure. This was time dependent with a further increase in expression at 15 minutes to 4.4 times that of control cells (P=0.046). Expression peaked at 30 minutes with an average 5.2 fold increase (P=0.013). Phosphorylation remained high but began to return to basal levels with a 4.2 fold increase compared to control at 60 minutes (P=0.064) and a 3.3 fold at 120 minutes (P=0.232). Levels of p65, IKK α and IKK β were unchanged by TNF α exposure.

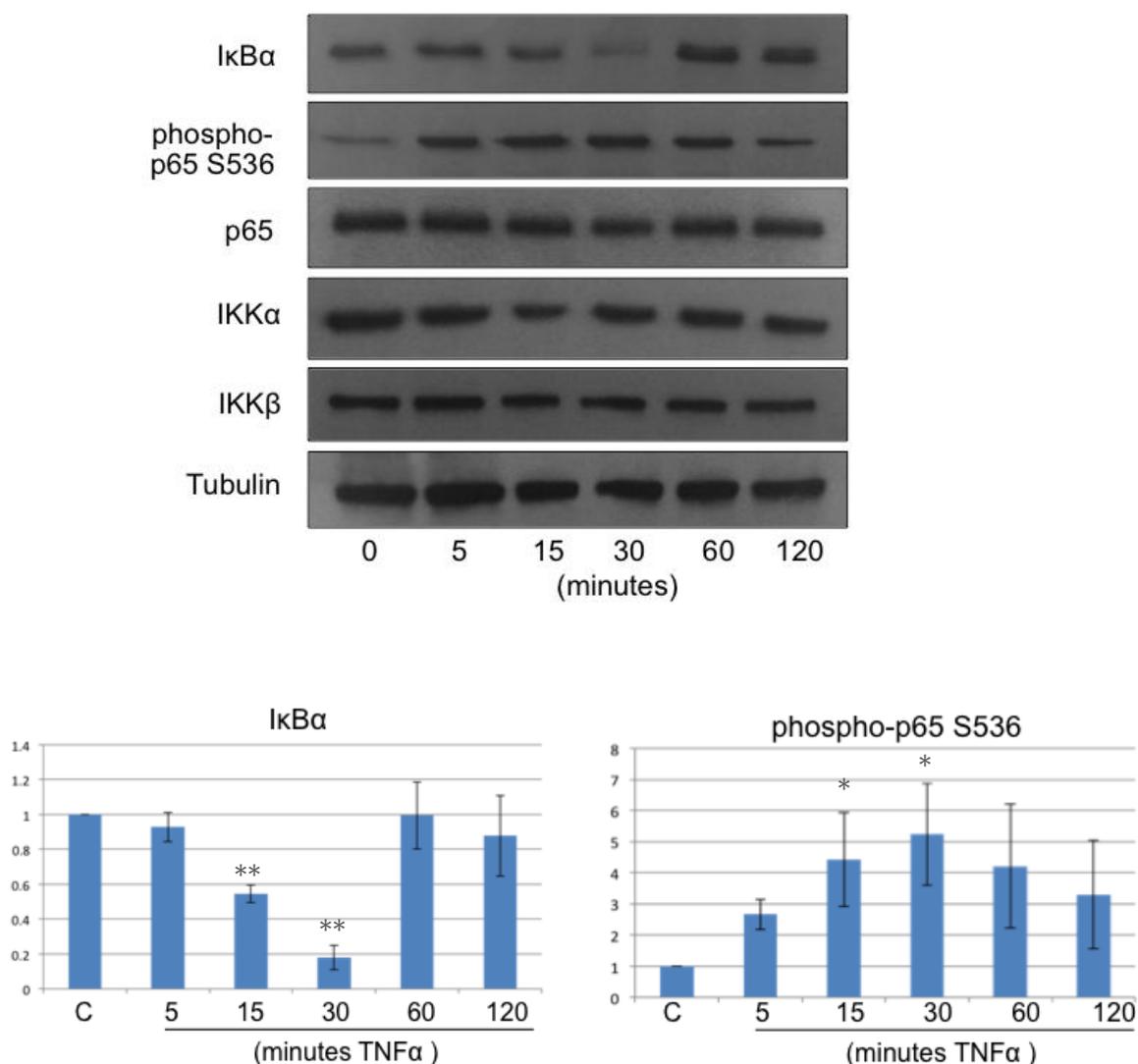


Figure 6.2: Expression of members of the canonical NF- κ B pathway in MCF7 breast cancer cells following TNF α exposure. MCF7 cells were exposed to 20ng/ml of TNF α for various time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of I κ B α , p65 and its phosphorylation status at serine 536, IKK β and IKK α . β -tubulin was used as a loading control. This is representative of 3 experiments. ImageJ was used to quantify expression of I κ B α and phospho-p65 and levels in TNF α stimulated cells were compared to unstimulated cells, displayed as fold change to control in the bar graphs. Error bars represent standard deviation. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. * signifies significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

6.2.3 TNF α exposure and expression of components of the canonical NF- κ B pathway in MDA-MB-231 cells

High basal levels of phosphorylation of p65 at serine 536 was observed in ER negative MDA-MB-231 cells, but degradation of I κ B α occurred following TNF α exposure (Figure 6.3). Phosphorylation of p65 was high at all time points indicating constitutive activation of the canonical pathway. Expression of phospho-p65 was increased slightly, but not significantly, at 15 minutes with around a 25% increase on average and at 30 minutes was 15% higher than unstimulated MDA-MB-231 cells. I κ B α degradation was evident in the MDA-MB-231 cells with approximately 50 % reduction at 15 minutes (P=0.01) and a minimal level at 30 minutes, where expression was reduced by 63% compare to basal levels (P=0.002). Levels of I κ B α began to return to basal levels at 60 minutes, where expression was 61% of control cells (P=0.044). Levels of p65, IKK α and IKK β were unchanged by TNF α stimulation.

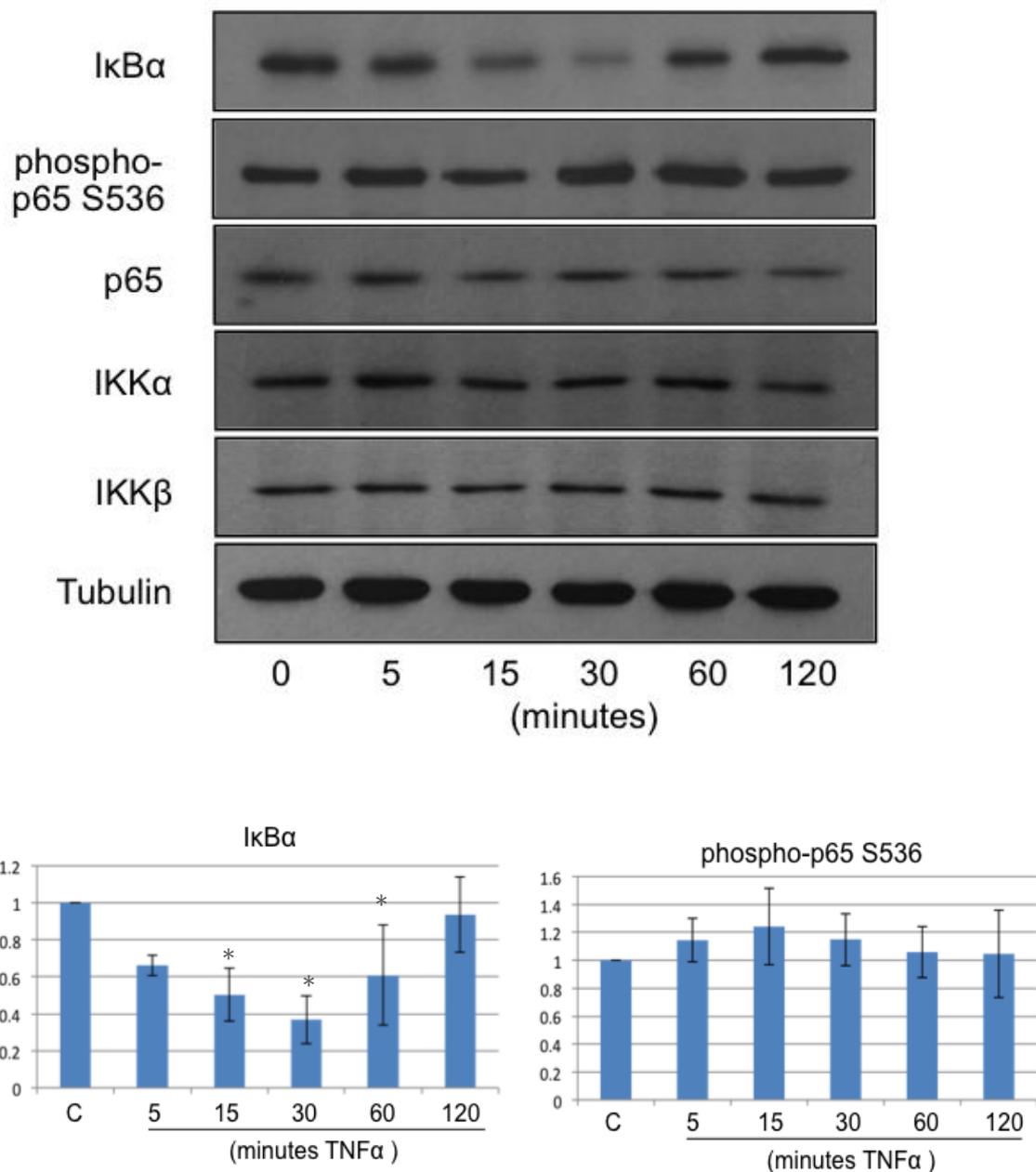


Figure 6.3: Expression of members of the canonical NF- κ B pathway in MDA-MB-231 breast cancer cells following TNF α stimulation. MDA-MB-231 cells were exposed to 20ng/ml of TNF α for various time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of I κ B α , p65 and its phosphorylation status at serine 536, IKK β and IKK α . β -tubulin was used as a loading control. This is representative of 3 experiments. ImageJ was used to quantify expression of I κ B α and phospho-p65 and levels in cells exposed to TNF α were compared to untreated cells, displayed as fold change to control in the bar graphs. Error bars represent standard deviation. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

6.2.4 Cellular location of p65 in MCF7 cells following exposure to TNF α

Cell pellets of MCF7 cells exposed to 20ng/ml TNF α for 15 minutes show nuclear translocation when IHC was performed using an antibody that detects the p65 subunit or p65 phosphorylated at residue 536 (Figure 6.4).

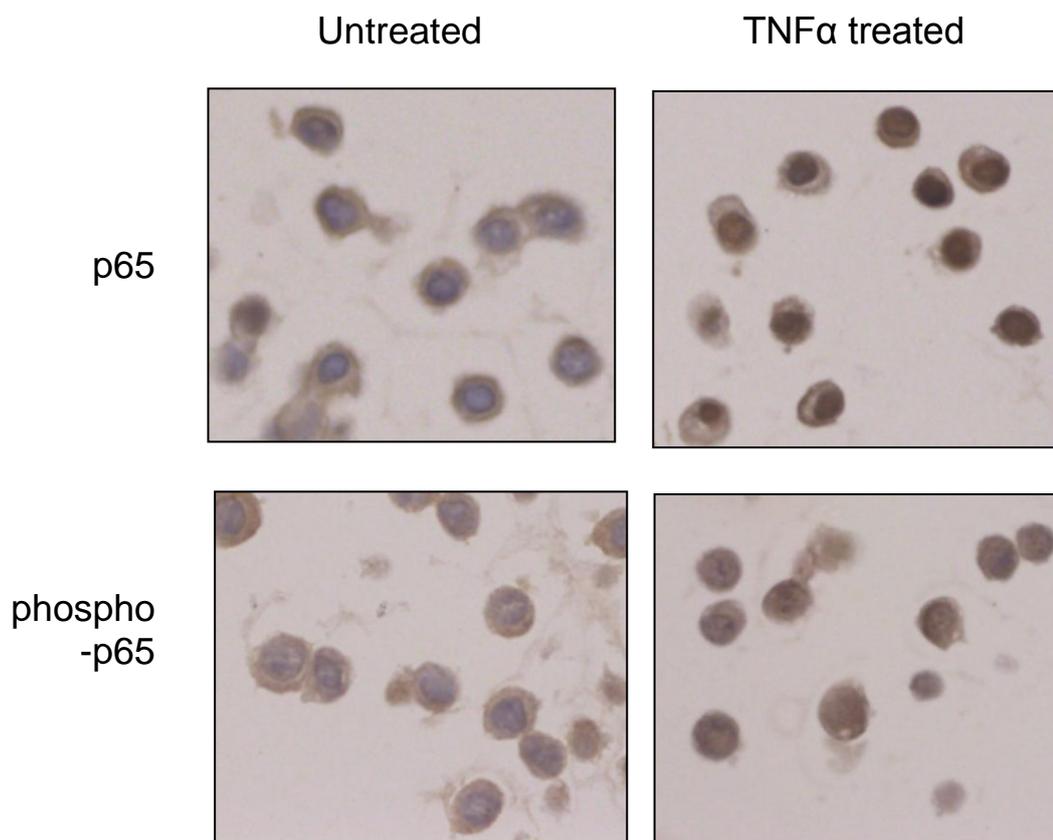


Figure 6.4: Expression of the p65 subunit in MCF7 cell pellets following TNF α stimulation. MCF7 cells were exposed to 20ng/ml of TNF α for 15 minutes before formalin fixation and wax embedding. IHC was performed to assess cellular location of p65 and phosphorylated p65 (N=1). Images were taken at magnification of 1000x. This figure was previously displayed as part of Figure 4.2 to show antibody specificity.

6.3 Activation of the non-canonical NF- κ B pathway in breast cancer cell lines

Expression of members of the non-canonical NF- κ B pathway was investigated including p100/p52, p100 phosphorylated at serine residues 866/870 and IKK α . A pilot experiment was performed to observe the expression of these proteins at several time points following treatment with various ligands. Exposure of MCF7 and MDA-MB-231 cells to IL-1 β , TNF α , RANK-L or lymphotoxin $\alpha_1\beta_2$ is shown in Figure 6.5. In order to compare the amount of protein in each sample β -tubulin was used as a loading control. This experiment was only performed once to inform on future use of ligands and quantification of the blots was therefore not performed in this instance

6.3.1 Activation of the non-canonical NF- κ B pathway in MCF7 and MDA-MB-231 cells

In ER positive MCF7 cells (Figure 6.5A), phosphorylation of p100 did not appear to be increased by RANK-L but did increase upon lymphotoxin exposure in comparison to unstimulated cells. No increase in p52 is obvious and a method of examining nuclear expression such as western blots of nuclear fractions or IHC on cell pellets may be required to visualise changes in p52 expression. TNF α and IL-1 β exposure both also appear to increase expression of phospho-p100 indicating IKK β driven activation of the non-canonical pathway.

In ER negative MDA-MB-231 cells (Figure 6.5B), phosphorylation of p100 appears low in the control cells, although β -tubulin shows less protein present in this sample. TNF α also increased expression of phospho-p100 in these cells.

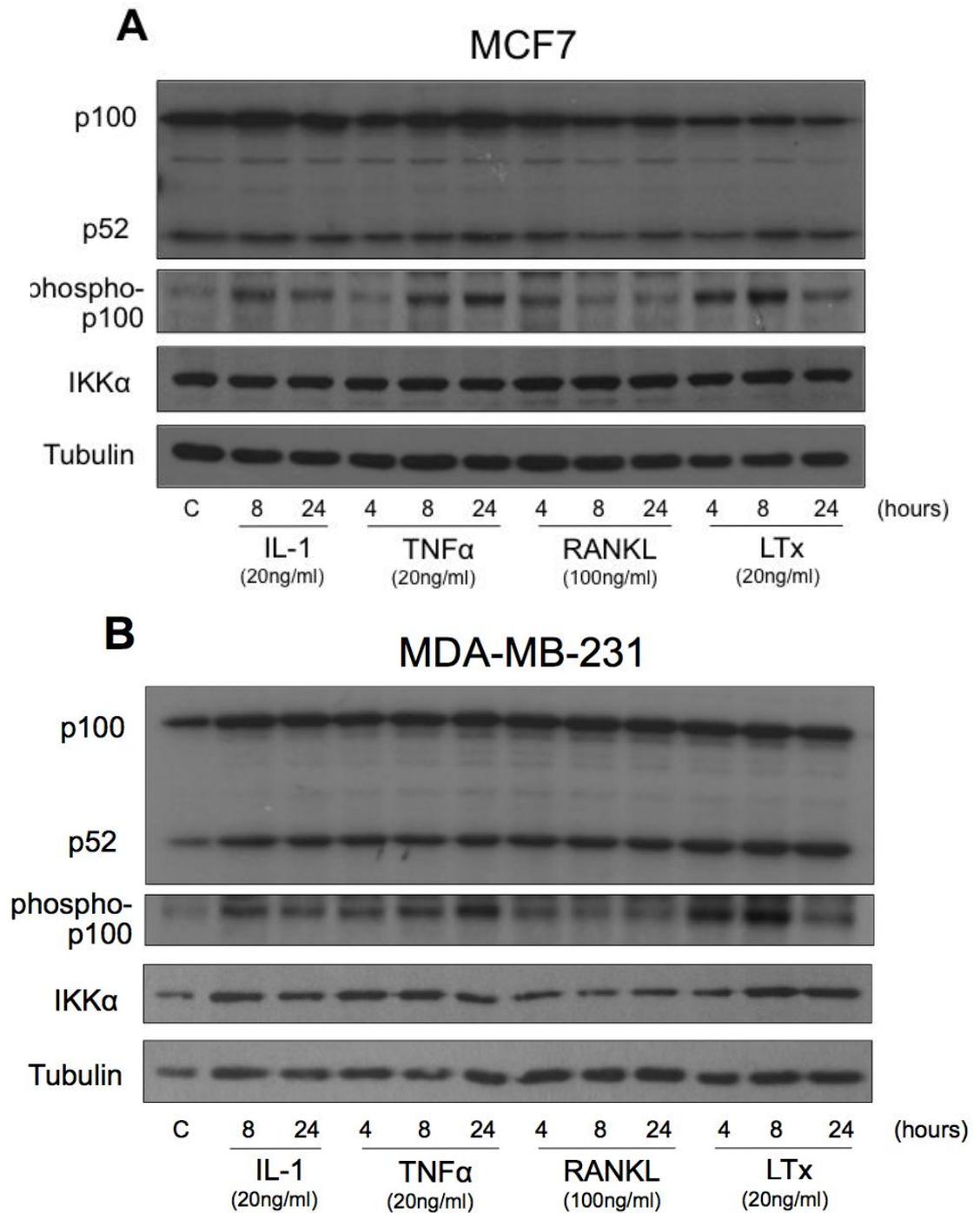


Figure 6.5: Expression of members of the non-canonical NF- κ B pathway in MCF7 and MDA-MB-231 breast cancer cell lines following TNF α , IL-1 RANK-L or lymphotoxin exposure. MCF7 and MDA-MB-231 cells were exposed to 20ng/ml of TNF α , IL-1 or lymphotoxin $\alpha_1\beta_2$ (LTx) or 100ng/ml RANK-L for various time periods (N=1). Protein was then extracted, separated by SDS-PAGE and assessed for expression of p100/p52, phosphorylated p100 and IKK α . β -tubulin was used as a loading control.

