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# The role of IKKalpha and IKKalpha phosphorylation in breast cancer

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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.

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August 2021

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# Summary

Breast cancer is the most frequently diagnosed cancer in women in the UK. Although several targeted therapies have been approved for patients with oestrogen-positive and HER2-positive breast cancer, chemotherapy remains the standard systemic option for patients with triple-negative breast cancer (TNBC). The molecular mechanism driving tumour heterogeneity of TNBC has not been fully elucidated. Understanding molecular pathways and identifying biomarkers allow the discovery of the potential therapeutic targets for the treatment of this disease.

The NF-κB pathway is a crucial link between inflammation and cancer. IKKα is an essential kinase of the pathway demonstrated to be involved in breast cancer progression through the NF-kB-dependent and NF-κB-independent functions. The current study aimed to investigate the role of IKKα, its phosphorylation at serine 176 (pIKKα-Ser176) and threonine 23 (pIKKα-Thr23), and their upstream kinases, NIK and pAKT in different breast cancer subtypes. Since it has been suggested that crosstalk exists between IKKα and androgen receptor (AR) in TNBC, expression of AR was also investigated.

Immunohistochemistry was employed on a tissue microarray (TMA) of patients with breast cancer to assess the expression of IKKα, pIKKα-Ser176, pIKKα-Thr23, pAKT, NIK and AR. Overall, the findings reported here suggest that the prognostic role of these potential biomarkers differed among breasts cancer subtypes and differed depending on their cellular locations. Inconsistent results between the cohort were also observed. Several limitations need to be noted, including using the IHC-based classification instead of gene expression subtyping, which might affect the outcome. Using old TMAs was another limitation that might affect protein expressions. Using the multiple cut-off values to classify tumours with low and high expression of each protein might lead to an inconsistent result among the cohorts. Further study is needed, using gene expression subtyping, freshly cut TMAs and an appropriated method for determining a cut-off value.

Another aim of the present study was to investigate differentially expressed gene profiles for tumours with low versus high cytoplasmic IKKα, cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23 in ER-negative tumours. We hypothesise that such DEGs are associated with distinct biological functions, representing a common mechanism of tumours with high IKKa, pIKKα-Ser176 and pIKKα-Thr23. 14 and 3 significant DEGs were observed in the IKKα and the pIKKα-Thr23 group. None was found in the pIKKα-Ser176 group. Several genes in

the IKKα group have been reported to involve in breast cancer progression, including ACTA2, ALCAM, PDCD6IP, CD9, MVP, and PRSS8 (1-6). As the DEGs analysis was based on small sample size, the present study did not confirm the hypothesis regarding DEGs between tumours with high and low expression of the proteins of interest. The study should be repeated using an adequate number of samples.

The impact of pharmacologically inhibiting IKKα in two ER-negative breast cancer cells with differing AR status was investigated. Inhibition on p52 processing was assessed by western blotting and phenotypic response was assessed by changes in cell viability. The impact of pharmacologically inhibiting IKKα in two ER-negative breast cancer cells with differing AR statuses was investigated. Inhibition on p52 processing was assessed by western blotting, and the phenotypic response was assessed by changes in cell viability. Using the western blot, the present study was unable to demonstrate an inhibitory effect on p52 by using first-in-class IKKα inhibitors. Future work is needed to repeat and perform this experiment in triplicate, which was not possible due