6.3.2 Lymphotoxin exposure and expression of components of the non-canonical NF- κ B pathway in MCF7 cells

Lymphotoxin was selected for stimulation of MCF7 and MDA-MB-231 cells in 3 separate experiments to allow quantification of expression levels of p100/p52 and phosphorylated p100. Cells were exposed to 20ng/ml lymphotoxin $\alpha_1\beta_2$ for various time periods before protein was extracted, separated by SDS-PAGE and expression of members of the pathway was visualised. Images were quantified using ImageJ and protein expression was normalised to the β -tubulin loading control. Differences in expression upon lymphotoxin stimulation were calculated using fold change to untreated cells.

Figure 6.6 shows a representative image of lymphotoxin stimulation in MCF7 cells and graphs showing the average fold change compared to untreated cells from the three experiments. On average lymphotoxin increased p52 expression 1.4 fold at 4 and 8 hours stimulation and 2.1 fold after 24 hours ($P=0.049$). Variable results were however observed and investigating the expression of p52 in the nucleus alone may achieve more reliable results. An increase in p100 phosphorylation was clearer in MCF7 cells. At 4 hours lymphotoxin incubation, phosphorylation of p100 increased on average 5.2 fold. A further increase was observed to around 6.8 fold compared to control cells after 8 hours ($P=0.024$). Phosphorylation of p100 was highest at 24 hours exposure with an average 8.8 fold increase in expression compared to unstimulated MCF7 cells ($P=0.005$). Levels of IKK α remained constant between unstimulated and stimulated cells.

6.3.3 Lymphotoxin exposure and expression of components of the non-canonical NF- κ B pathway in MDA-MB-231 cells

Figure 6.7 shows a representative image of three separate experiments investigating expression of p100/p52, phospho-p100 and IKK α in MDA-MB-231 cells. Constitutive expression of phospho-p100 is observed, with little different between control and stimulated cells. No increase in p52 is evident with lymphotoxin exposure. IKK α levels were unchanged by stimulation with lymphotoxin.

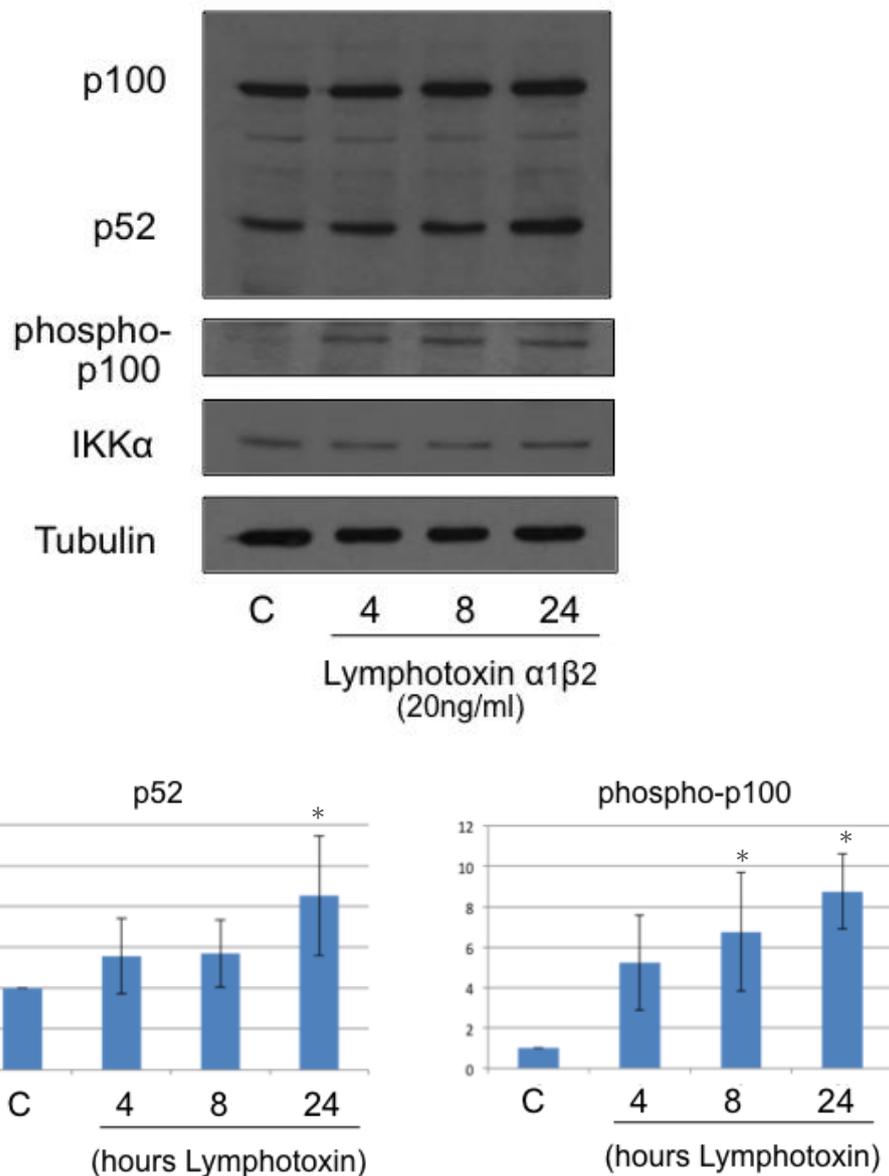


Figure 6.6: Expression of members of the non-canonical NF- κ B pathway in MCF7 breast cancer cell lines following lymphotoxin exposure. MCF7 cells were stimulated with 20ng/ml lymphotoxin $\alpha_1\beta_2$ (LTx) for various time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of p100/p52, phosphorylated p100 and IKK α . β -tubulin was used as a loading control. This is representative of 3 experiments. ImageJ was used to quantify expression of p100, p52, phospho-p100 and IKK α . Expression levels in cells exposed to lymphotoxin were compared to untreated cells, displayed as fold change to control in the bar graphs. Error bars represent standard deviation. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

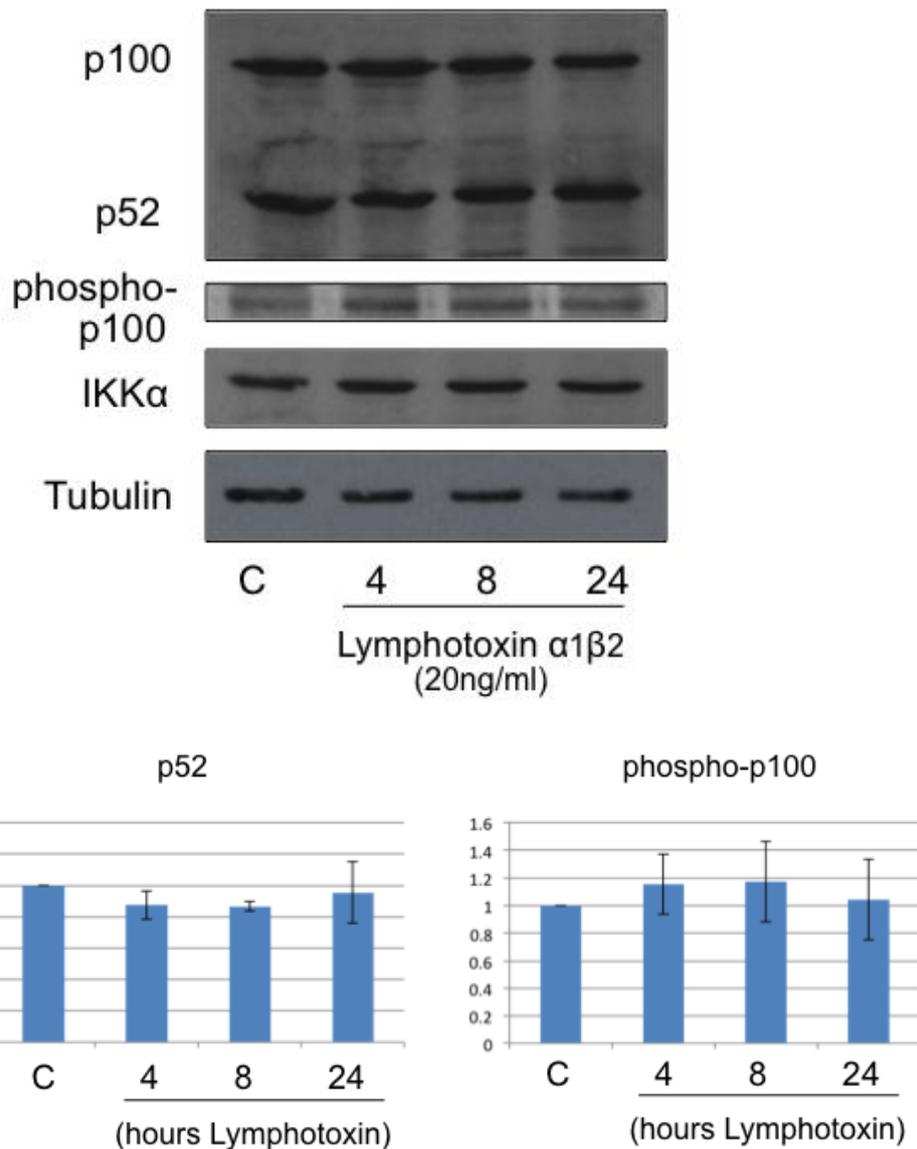


Figure 6.7: Expression of members of the non-canonical NF- κ B pathway in MDA-MB-231 breast cancer cell lines following lymphotoxin exposure. *MDA-MB-231 cells were stimulated with 20ng/ml lymphotoxin $\alpha_1\beta_2$ for various time periods. Protein was then extracted, separated by SDS-PAGE and assessed for expression of p100/p52, phosphorylated p100 and IKK α . β -tubulin was used as a loading control. This is representative of 3 experiments. ImageJ was used to quantify expression of p100, p52, phospho-p100 and IKK α . Expression levels in cells exposed to lymphotoxin were compared to untreated cells, displayed as fold change to control in the bar graphs. Error bars represent standard deviation. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. * signifies significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.*

6.4 Inhibition of IKK α and IKK β

In order to block activation of the canonical and non-canonical NF- κ B pathways, inhibition of IKK α and IKK β was explored in breast cancer cells by reducing expression or altering function.

6.4.1 siRNA silencing of IKK α and IKK β in MCF7 cells

Using a lipofection based method, small interfering RNA (siRNA) was delivered to the cells to reduce expression of IKK α or IKK β . To optimise the efficiency of the siRNA, different concentrations of siRNA were transfected into the cells and optimal duration was established using both 48 and 72 hours silencing. Non-targeting (NT) siRNA was used as a negative control to ensure observations were not due to the transfection procedure. Additionally, expression of the proteins after addition of lipofectamine alone was assessed. After silencing, protein was extracted from cells and protein expression assessed via western blotting.

Figure 6.8 shows expression of IKK α and IKK β in MCF7 cells after 48 hours (Figure 6.8A) and 72 hours (Figure 6.8B) silencing. At both 48 and 72 hours siRNA targeted to IKK α effectively reduced expression of IKK α at all concentrations. Similarly, reduction of IKK β was observed at all concentrations at both 48 hours and 72 hours after transfection with IKK β siRNA. Figure 6.8C shows the level of IKK α and IKK β 48 hours after transfection with 200nM siRNA. IKK α expression was reduced to 7.5% and IKK β to 4% of expression in untreated cells.

6.4.2 siRNA silencing of IKK α and IKK β in MDA-MB-231 cells

In 231 cells, expression of IKK α and IKK β was greatly reduced after 48 hours silencing at all concentrations (Figure 6.9). The graphs in Figure 6.9 depict levels of IKK α and IKK β 48 hours after transfection with 200nM siRNA. Levels of IKK α were reduced to less than 2% and IKK β expression following silencing was 3.6% of that in untreated cells.

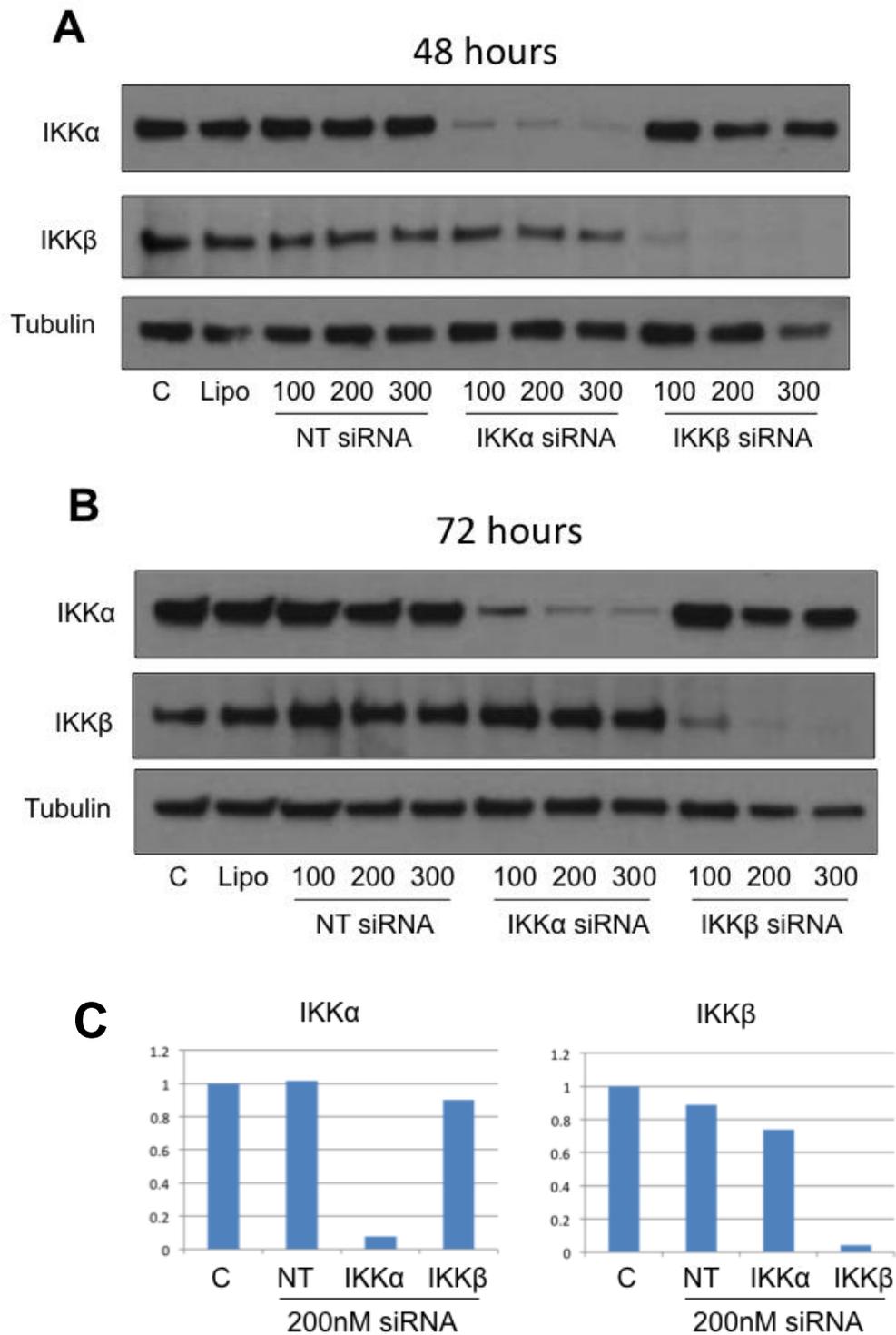


Figure 6.8: Expression of IKK α and IKK β after siRNA transfection in MCF7 cells. MCF7 cells were transfected with siRNA for IKK α and IKK β at various concentrations (100, 200 and 300nM). Non-targeting (NT) siRNA was used as a negative control. After 48 (A) or 72 (B) hours protein was extracted, separated by SDS-PAGE and assessed for expression IKK α and IKK β (N=1). β -tubulin was used as a loading control. (C) Fold change to control was calculated after images were quantified. Graphs show levels of IKK α or IKK β after 48 hours silencing with 200nM of siRNA (NT, IKK α or IKK β).

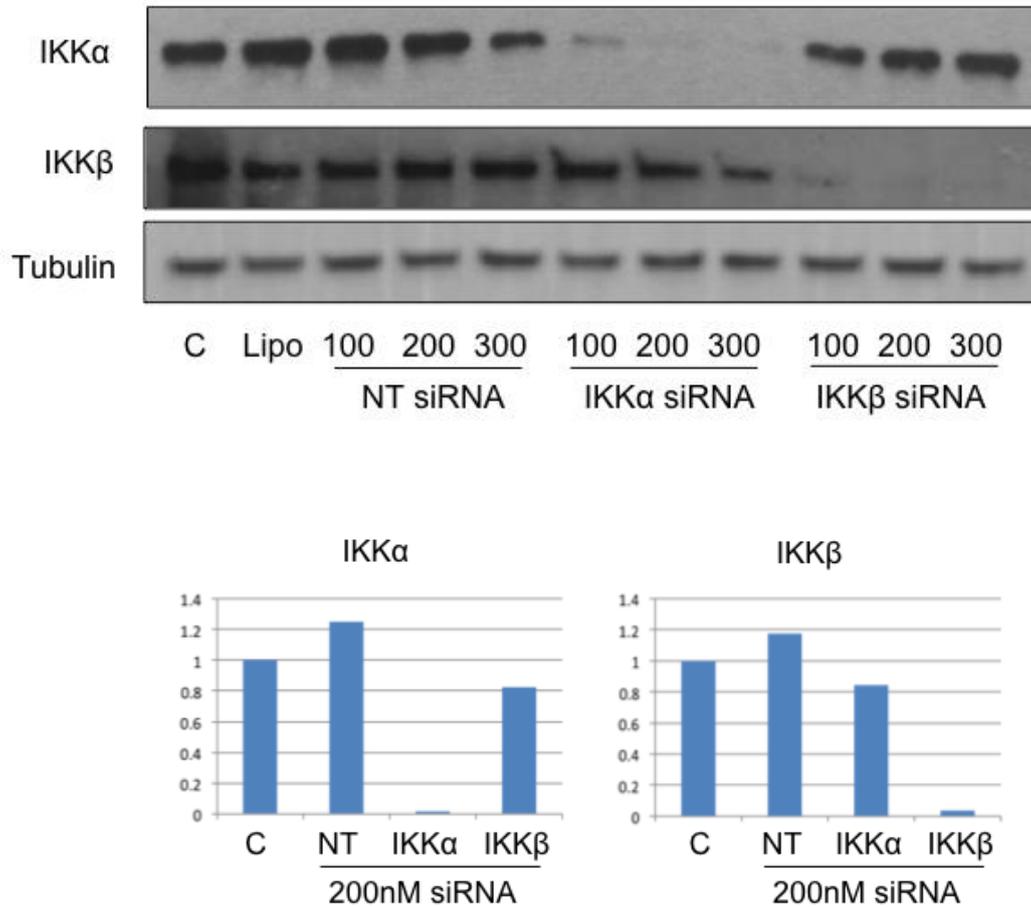


Figure 6.9: Expression of IKK α and IKK β after siRNA transfection in MDA-MB-231 cells. MDA-MB-231 cells were transfected with siRNA for IKK α and IKK β at various concentrations (100, 200 and 300nM). Non-targeting (NT) siRNA was used as a negative control. After 48 hours protein was extracted, separated by SDS-PAGE and assessed for expression IKK α and IKK β (N=1). β -tubulin was used as a loading control. Fold change to control was calculated after images were quantified. Graphs show levels of IKK α or IKK β after 48 hours silencing with 200nM of siRNA (NT, IKK α or IKK β).

6.4.3 Infection with Adv.DN-IKK β in MCF7 and MDA-MB-231 cells

Another method of manipulation of the pathway via inhibition of IKK function is the use of dominant negative (DN) versions of the kinases. DN-IKK β is catalytically inactive and therefore cannot function as a kinase and overexpression results in reduction in activation of the pathway. Adenovirus was used to deliver DN-IKK β to the cells. To establish the most appropriate multiplicity of infection (MOI) various concentrations were used, measured by plaque forming units (pfu). Adenovirus expressing β -galactosidase (β -gal) was used as a negative control. Figure 6.10 shows the expression of IKK β in MCF7 cells and MDA-MB-231 cells following infection with Adv.DN-IKK β .

In MCF7 cells, IKK β expression increased in a concentration dependent manner (Figure 6.10A). An increase of 2.6 fold compared to control was observed with 25 pfu, 2.9 fold with 50 pfu and 3.4 fold with 100 pfu. The largest increase was observed with 200 pfu with a 4.8 fold increase in IKK β expression compared to control MCF7 cells.

A concentration dependent increase was also observed with Adv.DN-IKK β infection in MDA-MB-231 cells (Figure 6.10B). Expression of IKK β increased only slightly (1.4 fold) with 25 pfu and (1.8 fold) with 50 pfu. Infection with 100 pfu Adv.DN-IKK β increased expression of IKK β 2.4 fold compared to control. The highest levels of IKK β were observed with 200 pfu of Adv.DN-IKK β , with an increase in expression of 2.9 fold compared to control MDA-MB-231 cells. Unfortunately due to time constraints and availability this was not able to be optimised for IKK α , therefore for all further experiments in which the pathways were inhibited used siRNA to silence IKK α or IKK β . This method should, however, be employed in studies continuing from this work to investigate the impact of loss of the IKK catalytic functions, while maintaining their scaffolding properties, more closely mimicking the use of inhibitors. It appears the breast cell lines used will be amenable to this form of manipulation and further experiments should be conducted using ligands to investigate the effects downstream.

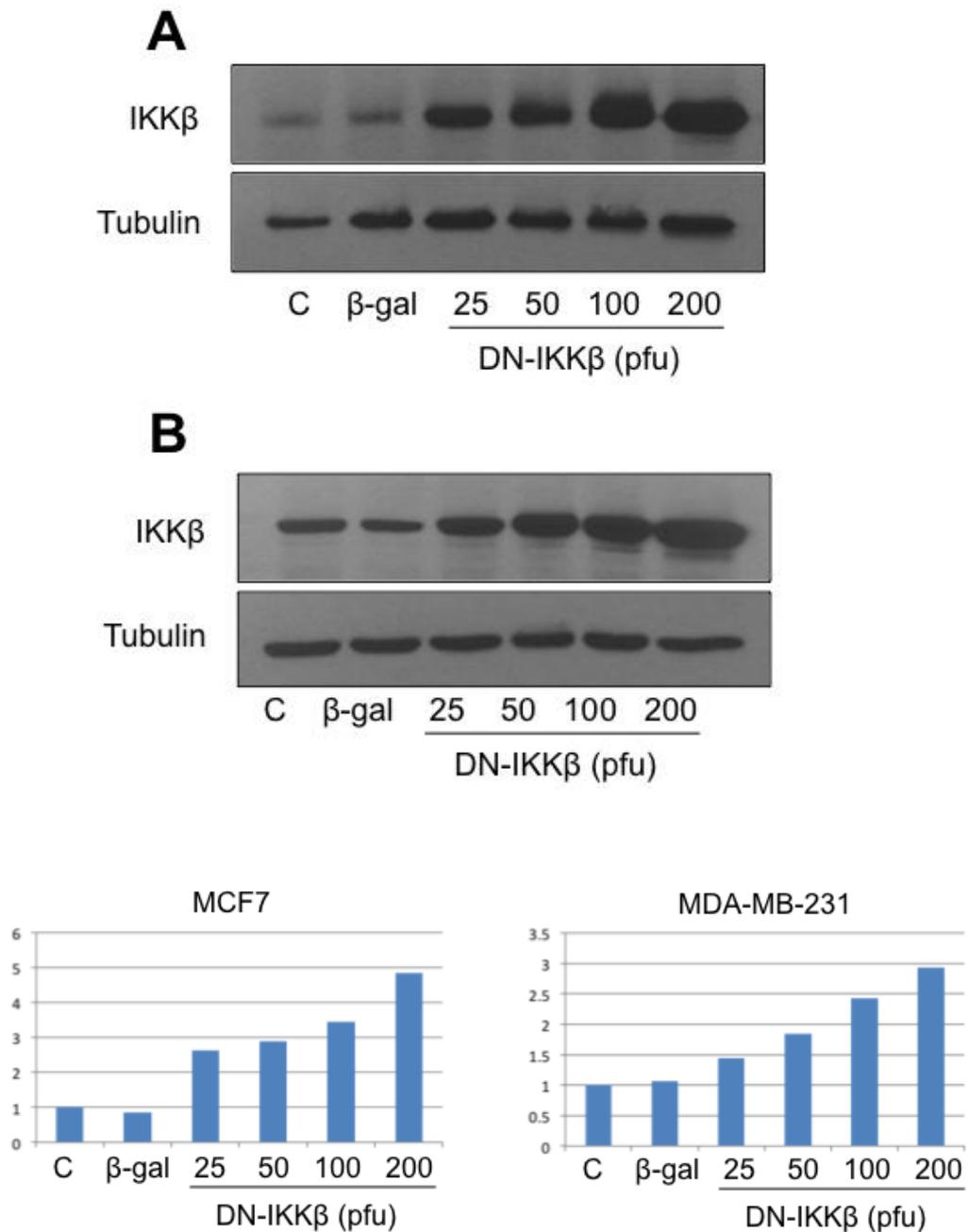


Figure 6.10: Expression of IKKβ in MCF7 and MDA-MB-231 cells infected with Adv.DN-IKKβ. MCF7 (A) and MDA-MB-231 (B) cells were infected with adenovirus with DN-IKKβ at various concentrations (25, 50, 100 and 200 pfu, N=1) for 40 hours. Infection with adenovirus with β-gal was used as a negative control. β-tubulin was used as a loading control. Fold change compared to control (shown in graphs) was calculated after images were quantified.

6.5 Effect of siRNA silencing of IKK α and IKK β upon gene expression in MCF7 cells.

Due to the problems reported with phospho-specific antibodies and difference observed between cohorts in levels of phospho-p65 from the canonical pathways and the lack of specific markers of activation of the non-canonical pathway, experiments were carried out to identify selective markers of these pathways. In order to compare gene expression between control samples and samples with silenced IKK α or IKK β and to attempt to establish selective downstream targets whose expression was altered following IKK α or IKK β silencing, quantitative real time PCR (qPCR) was used. MCF7 cells were treated with siRNA and RNA extracted, cDNA made and qPCR performed.

A panel of genes was selected as well as *CHUK* (IKK α) and *IKBKB* (IKK β) to confirm silencing of expression at the mRNA level. The panel of genes tested contained genes that have been demonstrated in other cancer models to be regulated by IKK α and/or IKK β , and one additional gene as a control that was reported to be IKK α and IKK β independent. Genes employed for this part of the study were *CCND1* (cyclin D1, previously found to be IKK α dependent), *NCOR2* (nuclear receptor corepressor 2, demonstrated to be IKK α dependent in other cancer models), *CXCL10* (chemokine CXC ligand 10, dependent on both IKK α and IKK β in previous studies), and *CRLF1* (chemokine receptor-like factor 1, independent of IKK α and IKK β). Levels of mRNA were normalised to those obtained for housekeeping gene *18S* and the comparative ($\Delta\Delta Ct$) method used to quantify relative gene expression compared to control and results are presented in fold change in the target gene relative to 18S endogenous control. (Fold Change = $2^{-\Delta(\Delta Ct)}$ where $\Delta Ct = Ct, target - Ct, 18S$ and $\Delta(\Delta Ct) = \Delta Ct, stimulated - \Delta Ct, control$). A 2 fold change compared to control was considered as a relevant change in expression.

Figure 6.10 shows gene expression in control cells and cells treated with NT, IKK α and IKK β siRNA. Silencing of IKK α and IKK β were confirmed at the mRNA level. Using siRNA for IKK α resulted in a decrease in mRNA expression levels of *CHUK* to 28% of control cells and siRNA for IKK β resulted in a decrease in expression of *IKBKB* to 46% of that in control cells. A greater efficiency in silencing was therefore achieved with IKK α siRNA than IKK β . In IKK β silenced cells, mRNA expression of IKK α increased over 8 fold, suggesting a compensatory role in the absence of IKK β . However, this was not confirmed at the protein level as no increase in IKK α protein is observed in lysates from IKK β silenced cells (Figure 6.8). No increase in IKK β mRNA expression was observed in IKK α silenced cells.

Levels of *CCND1* were increased 2.8 fold in $\text{IKK}\alpha$ silenced cells and therefore a significant increase in gene expression was observed. In $\text{IKK}\beta$ silenced cells this only increased 1.8 fold and cyclin D1 therefore appears to be a more $\text{IKK}\alpha$ dependent marker than $\text{IKK}\beta$. Expression of *NCOR2* was decreased to 45% following $\text{IKK}\alpha$ silencing and to 78% in $\text{IKK}\beta$ silenced samples, however levels in cells treated with NT siRNA were also decreased to 35% fold compared to control and therefore therefore it is not possible to comment using these results if *NCOR2* is a $\text{IKK}\alpha$ and/or $\text{IKK}\beta$ dependent marker in breast cancer. Therefore this experiment is required to be performed again before any conclusion could be drawn.

CXCL10 levels increased 15.4 fold compared to controls following $\text{IKK}\beta$ silencing and to 5.6 fold in $\text{IKK}\alpha$ silenced MCF7 cells. This target is therefore not selective for either $\text{IKK}\alpha$ or $\text{IKK}\beta$ but is affected by expression of both of these IKKs, although more strongly with $\text{IKK}\beta$. Levels of *CRLF1* were not significantly altered in $\text{IKK}\alpha$ or $\text{IKK}\beta$ silenced cells with 0.98 and 1.4 respective changes observed, therefore confirming previous observations from other cancer models investigated within our laboratory.

Unfortunately, this experiment was only completed once and only MCF7 cells were investigated and therefore no conclusions can be drawn from this. Several repeat experiments are required to ensure reliable and reproducible results are achieved and confirm if any are $\text{IKK}\alpha$ or $\text{IKK}\beta$ downstream targets in breast cancer cells. However this does highlight that the markers identified in prostate cancer are not applicable in breast cancer and it appears that further work using a different method exploring more targets is required to identify $\text{IKK}\alpha$ and $\text{IKK}\beta$ selective markers in breast cancer.

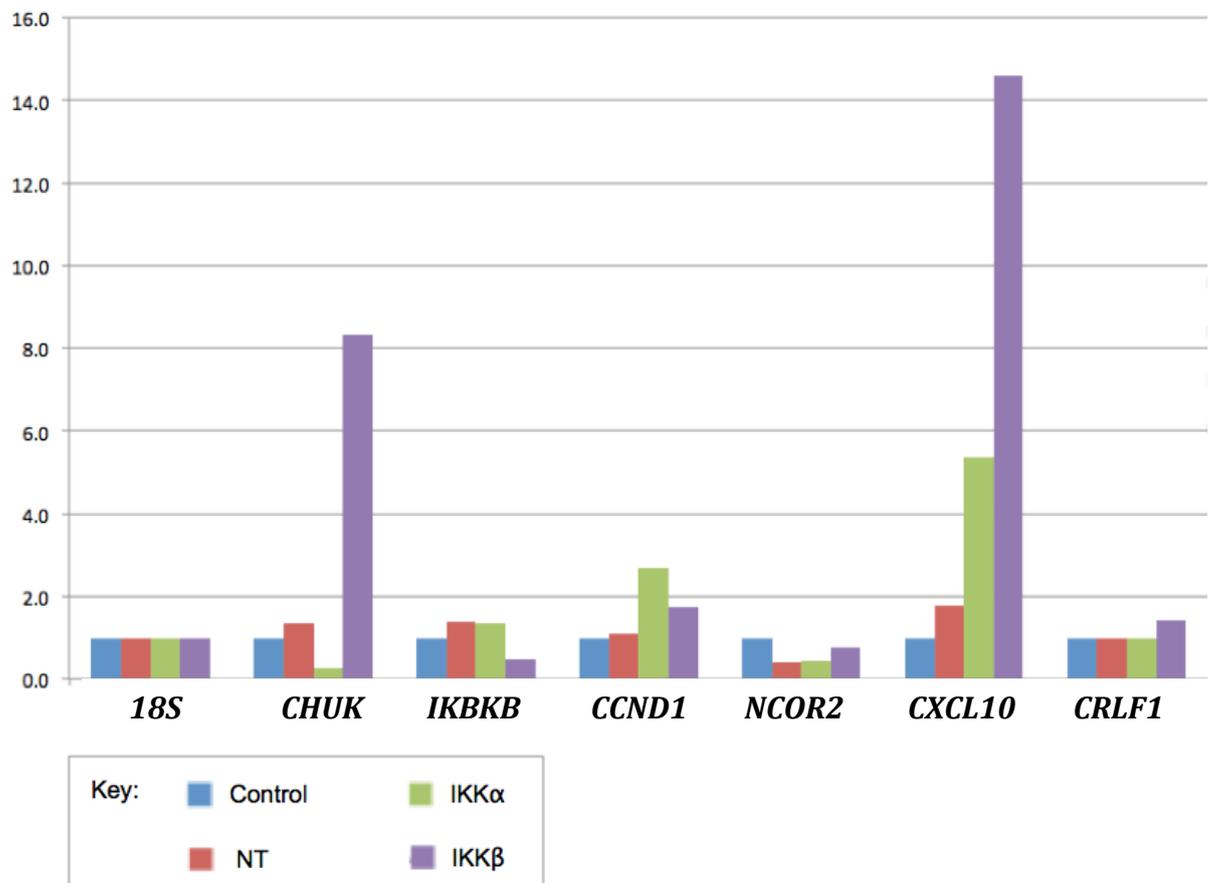


Figure 6.11: Expression of IKK α and IKK β after siRNA transfection in MCF7 cells. MCF7 cells were transfected with 200nM siRNA for IKK α and IKK β (N=1). Non-targeting (NT) siRNA was used as a negative control. After 48 hours RNA was extracted, and used to make cDNA. Quantitative Real Time-PCR (qPCR) was then performed to assess gene expression of IKK α (CHUK), IKK β (IKKB) as well as four other genes, CCND1, NCOR2, CXCL10 and CRLF1. Levels of mRNA were normalised to those obtained for housekeeping gene 18S and the comparative ($\Delta\Delta C_t$) method used to quantify relative gene expression compared to control.

6.6 Discussion

Data from the clinical cohort suggests a role for the NF- κ B pathways in the progression of breast cancer with differences in ER positive and negative tumours evident; *in vitro* studies were therefore performed to investigate expression of members of the NF- κ B pathways in ER positive MCF7 cells and ER negative MDA-MB-231 cells.

Expression of members of the canonical NF- κ B pathway (I κ B α , p65 and its phosphorylation status at serine 536, IKK β and IKK α) were evaluated in the breast cancer cell lines by measuring protein levels via Western blotting and changes in expression following exposure to TNF α calculated. In MCF7 cells, TNF α exposure stimulated activation of the pathway, as demonstrated by degradation of I κ B α and an increase in p65 phosphorylation at serine 536. MDA-MB-231 ER negative cells were observed to have constitutive activation of the canonical NF- κ B pathway with high levels of p65 phosphorylation in untreated cells. This is in agreement with other studies that have reported elevated levels of NF- κ B DNA binding activity in ER negative cells in comparison to ER positive cells [142]. Additionally, cellular location of p65 and phosphorylated p65 was examined using IHC on cell pellets and nuclear translocation was observed upon TNF α exposure. Although this is the required approach for validation of antibodies used in IHC due to cells being subject to formalin fixation, similar to the tissue cohorts, alternative approaches may be more appropriate for localisation studies. In hindsight immunocytochemistry, in which the cells are not subject to the same processing as in cell pellets, may have been a better method to select to study the localisation of the p65 subunit following TNF α exposure. Other methods that could have been employed include immunofluorescence and Western blotting of fractionated protein samples.

Similar results were observed with the non-canonical NF- κ B pathway with induction of phosphorylation of p100 observed in MCF7 cells following exposure to lymphotoxin $\alpha_1\beta_2$ and high levels demonstrated in MDA-MB-231 cells even before stimulation. Wang *et al* reported elevated levels of RelB, another component of the non-canonical NF- κ B pathway, in a panel of ER negative breast cancer cells including MDA-MB-231 [184]. A difference in ER positive and ER negative cells is therefore evident for both canonical and non-canonical NF- κ B activity.

Expression of IKK α and IKK β was then silenced in both MCF7 and MDA-MB-231 cells and levels of both kinases assessed to test efficacy. Silencing reduced expression of IKK α and IKK β at all concentrations in both MCF7 and MDA-MB-231 cells. Silencing was confirmed at the mRNA level with expression of *CHUK* (IKK α) and *IKBKB* (IKK β) in MCF7 cells.

Transfection with siRNA was therefore efficient at silencing IKK α and IKK β expression and optimal conditions were established.

Expression of a panel of genes was also assessed in MCF7 cells using qPCR in an endeavour to identify selective downstream markers including *CCND1*, *CXCL10*, *CRFL1* and *NCOR2*. Several reports have suggested cyclin D1, a protein involved in the G1/S transition in the cell cycle, as a target of NF- κ B [185-186]. Expression of *CCND1*, which encodes for the cyclin D1 protein, was altered to a greater extent following IKK α silencing and to a lesser extent with IKK β silencing. In knock-in mice where IKK α has serine residues that ordinarily undergo phosphorylation during activation of the pathway replaced with alanine, NF- κ B activation was found to be defective and cyclin D1 expression was reduced [121]. Additionally, IKK α has been reported to regulate cell cycle through cyclin D1 expression in association with Wnt signalling [120]. This supports a role for both canonical and non-canonical NF- κ B regulation of cyclin D1 expression as observed by the present study. As the literature suggests that IKK α induces cyclin D1 expression, it would have been expected that cyclin D1 would be reduced when IKK α was silenced. This was however not what was observed in this initial experiment or in studies in other cancer models in the host laboratory. Further experiments are however required to confirm our findings. In the host laboratory a gene card experiment using prostate cancer cell lines identified *CCND1* as IKK α dependent but this does not appear to have translated to our breast cancer work as both IKK α and IKK β were observed to influence *CCND1* expression, albeit to much lesser extent with IKK β compared to IKK α .

CXCL10, also known as Interferon gamma-induced protein 10 or eotaxin, is a chemokine whose expression has been reported to be induced by pro-inflammatory stimuli such as TNF α through activation of NF- κ B [187]. *CXCL10* expression has been demonstrated to be IKK β , but not IKK α , dependent [188]. *CXCL10* levels increased over 15 fold compared to controls following IKK β silencing and to over 5 fold in IKK α silenced MCF7 cells. It was therefore found this target is not selective for either IKK α or IKK β . Again this gene was chosen as in the host laboratory this was observed to be IKK β dependent in prostate cancer cells. This has again not been translated into this initial experiment using breast cancer cell lines, although expression of *CXCL10* appears to be affected to a greater degree by IKK β absence.

Expression of *CRLF1*, which has to our knowledge not been reported to be associated with IKK α or IKK β and was found to be independent of both in previous studies using prostate cancer cell lines, was assessed as an additional control. Expression was not significantly altered in IKK α or IKK β silenced cells. The transcriptional coregulatory protein SMRT (also known as NCOR2) is phosphorylated by IKK α , contributing to its dissociation from chromatin. IKK β was, however, not associated with derepression of SMRT [118]. Expression of *NCOR2* decreased significantly following IKK α silencing but not IKK β silencing and would therefore appear to be an IKK α selective marker. Levels in cells treated with NT siRNA were however decreased compared to control and repeated experiments are required. Although only conducted once and in one cell line, this preliminary study does however show that the markers identified as specific in prostate cancer are not likely to translate to breast cancer and therefore other methods to look at larger panels of genes should be explored in future studies.

Although silencing was found to be efficient in reducing levels of IKK α and IKK β , one potential problem with siRNA is off targeting effects where silencing of partially complementary transcripts occurs. In order to overcome this ON-TARGET plus which has position-specific sequence-independent chemical modifications to reduce off target effects was employed [189]. Additionally, compensation was evident between IKK α and IKK β . When IKK β was silenced in MCF7 cells, qPCR showed mRNA levels of IKK α increased over 8 fold. Furthermore, western blots show TNF α and IL-1 β were able to induce phosphorylation of p100, therefore demonstrating IKK β driven induction of the non-canonical NF- κ B pathway. The role of IKK α and IKK β appears to not solely involve IKK β in the canonical and IKK α in the non-canonical NF- κ B pathway, with compensation between both kinases apparent. IKK α has been demonstrated to play a compensatory role in the canonical NF- κ B pathway upon IKK β inhibition in a subtype of B-cell lymphoma [190]. Another method of inhibition of the IKKs is to instead use adenovirus-mediated delivery of dominant negative mutants of IKK α and IKK β to inhibit activation of the pathways. This method allows the protein to maintain scaffolding function but as these mutants are catalytically inactive, they are unable to perform as a kinase and induction of the pathway is therefore blocked. Degradation of I κ B α following TNF α exposure was inhibited in HUVEC cells following expression of DN-IKK β [111]. Expression of DN-IKK α resulted in inhibition of p100 processing and a reduction in p52 formation [191]. The use of DN isoforms of IKK α and IKK β therefore appear to prevent activation of NF- κ B signalling and may offer an alternative approach to inhibition in breast cancer cells. In both MCF7 and MDA-MB-231 cells infected with DN-IKK β adenovirus, overexpression of IKK β was observed and the breast cancer cells

therefore appear to be responsive to this method of inhibition.

It has therefore been demonstrated that breast cancer cells, particularly ER positive MCF7 cells, are amenable to manipulation of the NF- κ B pathways. This was achieved either by exposure to ligands to drive expression of family members or by inhibiting expression of the kinases IKK α and IKK β which are required for activation of the pathways. This will be further explored in the next chapter to investigate the impact of stimulating or inhibiting the NF- κ B pathways on cell growth and viability.

In conclusion, a difference in the NF- κ B pathways is observed between ER positive and ER negative breast cancer in these *in vitro* studies assessing the expression of certain members of the canonical and non-canonical pathway. In ER positive MCF7 cells degradation of I κ B α and phosphorylation of p65 (as markers of activation of the canonical pathway) and phosphorylation of p100 (as a marker of activation of the non-canonical pathway) were inducible by exposure to ligands that stimulate these pathways. In ER negative MDA-MB-231 cells activation was observed in untreated cells indicating constitutive activation of these pathways.

Chapter 7:

Phenotypic impact of stimulating or inhibiting the NF- κ B pathways in breast cancer cell lines

7.1 Introduction

As discussed previously, we investigated the role of proliferation and apoptosis in breast cancer clinical cohorts and found both to be associated with clinical outcome. In addition, we observed that high expression of the canonical and non-canonical pathways are associated with poorer clinical outcome. Furthermore, the previous chapter has demonstrated that breast cancer cell lines are amenable to manipulation of NF- κ B signalling.

We hypothesised that high expression of the pathways would be associated with poorer clinical outcome and that inhibition of the IKKs would reduce the viability of breast cancer cells. In order to address these hypotheses and explore the potential of the IKKs as novel targets in breast cancer, siRNA was employed to knock down expression of both kinases and the effect on cell viability and apoptosis was assessed. Due to differences in clinical outcome measures observed in the canonical and non-canonical NF- κ B pathways between ER negative and positive breast cancer, and when investigating the signalling of these pathways in ER negative and positive breast cancer cell lines, differences in results between ER negative MDA-MB-231 cells and ER positive MCF7 cells were compared.

7.2 Impact of stimulation of the canonical and non-canonical pathways on cell growth and viability

The NF- κ B pathways regulate genes involved in several aspects of cancer development and progression including cell death and growth [104]. In order to explore the consequence of activation of the pathways, cells were rendered quiescent in serum free media before stimulation of the canonical or non-canonical pathways by exposure to TNF α or lymphotoxin respectively. Using several assays the impact of activation of the pathways on cell growth and viability was assessed.

7.2.1 Assessment of apoptosis in breast cancer cells following stimulation of the NF- κ B pathways

Apoptosis levels were measured using a commercially available ELISA kit to detect cell death after stimulation. This one step sandwich ELISA quantifies levels of DNA fragmentation. The amount of dye, which is proportional to the amount of DNA fragmentation, is then measured using a spectrophotometer and the levels of apoptosis calculated.

Again, cells were exposed to 20ng/ml of TNF α or lymphotoxin for various lengths of time (24, 48 and 72 hours) and the ELISA to detect cell death was then performed. Cells were incubated in the vehicle used to deliver the ligand (distilled water for TNF α and PBS with

0.1% BSA for lymphotoxin) as a negative control to ensure this was not responsible for any effects observed. Statistical analysis was performed on the results from three experiments using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels.

7.2.1.1 Apoptosis in MCF7 cells following stimulation of the NF- κ B pathways

TNF α stimulation increased apoptosis in ER positive MCF7 cells after 24 hours incubation ($P=4.2 \times 10^{-4}$, Figure 7.1A). However 48 and 72 hours TNF α incubation did not significantly alter apoptosis levels ($P=0.065$ and $P=0.237$ respectively, Figure 7.1A). Incubation in the vehicles did not affect the number of apoptotic cells. Lymphotoxin significantly increased apoptosis in MCF7 cells at all incubation periods examined (24 hours $P=6.0 \times 10^{-6}$, 48 hours $P=0.008$, 72 hours $P=3.0 \times 10^{-6}$, Figure 7.1B).

As an additional control for the assay conditions, apoptosis was measured in cells incubated in full serum media for 24 hours, 48 hours and 72 hours following serum starvation. Full serum media rescued the cells from cell death and a decrease in apoptosis was observed at all time periods (24 hours $P=4.0 \times 10^{-6}$, 48 hours $P=6.6 \times 10^{-8}$, 72 hours $P=1.4 \times 10^{-7}$, Figure 7.1C).

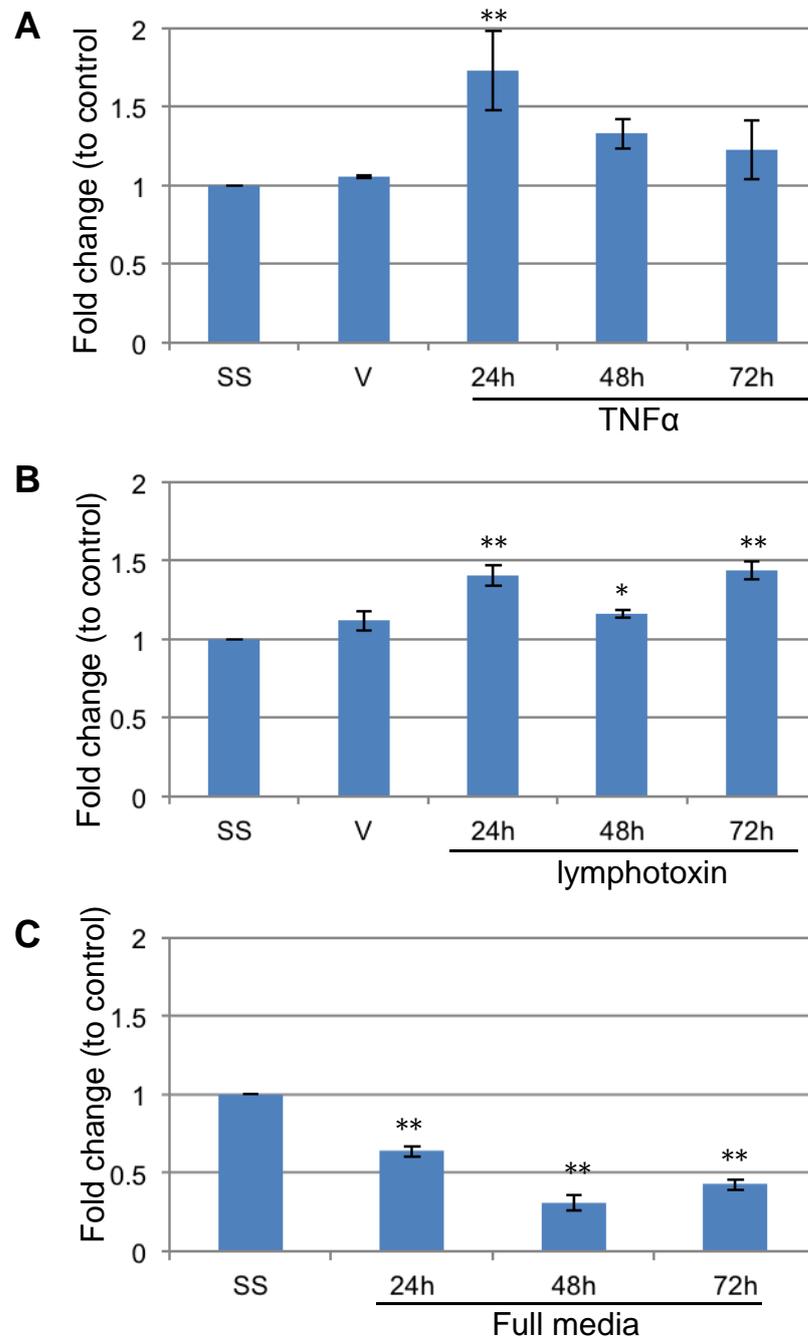


Figure 7.1: Apoptosis in MCF7 cells following stimulation of the canonical and non-canonical NF- κ B pathways. Levels of apoptosis were measured using a Cell Death Detection ELISA kit. Cells were quiesced in serum free media then incubated in 20ng/ml TNF α (A), 20ng/ml lymphotoxin $\alpha_1\beta_2$ (B) or full serum media (C) before the apoptosis assay was performed. SS = serum starved, V = vehicle (distilled water for TNF α and PBS with 0.1% BSA for lymphotoxin). Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.2.1.1 Apoptosis in MDA-MB-231 cells following stimulation of the NF- κ B pathways

In ER negative MDA-MB-231 cells TNF α did not induce apoptosis at any time point (24 hours P=0.973, 48 hours P=1.0, 72 hours P=0.369, Figure 7.2A). Similarly, incubation in lymphotoxin did not affect apoptosis levels in these cells (24 hours P=0.125, 48 hours P=1.0, 72 hours P=0.57, Figure 7.2B). Incubation in either vehicle (distilled water or PBS with 0.1% BSA) did not affect apoptosis. To rescue the cells from the level of apoptosis observed with serum starvation cells were treated with incubation in full media, but 24 hours incubation did not affect levels of apoptosis (P=1.0, Figure 7.2C). However, by 48 hours and 72 hours (both P<1.0x10⁻⁶) full media reduced the number of apoptotic cells.

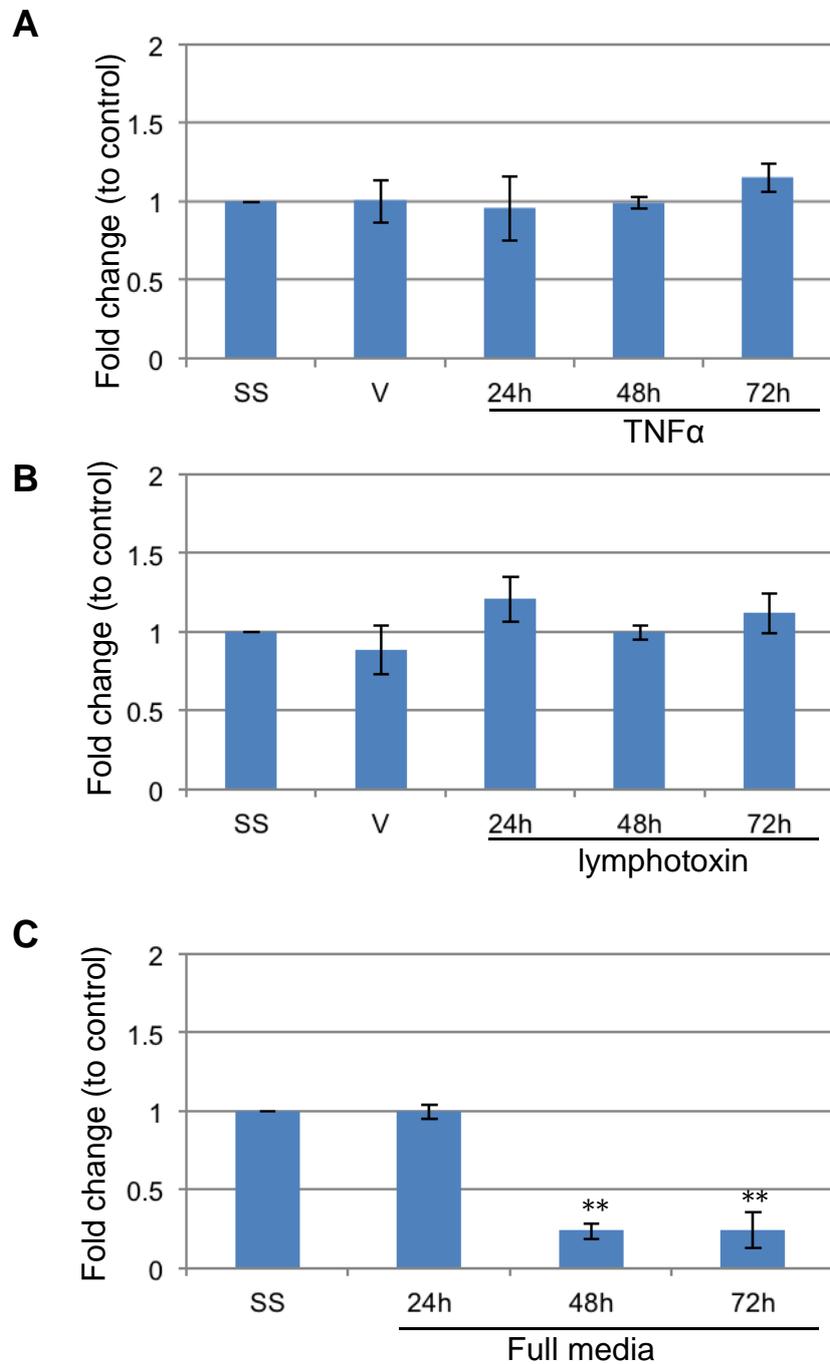


Figure 7.2: Apoptosis in MDA-MB-231 cells following stimulation of the canonical and non-canonical NF- κ B pathways. Levels of apoptosis were measured using a Cell Death Detection ELISA kit. Cells were quiesced in serum free media then incubated in 20ng/ml TNF α (A), 20ng/ml lymphotoxin $\alpha_1\beta_2$ (B) or full serum media (C) before the WST assay was performed. SS = serum starved, V = vehicle (distilled water for TNF α and PBS with 0.1% BSA for lymphotoxin). Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.2.2 Assessment of viability in breast cancer cells following stimulation of the NF- κ B pathways by WST-1

Cell viability was assessed using a WST-1 based assay. This colourimetric assay is based on cleavage of WST-1 to formazan. More activity of mitochondrial dehydrogenases, which corresponds to the number of viable cells, results in an increase in formation of formazan dye. The amount of dye is then measured using a spectrophotometer and the fold change compared to the control was calculated.

Cells were treated with 20ng/ml of TNF α or lymphotoxin to stimulate the canonical or non-canonical NF- κ B pathway and then the WST-1 assay performed. Additionally, as a negative control cells were incubated in the vehicle used to deliver the ligand (distilled water for TNF α and PBS with 0.1% BSA for lymphotoxin). Experiments were repeated three times to allow statistical analysis using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels.

7.2.1.1 Cell viability, by WST-1, in MCF7 cells following stimulation of the NF- κ B pathways

In MCF7 cells, TNF α exposure did not impact on cell viability at any of the time points examined (24 hours P=0.316, 48 hours P=0.673, 72 hours P=0.972, Figure 7.3A). Incubation in lymphotoxin for 24 hours resulted in a slight increase in viability (P=0.020, Figure 7.3B) but 48 hours and 72 hours incubation did not significantly alter viability (P=0.051 and P=0.052 respectively, Figure 7.3B). Incubation in either vehicle did not affect cell viability. As an additional control for the assay conditions, viability was measured in cells incubated in growth media containing full serum for 24 hours, 48 hours and 72 hours following serum starvation. Incubation in growth media containing serum for 24 hours significantly enhanced cell viability (P=0.001, Figure 7.3C). A time dependent increase was observed, with highly significant increase in viability at 48 hours and 72 hours incubation (P=1.2x10⁻⁵ and P=1.0x10⁻⁶, respectively, Figure 7.3C).

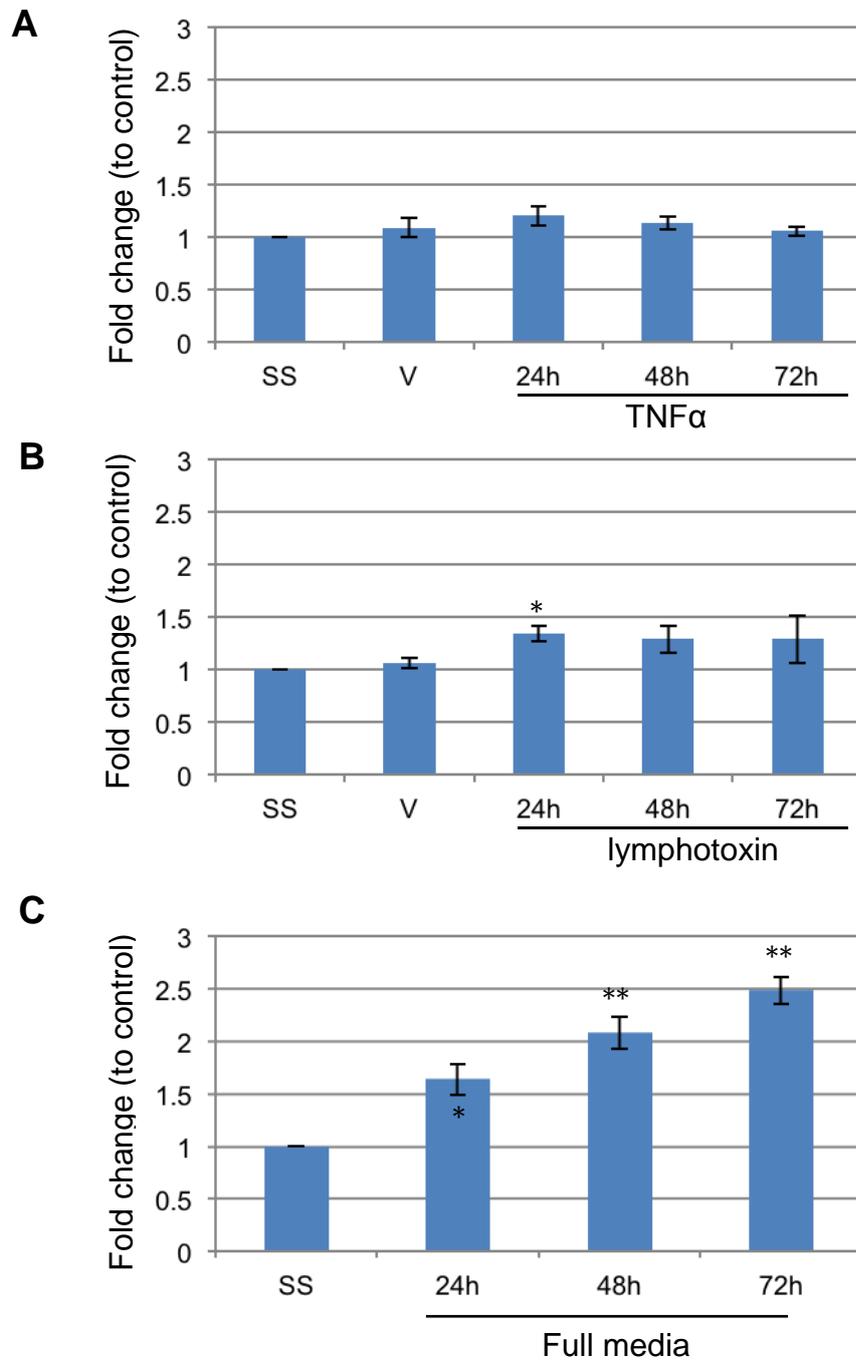


Figure 7.3: Cell viability, assessed by WST-1, in MCF7 cells following stimulation of the canonical and non-canonical NF- κ B pathways. Cell viability was measured using a WST-1 assay. Cells were quiesced in serum free media then incubated in 20ng/ml TNF α (A), 20ng/ml lymphotoxin $\alpha_1\beta_2$ (B) or full serum media (C) before the WST assay was performed. SS = serum starved, V = vehicle (distilled water for TNF α and PBS with 0.1% BSA for lymphotoxin). Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.2.2.2 Cell viability, by WST-1, in MDA-MB-231 cells following stimulation of the NF- κ B pathways

TNF α exposure increased cell viability in MDA-MB-231 cells after 24 hours and 48 hours incubation ($P=1.0 \times 10^{-6}$ and $P=3.0 \times 10^{-6}$, respectively, Figure 7.4A). 72 hours TNF α incubation did not significantly alter cell viability ($P=0.998$, Figure 7.4A). However, incubation in the vehicle (distilled water) also increased cell viability ($P=0.002$, Figure 7.4A). Incubation in the lymphotoxin vehicle (PBS with 0.1% BSA) did not affect cell viability ($P=0.824$, Figure 7.4B) and lymphotoxin did not significantly affect viability in MDA-MB-231 cells at any of the incubation periods examined (24 hours $P=0.359$, 48 hours $P=0.234$, 72 hours $P=0.995$, Figure 7.4B). As an additional control for the assay conditions, viability was measured in cells incubated in full serum media for 24 hours, 48 hours and 72 hours following serum starvation. Incubation in growth media containing full serum for 24 hours did not significantly impact on cell viability ($P=0.180$, Figure 7.4C). An increase in viability was observed at 48 hours and 72 hours incubation in full serum media (both $P=0.001$, Figure 7.4C).

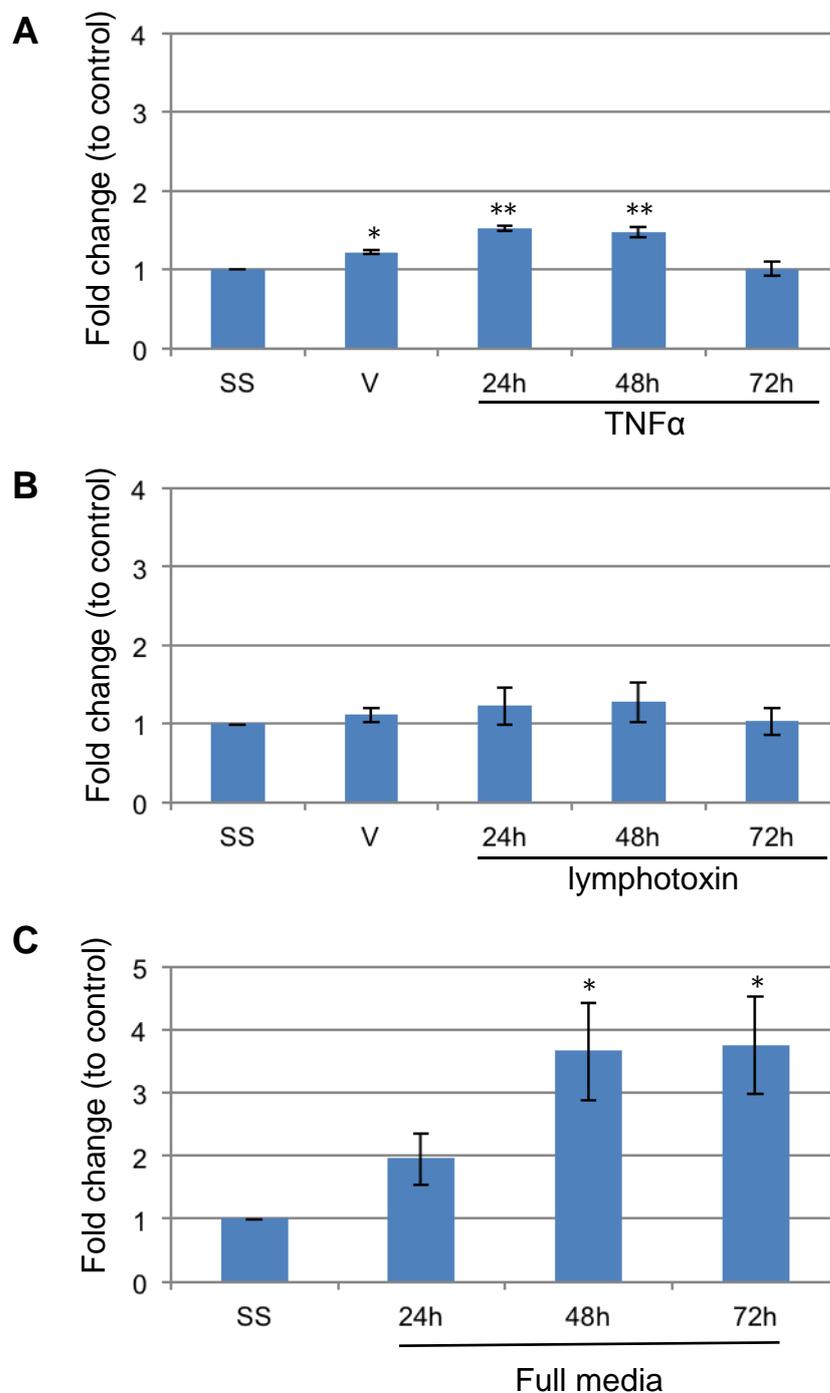


Figure 7.4: Cell viability, assessed by WST-1, in MDA-MB-231 cells following stimulation of the canonical and non-canonical NF- κ B pathways. Cell viability was measured using a WST-1 assay. Cells were quiesced in serum free media then incubated in 20ng/ml TNF α (A), 20ng/ml lymphotoxin $\alpha_1\beta_2$ (B) or full serum media (C) before the WST assay was performed. SS = serum starved, V = vehicle (distilled water for TNF α and PBS with 0.1% BSA for lymphotoxin). Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.2.3 Assessment of cell viability in breast cancer cells following stimulation of the NF- κ B pathways using xCELLigence

The xCELLigence machine was used to display cell growth and viability in real time [192]. Using special plates with microelectrodes, the impedance of each well was measured continuously in a non-invasive manner, allowing real time growth curves to be plotted. Measurements are produced using “Cell Index” which represents the number of viable cells in each well. Cells were serum starved for 24 hours before exposure to 20ng/ml TNF α /lymphotoxin or serum. The cell index for each condition was normalised at 0 hours treatment and experiments were continued 72 hours post treatment. For each treatment triplicate wells were included and each experiment was repeated independently to an N=2.

7.2.3.1 Cell viability, by xCELLigence, in MCF7 cells following stimulation of the NF- κ B pathways

Results from xCELLigence show that lymphotoxin did not affect cell viability in MCF7 cells (Figure 7.5B). TNF α treatment, however, resulted in reduced cell viability from 24 hours post treatment, which continued to decrease at 48 and 72 hours incubation (Figure 7.5A). As expected, MCF7 cells incubated in media containing 10% serum had increased viability in comparison to cells that were deprived of serum.

7.2.3.2 Cell viability, by xCELLigence, in MDA-MB-231 cells following stimulation of the NF- κ B pathways

Neither TNF α nor lymphotoxin appear to affect cell viability in MDA-MB-231 cells (Figure 7.6A and Figure 7.6B). Incubation in media containing 10% serum greatly increased viability of MDA-MB-231 cells in comparison to cells that were deprived of serum.

Results obtained from the different assays upon stimulation of the pathways with ligands appear to be variable. However, as expected, serum induced proliferation and reduced apoptosis resulting in increased cell viability. This was consistent across all assays for both MCF7 and MDA-MB-231 cells. Hence, comparison between cells that were grown in normal full serum media and those that had been starved of serum was used as a control for further assays.

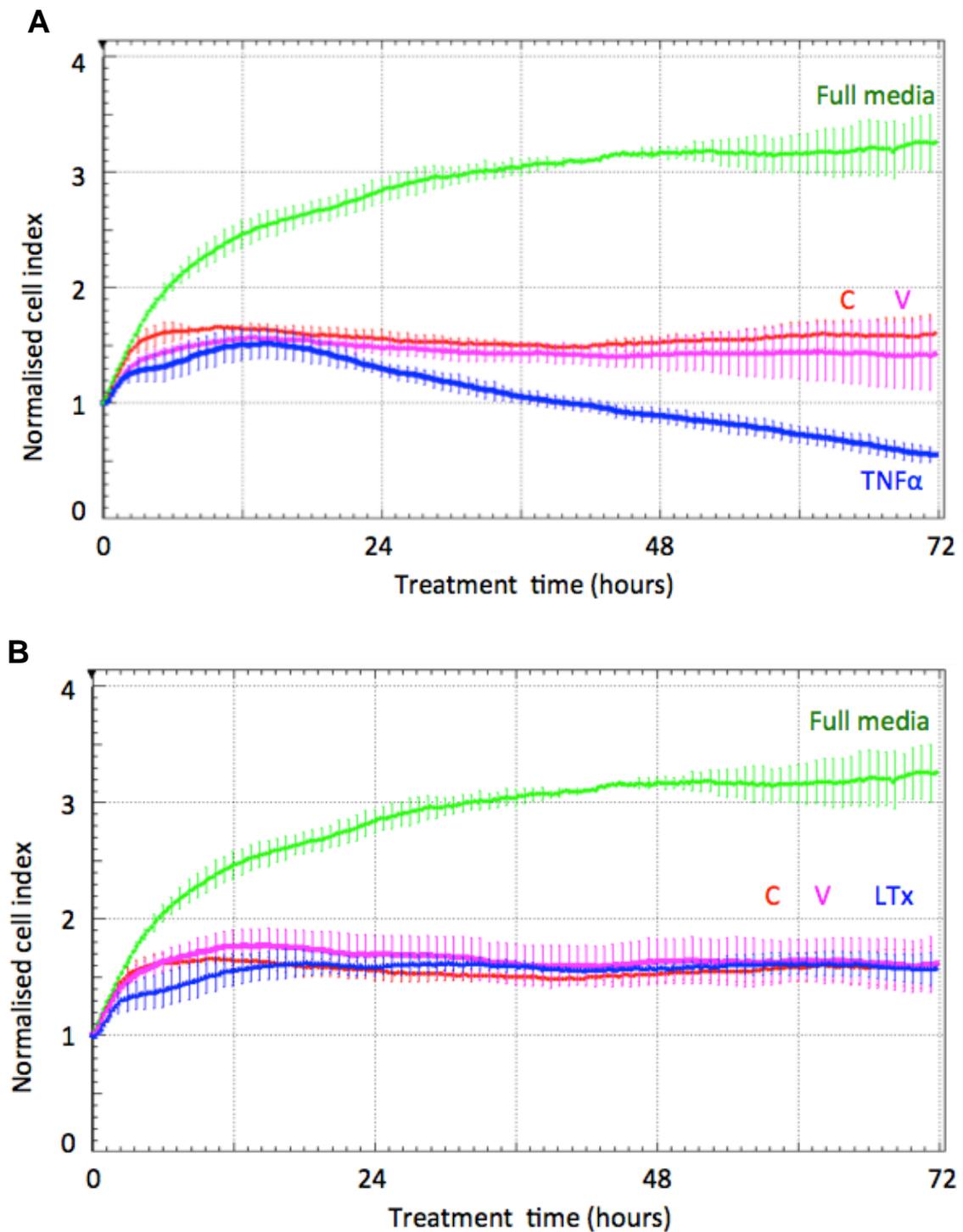


Figure 7.5: Cell viability in MCF7 cells following stimulation of the canonical and non-canonical NF- κ B pathways. The xCELLigence was used to measure cell viability (expressed as cell index) and generate real time plots of these cells following stimulation of the NF- κ B pathways. Cells were quiesced in serum free media then incubated in 20ng/ml TNF α (A), 20ng/ml lymphotoxin $\alpha_1\beta_2$ (B). Media with 10% serum (full media) was used as a positive control. V = vehicle (distilled water for TNF α and PBS with 0.1% BSA for lymphotoxin). Graphs shown are representative of two separate experiments.

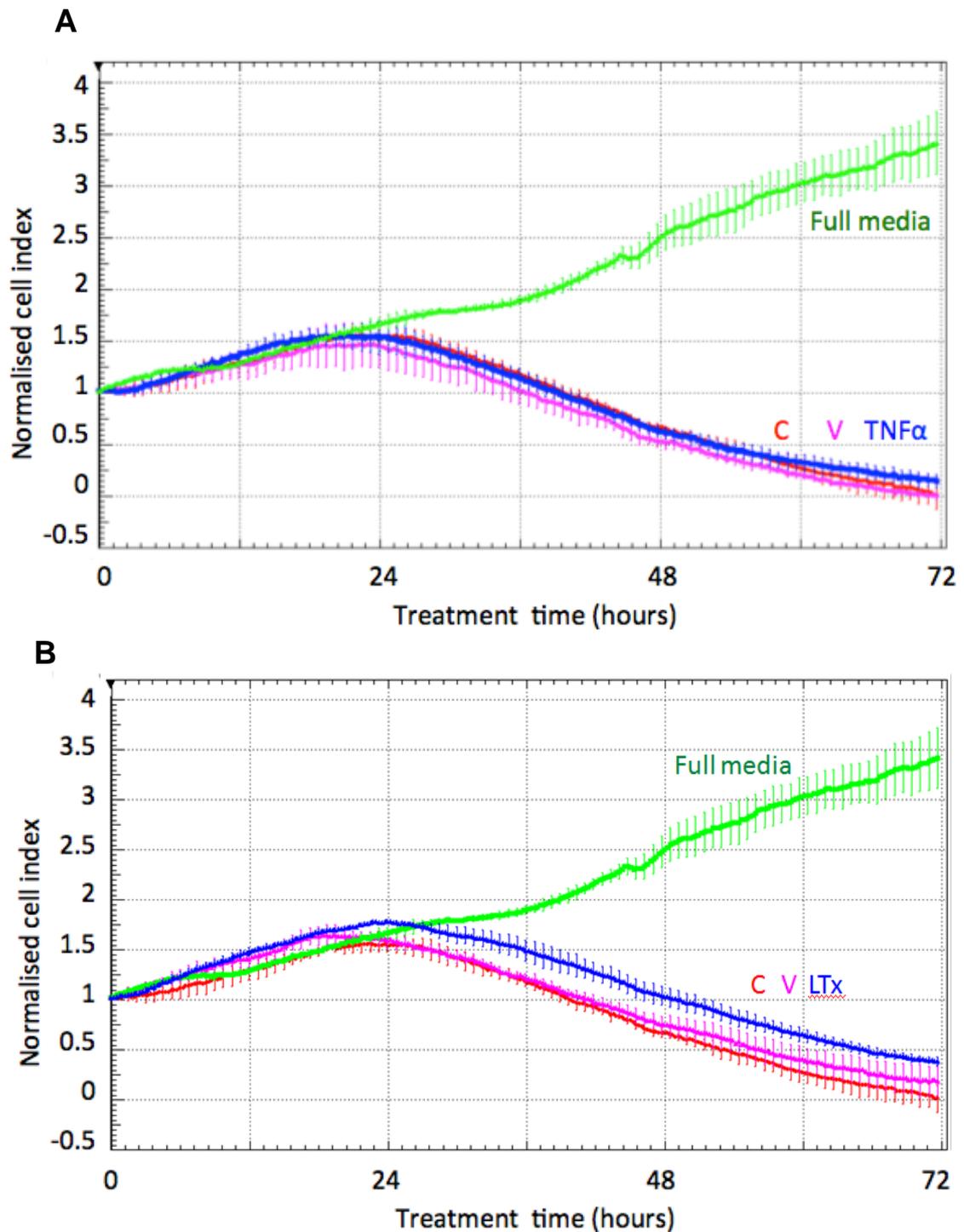


Figure 7.6: Cell viability in MDA-MB-231 cells following stimulation of the canonical and non-canonical NF- κ B pathways. The xCELLigence was used to measure cell viability (expressed as cell index) and generate real time plots of these cells following stimulation of the NF- κ B pathways. Cells were quiesced in serum free media then incubated in 20ng/ml TNF α (A), 20ng/ml lymphotoxin $\alpha_1\beta_2$ (B). Media with 10% serum (full media) was used as a positive control. V = vehicle (distilled water for TNF α and PBS with 0.1% BSA for lymphotoxin). Graphs shown are representative of two separate experiments.

7.3 Impact of silencing the IKKs on cell growth and viability

To examine the effect of inhibition of the IKKs and further explore these as targets in breast cancer, we used RNA interference to knock down expression of both the kinases to observe the effect on cell growth and viability. Optimal conditions for siRNA transfection were previously established by visualisation of protein expression using western blotting, as discussed in chapter 6.

7.3.2 Assessment of apoptosis in breast cancer cells following silencing of IKK α and IKK β

Levels of apoptosis were again assessed using an ELISA kit, which measures DNA fragmentation. MCF7 and MDA-MB-231 cells were transfected with 200nM IKK α and IKK β siRNA, as well as NT siRNA as a negative control, and 48 hours post treatment the apoptosis assay was performed. Serum starvation, which as previously described induces apoptosis, was used as an additional control.

7.3.2.1 Apoptosis in MCF7 cells following silencing of IKK α and IKK β

ER positive MCF7 cells treated with NT siRNA (as a negative control) did not show a significant difference in apoptosis when compared to untreated control cells ($P=0.992$, Figure 7.9). A significant increase was observed in levels of apoptosis when cells were treated with siRNA to silence expression of IKK α and IKK β . Compared to control cells, IKK α silencing resulted in a 5 fold increase on average ($P=2.1 \times 10^{-5}$, Figure 7.7). Silencing IKK β increased apoptosis almost 7 fold ($P=1.0 \times 10^{-6}$, Figure 7.7). Additionally, consistent with previous findings, serum starvation significantly induced apoptosis resulting in over 2 fold increase compared to untreated cells ($P=0.038$, Figure 7.7).

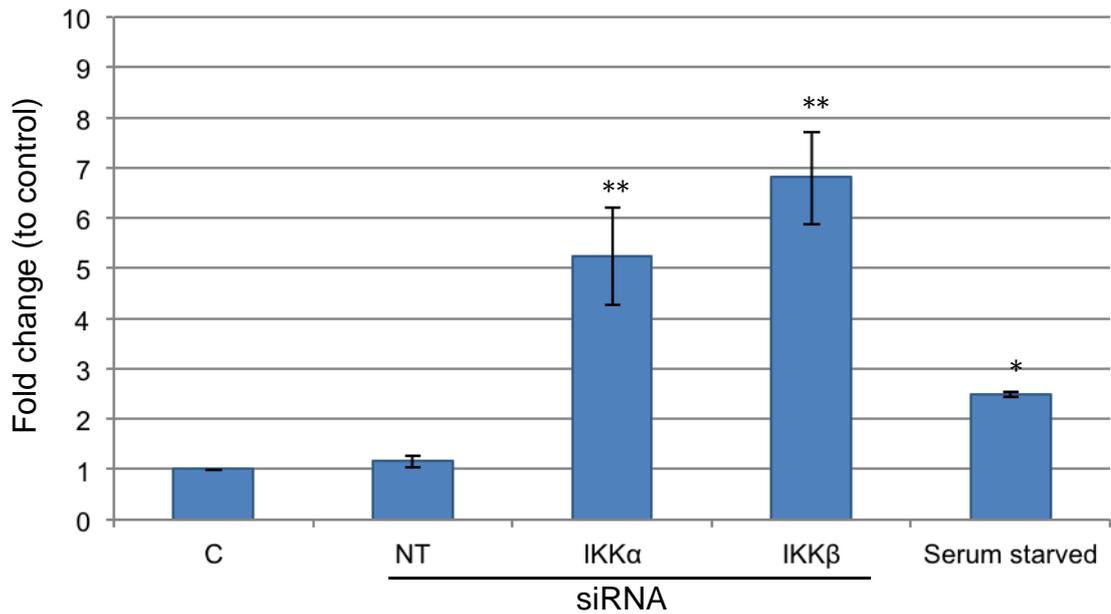


Figure 7.7: Apoptosis in MCF7 cells following silencing of IKK α or IKK β . Using a lipofection based method, ER positive MCF7 cells were transfected with 200nM siRNA for 48 hours and the ELISA for cell death was then performed to assess apoptosis levels. NT = non-targeting siRNA, negative control. As an internal assay control, cells were serum starved to induce apoptosis. Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.3.2.2 Apoptosis in MDA-MB-231 cells following silencing of IKK α and IKK β

ER negative MDA-MB-231 cells treated with NT siRNA (as a negative control) did not show a significant difference in apoptosis when compared to untreated control cells ($P=1.0$, Figure 7.8). A significant increase was observed in levels of apoptosis when cells were treated with siRNA to silence expression of IKK β with an almost 5 fold increase on average compared to untreated cells ($P=2.1 \times 10^{-4}$, Figure 7.8). Silencing IKK α expression, however, did not significantly affect levels of apoptosis ($P=0.999$, Figure 7.8). As expected, serum starvation significantly induced apoptosis resulting in an 8 fold increase compared to untreated cells ($P=1.0 \times 10^{-6}$, Figure 7.8).

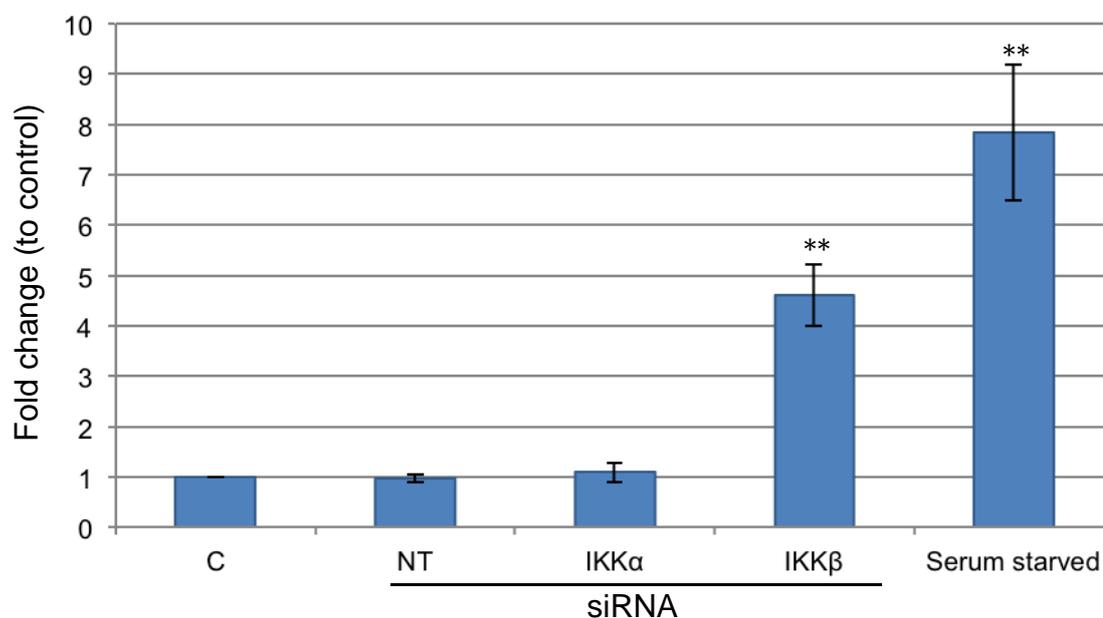


Figure 7.8: Apoptosis in MDA-MB-231 cells following silencing of IKK α or IKK β . Using a lipofection based method, ER negative MDA-MB-231 cells were transfected with 200nM siRNA for 48 hours and the ELISA for cell death detection was then performed to assess apoptosis levels. NT = non-targeting siRNA, negative control. As an internal assay control, cells were serum starved to induce apoptosis. Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * shows significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.3.1 Assessment of cell viability in breast cancer cells following silencing of IKK α and IKK β using WST-1

Similar to stimulation experiments, proliferation was assessed via a WST-1 assay. Non-targeting siRNA (NT) was used as a negative control to ensure results obtained were not due to the transfection procedure. Cells were treated with 200nM of siRNA for 48 hours before the assay was performed, and confirm via western blot that a decrease in expression had been achieved. Experiments were repeated to an N=3 to allow statistical analysis of the data.

7.3.1.1 Cell viability, by WST-1, in MCF7 cells following silencing of IKK α and IKK β

In MCF7 cells treated with NT siRNA (as a negative control), cell viability were not significantly different to untreated control cells ($P=0.773$, Figure 7.9). A decrease in cell viability was observed when cells were treated with siRNA to silence expression of IKK α and IKK β ($P=0.001$ and $P=6.8 \times 10^{-5}$ respectively, Figure 7.9). Additionally, consistent with previous findings, serum starvation significantly reduced cell viability compared to untreated control cells ($P=1.0 \times 10^{-6}$, Figure 7.9).

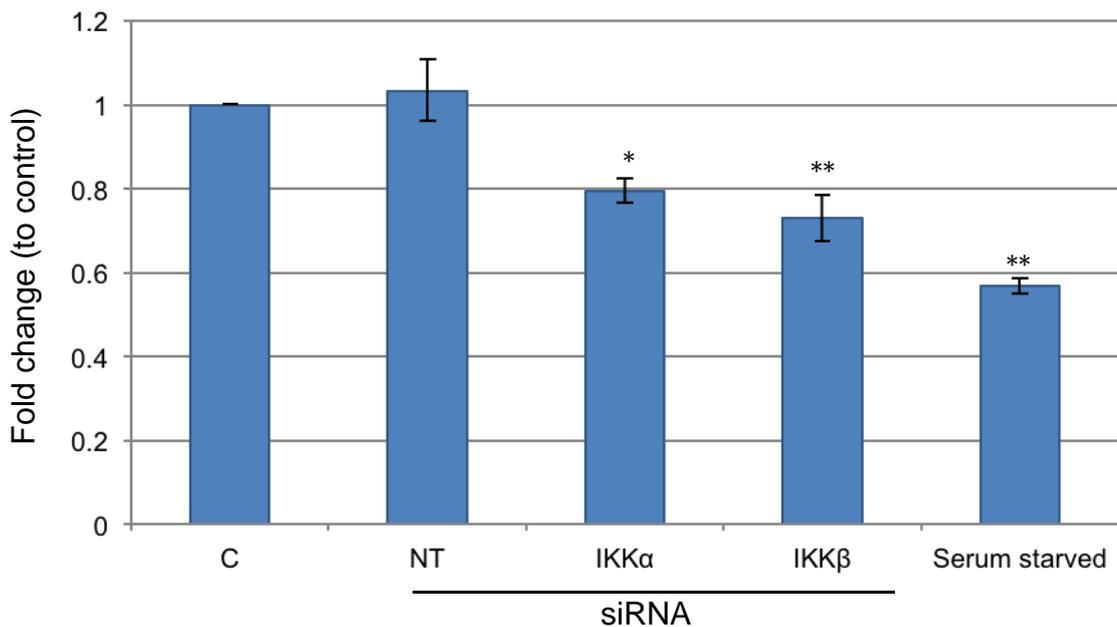


Figure 7.9: Cell viability, assessed by WST-1, in MCF7 cells following silencing of IKK α or IKK β . Using a lipofection based method, ER positive MCF7 cells were transfected with 200nM siRNA for 48 hours and the WST assay was then performed to assess viability. NT = non-targeting siRNA, negative control. As an internal assay control, cells were serum starved to inhibit proliferation. Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * signifies significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.3.1.2 Cell viability, by WST-1, in MDA-MB-231 cells following silencing of IKK α and IKK β

In ER negative MDA-MB-231 cells, cell viability were not significantly different in cells treated with NT siRNA than untreated control cells ($P=0.954$, Figure 7.10). A decrease in cell viability was observed when cells were treated with siRNA to silence expression of IKK β ($P=0.002$, Figure 7.10) but not IKK α ($P=0.872$, Figure 7.10). Additionally, consistent with previous findings, serum starvation significantly reduced cell viability compared to untreated control cells ($P=1.0 \times 10^{-6}$, Figure 7.10).

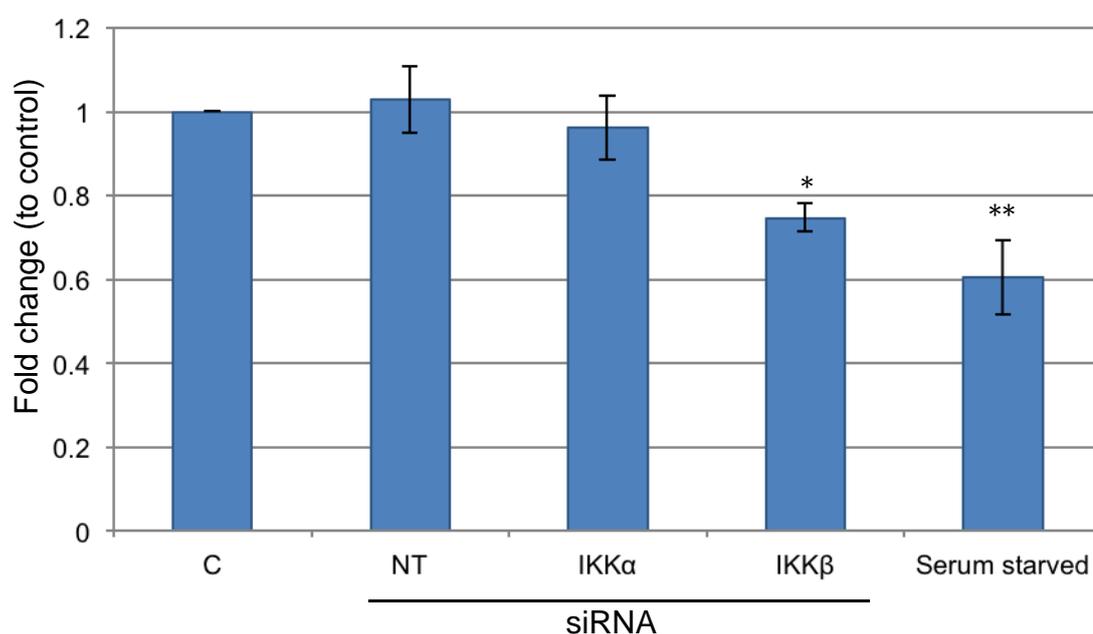


Figure 7.10: Cell viability, assessed by WST-1, in MDA-MB-231 cells following silencing of IKK α or IKK β . Using a lipofection based method, ER negative MDA-MB-231 cells were transfected with 200nM siRNA for 48 hours and the WST assay was then performed to assess viability. NT = non-targeting siRNA, negative control. As an internal assay control, cells were serum starved to inhibit proliferation. Within each experiment an internal triplicate (3 wells per condition) was included and each experiment was completed independently 3 times. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction and Dunnett's test to compare to control levels. Error bars represent standard deviation. * signifies significant difference of $P < 0.05$. ** indicates highly significant differences $P < 0.001$.

7.3.3 Assessment of cell viability in breast cancer cells following silencing of IKK α and IKK β using xCELLigence

Cell viability was also measured using the xCELLigence machine, as previously described. MCF7 and MDA-MB-231 cells were seeded in special plates with electrodes on the bottom and real time viability measurements recorded. Cells were treated with 200nM siRNA targeting either IKK α or IKK β . NT (non-targeting) siRNA was used as a negative control to ensure observations were not due to the transfection procedure. Media was replaced after 8 hours and cell index was normalised at this point. Real time plots were generated to 80 hours post-treatment. Although this was only performed once, the results are consistent with those obtained with the WST-1 viability assay and therefore add to the evidence of the role of these kinases in cell viability in the different ER backgrounds.

7.3.3.1 Cell viability, by xCELLigence, in MCF7 cells following silencing of IKK α and IKK β

Cell viability plots following silencing in MCF7 cells is shown in Figure 7.11A. Cell viability in cells transfected with NT siRNA, employed as a transfection control, did not appear to be different to that observed for untreated cells. Both IKK α and IKK β siRNA reduced cell viability in MCF7 cells.

7.3.2.2 Cell viability, by xCELLigence, in MDA-MB-231 cells following silencing of IKK α and IKK β

Figure 7.11B shows cell viability in MDA-MB-231 cells following treatment with siRNA. Cells transfected with NT or IKK α siRNA did not display any difference in cell viability to untreated cells. IKK β siRNA, however, reduced cell viability in MDA-MB-231 cells.

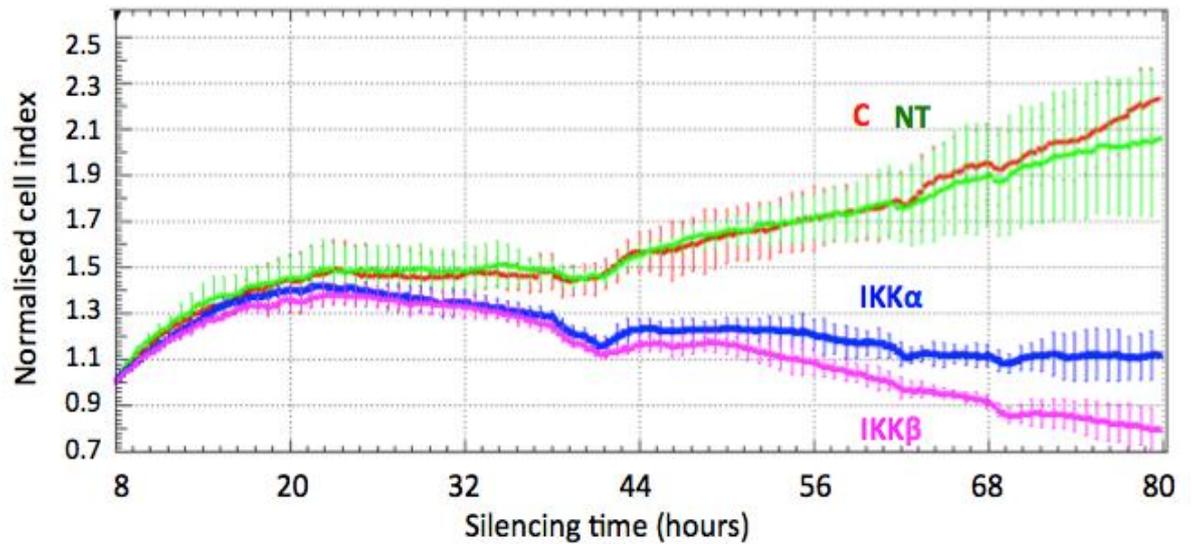
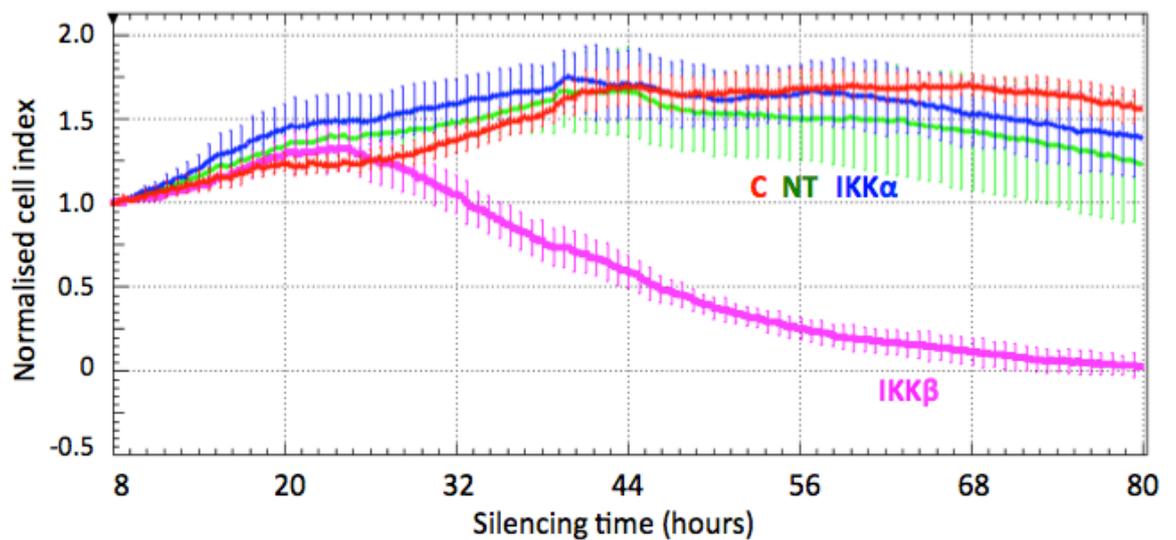
A**B**

Figure 7.11: Cell viability, measured using xCELLigence, in breast cancer cell lines following silencing of IKK α or IKK β . The xCELLigence machine was used to measure cell viability and growth in real time in both ER positive MCF7 cells (A) and ER negative MDA-MB-231 cells (B). Cells were treated with 200nM siRNA to silence either IKK α or IKK β . NT (non-targeting) siRNA was used as a negative control to ensure observations were not due to the transfection procedure. Media was replaced after 8 hours, results normalised and real time plots generated to 80 hours post-treatment. $N=1$.

7.4 Discussion

Decreased cell growth and viability can reflect either a reduction in proliferation rates or an increase in apoptosis, or a combination of both mechanisms. Cell viability was measured through various methods including assessment of apoptosis using an ELISA, viability using a WST-1 assay real time growth and viability using xCELLigence. As observations from the clinical tissue implicated members of the pathway in the progression of ER positive breast cancer, the effect of stimulating the pathway or inhibiting activation through silencing of the relevant kinases was examined. Encouraging results were obtained when IKK α and IKK β were silenced suggesting these are possible targets for exploitation in treatment of breast cancer.

Variable results were obtained when ligands were applied to stimulate activation of the canonical and non-canonical pathways. Incubation with TNF α slightly induced apoptosis after 24 hours in ER positive MCF7 cells and a reduction in cell viability was observed when xCELLigence technique was employed. However no change was observed using the WST-1 assay at this time point. In ER negative MDA-MB-231 cells, TNF α increased viability slightly at 24 hours and 48 hour time points. Statistical analysis implied viability was also increased in the cells treated with vehicle. Although this effect was minimal, this however means that these results should be interpreted with caution. However, when the xCELLigence technique was employed no difference in cell viability between control cells or cells treated with vehicle or TNF α in MDA-MB-231 cells was observed. Although decreased viability is observed in MCF7 cells following exposure to TNF α , activation of NF- κ B is associated with suppression of apoptosis. The role of TNF α in the cell is complex as it has been demonstrated to both induce apoptosis via activation of caspases and to aid cell survival by stimulation of expression of anti-apoptotic genes via NF- κ B [193-194]. Therefore, although TNF α is often thought of as a ligand that induces apoptosis, it has a dual function and in the correct conditions can in fact inhibit apoptosis via NF- κ B. The anti-apoptotic role of NF- κ B was first observed in p65 knockout mice, which die due to hepatic apoptosis mid-gestation [109]. Further work has demonstrated activation of NF- κ B induces the transcription of anti apoptotic genes such as X-linked inhibitor of apoptosis (XIAP), and overexpression of XIAP has been reported to protect cells from TNF α induced apoptosis as part of a feedback mechanism [193]. A complex relationship between TNF α and cell survival and apoptosis is therefore evident and it is likely that the reduction in cell viability that we observe in our experiments follow TNF α treatment is due to activation of an alternative pathway and may be completely independent of NF- κ B.

Lymphotoxin was used to induce activation of the non-canonical pathway, as demonstrated in chapter 6. Incubation with lymphotoxin for 24 hours marginally increased both cell viability and apoptosis in MCF7 cells at all time points examined, although it is questionable if the levels observed would in fact have an overall effect as they were so small. No difference, however, was observed between control MCF7 cells or cells treated with the vehicle or lymphotoxin using the xCELLigence method. In MDA-MB-231 cells lymphotoxin did not affect cell viability or apoptosis in any of the assays used. Results obtained from the different assays upon stimulation with ligands therefore appear to be variable at times, likely due to activation of various other pathways involved in cell survival and apoptosis. Nevertheless, as expected serum induction of proliferation and reduction of apoptosis, resulting in increased cell viability, was observed in all assays used, confirming that the assays were indeed reliable and the lack of consistency observed between assay types was more likely to be a consequence of the ligands employed rather than the reliability of the assays themselves. As use of full serum media gave consistent results across all assays for both MCF7 and MDA-MB-231 cells this was subsequently employed as a control across all further assays.

Phosphorylation of p65 was previously observed to be associated with poor recurrence free interval in breast cancer patients and others have reported that NF- κ B activity is associated with reduced apoptosis and promotion of cell survival. It was therefore hypothesised that inhibition of the IKKs would induce apoptosis and diminish survival of breast cancer cells, making them possible targets for breast cancer treatment. This hypothesis was tested by silencing the IKKs and performing a series of phenotypic assays to assess cell viability and apoptosis. Due to the complications that we observed in the previous set of experiments when ligands were employed to stimulate the NF- κ B pathways, the experiments investigating silencing of IKKs utilised full serum media for pathway induction in preference to TNF α or lymphotoxin. IKK β , the kinase in the canonical pathway that is responsible for phosphorylation of p65 in response to certain stimuli, has been implicated in cell survival. A recent study in gastric cancer found expression of a microRNA that was upregulated in tissue and cell lines was associated with promotion of cell proliferation and prevention of apoptosis. This microRNA was found to be involved in regulation of NF- κ B and genes induced by NF- κ B activation and inhibition resulted in a reduction in proliferation and increase in apoptosis [195]. In both MCF7 and MDA-MB-231 breast cancer cell lines, it was observed that inhibition of IKK β using siRNA robustly induced apoptosis and reduced cell viability.

In keeping with results from the clinical specimens, IKK α however only had an impact in ER positive MCF7 breast cancer cells. Loss of IKK α expression increased cell death and inhibited

cell growth resulting in reduced numbers of viable MCF7 cells. It therefore appears IKK α selective inhibitors would be most beneficial to patients with ER positive tumours. IKK α has been associated with the regulation of oestrogen-dependent genes such as *cyclin D1* and *c-myc*, resulting in increased proliferation [119]. Oestrogen inhibits the Notch pathway and application of anti-oestrogens result in the activation of Notch [196]. Furthermore, the kinase activity of IKK α has been found to be associated with Notch in the activation of ER-dependent genes [197]. Along with the finding that high cytoplasmic IKK α results in increased recurrence and that in ER positive MCF7 cells silencing IKK α decreased cell viability, this suggests that IKK α is associated with the ER and is a worthwhile target to pursue investigating in ER positive breast cancer. Until recently there have been no IKK α selective inhibitors available, but through collaboration with Strathclyde University on a CRUK Drug Discovery programme, our laboratory has been involved in the testing of new ‘first in class’ IKK α inhibitors in prostate cancer and hope to employ these inhibitors as tools in our future breast cancer studies.

Although these experiments yielded interesting results that add to the evidence that IKK α may be a potential target in ER positive breast cancer, there are several limitations of the *in vitro* work conducted in this thesis. The ER positive cell line used in this research was MCF7, as these are a widely used and well categorised ER positive cell line. These cells are caspase 3 deficient due to a deletion in the *CASP-3* gene, a critical constituent of the apoptosis cascade [198]. Studies investigating apoptosis in MCF7 cells have, however, found that in response to certain stimuli (such as PBOX-6, a microtubule-targeting agent) DNA fragmentation and cell death still occur despite these cells lacking caspase 3 [199]. Despite the presence of apoptosis in these cells in our study, investigation into the effect of IKK α and IKK β silencing on cell viability and apoptosis on in another ER positive cell line, or ideally in several, would be extremely beneficial at strengthening the data generated in this study. Other ER positive cell lines have been identified that express caspase 3, such as BT-474 or T47D, and testing these findings in these cell lines would be beneficial for validation of results [200].

Additionally cell lines were unfortunately not authenticated, which is a necessary process to ensure data is not misleading if incorrect cell lines are used. Authentication is an extremely important verification to verify that no cross-contamination has ensued and that genetic drift has not occurred. This is done by short tandem repeat DNA typing [201], examining a set of loci and comparing the profile of the cell line to that of a reference library. Cell line authentication should be performed for all future work.

In conclusion, through various methods it has therefore been demonstrated that IKK β silencing increased apoptosis and reduced cell viability in both ER positive MCF7 cells and ER negative MDA-MB-231 cells. IKK α silencing, however, induced apoptosis and decreased cell viability solely in MCF7 cells. These results suggest IKK α may be a valid target for ER positive breast cancer. Further investigation is needed using a second siRNA, as well as using dominant negative versions of the IKKs, in multiple ER positive and ER negative cell lines.

Chapter 8:

General discussion

8. General discussion and future work

There are several hallmarks of cancer including self-sustained proliferation, resistance to growth suppressors and absence of apoptosis, all of which contribute to uncontrolled cell growth [123]. The NF- κ B pathways regulate the transcription of a wide range of genes involved in many of these processes, as well as inflammation and immune response, and NF- κ B is thought to represent a link between inflammation and cancer [104]. We hypothesised that increased activity of the NF- κ B pathways contributes to the recurrence of breast cancer. In order to address this hypothesis we aimed to establish whether members of the NF- κ B pathway were associated with clinical outcome and investigate the potential therapeutic benefit of inhibitors of the pathways in certain subgroups of patients.

First it was demonstrated that use of a MIB1 antibody to assess Ki67 expression via IHC was successfully able to stratify patients into groups with different outcome based on proliferation status in two independent cohorts. Additionally, in accordance with the study by Cheang *et al* [54] and recommendations by the expert panel at the St. Gallen Conference [48], it was found that Ki67 in addition to HER2, ER and PgR status appeared to appropriately identified luminal A, luminal B, HER2 enriched and triple negative tumours, allowing the use of a stratified approach for further investigations.

After specificity of all antibodies was confirmed, IHC was employed on a TMA of patients to assess whether expression of members of the canonical and non-canonical NF- κ B pathways were associated with recurrence. High levels of phospho-p65 and IKK α were both associated with shorter recurrence free interval, possibly implicating both NF- κ B pathways in the progression of breast cancer. When stratified by ER status, this relationship with both IKK α and phospho-p65 and recurrence free interval was retained in ER positive tumours but negated in ER negative tumours. Other studies have also reported an association with high NF- κ B activity, assessed by p50 DNA binding activity, in ER positive tumours [139]. Diverging roles were observed for each pathway however, with the canonical NF- κ B pathway associating with angiogenesis and recurrence in luminal B patients and expression of the non-canonical NF- κ B pathway associating with cell death (both necrosis and apoptosis) and recurrence in luminal A patients. These results add to the growing body of evidence that highlight the need to consider breast cancer not as one disease but a collection of breast diseases with different molecular profiles, allowing patients, where possible, to be treated based on these profiles.

A difference between ER positive and ER negative breast cancer was also reflected in our *in vitro* work using cell lines of different ER status. Consistent with other studies, which observed elevated levels of components of both the canonical and non-canonical NF- κ B pathways in ER negative cells [142], high basal expression levels of both phospho-p65 and phospho-p100 were observed in ER negative MDA-MB-231 cells. In contrast, although basal levels of both NF- κ B pathways were low in ER positive MCF7 cells, activation of both pathways was inducible.

The use of cell lines representative of each subtype would be greatly beneficial to investigate the relationship of phospho-p65 and IKK α with the different luminal tumours, however often these are simply defined as luminal rather than specifying which of the luminal subtypes it reflects [202-203]. Suggestions of cell lines representative of the luminal subtypes have defined MCF7 cells as luminal A based on their profile using immunohistochemistry and proposed BT474 cells as representative of luminal B tumours [204]. These immunoprofiles include ER, PgR and HER2, and the difference between luminal A and luminal B is therefore defined by HER2 status. However, upon further examination of the relationship with phospho-p65 and luminal B tumours it was found that this relationship was most significant in luminal B tumours with high Ki67 rather than those that were HER2 positive, although very small numbers were available for analysis and caution is required in interpretation of these results. The use of a cell line defined as luminal B based on HER2 status therefore does not appear to be the most appropriate for use in future studies continuing from this project. Additionally, when the molecular and phenotypic relation of a panel of breast cancer cells were evaluated in comparison to breast tumour samples, all of the intrinsic subtypes were represented with the exception of the luminal A subtype [205]. Furthermore, this study found that MCF7 cells were most similar to luminal B tumours [205]. There is therefore conflicting opinions on what cell lines best represent the different subtypes and subdividing breast cancer cell lines into luminal A and B remains difficult. In order to investigate the use of novel therapies and identify which patients are most likely to respond to these, *in vitro* preclinical models need to accurately represent the profile of tumours in different patients. One promising approach is the use of patient derived cell lines. These more accurately replicate the heterogeneity observed in breast cancer and models using patient derived xenografts are likely to be more useful in the translation of cancer therapies [206].

As an association was observed with both markers in ER positive breast cancer and recurrence in the first 5 years in ER positive patients receiving tamoxifen as endocrine therapy, a cohort of ER positive patients with information on recurrence on tamoxifen was

selected as the second independent cohort used to validate findings. In this cohort phospho-p65 was higher overall meaning the cut off established in the previous cohort was unable to be used due to too few numbers in the low, highlighting the difficulty often experienced with phosphorylation status antibodies. Nuclear expression of phospho-p65 was not associated with recurrence free interval or recurrence on tamoxifen but was associated with poorer breast cancer specific survival. Levels of IKK α were however, were consistent with the previous cohort and high cytoplasmic expression was found to be more significantly associated with clinical outcome with quicker recurrence on tamoxifen, shorter recurrence free interval and reduced breast cancer specific survival. However as this was found to be in luminal B tumours, where in the previous cohort it was with luminal A tumours, and therefore further investigations are needed to strengthen the implication that IKK α may be associated with recurrence. This cohort, although valuable to investigate recurrence on tamoxifen, unfortunately does not include patients treated with AIs. In order to determine whether NF- κ B and the IKKs appear to be involved in resistance to AIs, examination of expression of the pathways in a cohort of patients treated with AIs is required. The Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial is a phase 3 trial with two arms where arm 1 was treated with the aromatase inhibitor exemestane and arm 2 was treated with tamoxifen for five years followed by exemestane [207]. This cohort is an ideal platform to test whether the pathways are also associated with recurrence on AIs and with tissue available for 4500 patients, it has sufficient statistical power to validate appropriate biomarkers. Additionally, this cohort includes patients from nine different countries, meaning that this would be considered external validation of the role of NF- κ B in breast cancer progression, due to the inclusion of patients from a different geographical locations [182]. Work continuing on from the research presented in this thesis will investigate the identified markers in the TEAM trial cohort, in order to validate these initial findings and to further investigate them in the context of AI treatment. Investigations are needed into whether the relationship of these pathways with clinical outcome is due to a causative role.

Although tamoxifen and other endocrine therapies are effective for the treatment of ER positive tumours, endocrine resistance remains a major clinical problem. The mechanisms behind progression are not yet fully understood and the Gap Analysis Working Group identified mechanisms driving resistance as one of the major gaps in research that if filled would make the biggest clinical impact [84]. Further investigation is required to examine the role of the NF- κ B pathways in the transition from endocrine sensitive to endocrine resistant tumours. Studies exploring changes in other signalling pathways have used matched tissue of primary and recurrent endocrine resistant tumours. Using 77 matched

tumours, Drury *et al* assessed changes insulin-like growth factor receptor 1 (IGFR1) and the PI3K pathway in addition to ER, PgR and HER2 [208]. Through this approach the authors were able to confirm loss of ER and PgR as well as increase in HER2 expression in some of the tamoxifen resistant tumours. An increase in expression of stahmin, which has been identified as a marker of PI3K activation, was also detected, however no evidence of change in IGFR1 was observed [208]. Other studies within our laboratory in prostate cancer have examined the expression of the canonical NF- κ B pathway, as well as aspects of PI3K signalling, in matched tissue from hormone naïve and castrate resistant tumours [135]. Expression of components of both NF- κ B pathways in the transition from endocrine responsive breast cancer to resistant disease has yet to be assessed and investigations using matched tissue would be extremely valuable in assessing the role of these pathways in endocrine resistance. Additionally, mechanistic work is required in ER positive breast cancer cells that are resistant to endocrine treatment. In cellular models of tamoxifen resistance or oestrogen deprivation resistance, the effectiveness of a dual inhibitor of mTORC1/2 mTOR kinase inhibitor was assessed [209]. These cells allowed the authors to establish the impact of this inhibitor in acquired endocrine resistance. Use of this dual inhibitor was found to reduce proliferation, induce cell death and decrease migration and observations suggested the use of these inhibitors in combination with anti-oestrogen therapy may be an appropriate second-line treatment following endocrine resistance [209]. Use of similar *in vitro* models would be advantageous in the investigation of IKK α /NF- κ B signalling in endocrine resistance and the incorporation of these models should be considered in future studies.

Due to problems with phosphorylated antibodies and the lack of reliable markers of activation of the pathways we aimed to establish IKK α and IKK β selective markers. Using siRNA for IKK α and IKK β and qPCR, expression of a panel of markers used in previous studies in prostate cancer was examined. Of the genes investigated, however, no selective marker for either kinase was identified, although this experiment was only completed once. Markers used in prostate cancer were revealed using TaqMan Low Density Arrays (TLDA), which enable qPCR analysis of 384 markers in one experiment. The use of TLDA cards has been found to be a reproducible method with minimal variation when assessed for use in microRNA profiling [210]. The application of these arrays would be useful in future studies that endeavour to find both IKK α and IKK β selective markers.

Since the evidence from clinical specimens suggests high NF- κ B may be associated with a poorer outcome, the potential of targeting the IKKs in breast cancer was investigated in ER

positive and ER negative cells. Silencing of IKK β induced apoptosis and decreased cell viability in both MDA-MB-231 cells and MCF7 cells. On the other hand, increased apoptosis and reduced viability following silencing of IKK α was limited to ER positive MCF7 cells, suggesting inhibitors of IKK α would be beneficial for patients with ER positive tumours. A difference was observed in the luminal subtypes, with phospho-p65 associating with recurrence in luminal B subtype and IKK α associating with recurrence free interval and recurrence on tamoxifen in luminal A subtype. Patient selection may consequently be important in the use of NF- κ B inhibitors and drugs selectively targeting both canonical and non-canonical arms of the NF- κ B pathways may provide possible clinical benefit. Pharmacological intervention targeting NF- κ B has already been proposed as a possible therapeutic option in many cancers due to its role in regulation of genes involved in cell growth and apoptosis. In 2006 a review of the literature revealed 785 modulators of NF- κ B signalling at many different levels including those which stabilise and prevent degradation of I κ B or result in upregulation of I κ B expression as well as NF- κ B DNA-binding inhibitors [211]. The present study suggests that a successful approach would be to selectively target the kinases IKK α and IKK β , preventing activation of the pathway and therefore inhibiting downstream effects. Several compounds described as IKK β inhibitors have been developed, including SC-514 from Pfizer, TPCA-1 from GlaxoSmithKline, BMS-345541 from Bristol-Myers Squibb and ML120B from Millennium Pharmaceuticals [212]. However, many of these compounds also display inhibition of IKK α , although to a lesser extent and additionally, several of these inhibitors have displayed adverse effects [91]. There has therefore been no IKK α or IKK β selective inhibitors available, but through collaboration with Strathclyde University on a CRUK Drug Discovery programme, our laboratory has been involved in the testing of new 'first in class' IKK α inhibitors and IKK β inhibitors with a higher level of selectivity than previously available. These compounds require testing in breast cancer cell lines however initial studies involving prostate cancer models have yielded encouraging results. An exciting continuation of this project should therefore involve testing these novel compounds in *in vitro* breast cancer models.

In conclusion, results from the current study demonstrate a possible role of both NF- κ B pathways in the recurrence of breast cancer and expression of IKK α in the resistance to endocrine therapy. Additional thorough investigation, including both tissue studies and mechanistic work, is required to further elucidate the role of the NF- κ B pathways and IKKs in the progression of breast cancer, though involvement of these pathways in breast cancer recurrence appears to exist. Inhibitors of this pathway may therefore be a promising therapeutic approach in the future, once novel compounds are found to be selective and efficient in

preclinical models, and may be beneficial in the treatment of breast cancer, particularly following endocrine resistance.

Future work

Future studies continuing on from the research presented in this thesis should aim to validate results and further explore the role of the canonical and non-canonical pathways in the progression of breast cancer and test whether the upstream kinases are possible novel targets for breast cancer.

One of the most interesting and novel findings from this study was the examination of the different pathways in the molecular subtypes of breast cancer. However, although IKK α and phospho-p65 were associated with poorer outcome in both cohorts in ER positive tumours, within the each cohort IKK α was associated with poorer outcome in different luminal subtypes. This should be further explored in a larger cohort from outwith the west of Scotland, information on subtypes would be extremely beneficial. The TEAM trial would be an excellent resource for this study, as well as to explore the association of the markers with endocrine resistance [207].

This study only used one ER positive and one ER negative cell line, and as previously mentioned although MCF7 cells are widely used and well characterised, these are caspase 3 deficient [198] and may not be the most appropriate cell line to examine cell viability and apoptosis. Experiments should be repeated using a second ER positive cell line, for example T47D, which is not caspase 3 deficient or BT474, which is also not caspase 3 deficient and is HER2 positive, and another ER negative cell line, such as MDA-MB-330 or BT-20 cells. Additionally, repetition using a second siRNA for a different sequence from IKK α /IKK β would improve the validity of the study. In hindsight, the time spent completing the viability and apoptosis assays after exposure to ligands that activate the pathway may have been better spent addressing some of these issues, such as testing the results obtained with both a second ER positive cell line and also a second siRNA. This should be performed in studies carrying on from the research presented in this thesis. Investigations using DN-IKK α and IKK β should be carried out, as it has been shown that the breast cancer cells appear amenable to this manipulation but further examination of the effects on the signaling of the pathway is necessary. This should be followed by assays to test the affects of loss of IKK α and IKK β in the presence of a ligand that drives each pathway. Additionally, the novel compounds should

be screened in breast cancer cells to investigate the effect these have on NF- κ B signaling as well as cell viability.

Other future *in vitro* work could also include a longer term viability assay to observe effects of loss of IKK α and IKK β function, such as a clonogenic assay to measure the ability of single cells to form colonies of over 50 cells [213]. In order to address the lack of specific markers of the non-canonical pathway and the variation observed with the phosphorylation of p65, selective downstream markers are required. From the initial qPCR experiment performed in this study, it appears that markers identified as specific in other cancer models do not translate to breast cancer cells. Another method should be used to look at a much larger set of downstream markers to find ones selective for both pathways in breast cancer. This could include the TLDA cards, which were used previously in our laboratory to identify markers in other cancer models.

Conclusion

We hypothesised that high expression of the NF- κ B pathways would result in poorer outcome in breast cancer patients and that these pathways may be involved in the development of endocrine resistance. Results presented in this thesis using two patient tissue cohorts suggest that both phosphorylation of p65, from the canonical pathway, and IKK α , from the non-canonical pathway, may be associated with shorter recurrence free interval in ER positive breast cancer. Additionally, results from the second cohort also show that IKK α expression may be associated with recurrence on tamoxifen. Further investigation is needed in larger cohorts from another location in addition to studies that would provide evidence of a direct involvement, rather than association, of the pathways in the progression of breast cancer. It was also speculated that inhibition of the IKKs would reduce the viability of breast cancer cells. This was tested using siRNA to target IKK α and IKK β in both ER positive MCF7 cells and ER negative MDA-MB-231 cells. It was found that loss of IKK β expression resulted in reduced viability in ER positive and ER negative cells however IKK α silencing only reduced viability in ER positive cells. More mechanistic work is required, however it appears these may be possible targets, particularly in ER positive breast cancer, and further investigation into this is warranted.

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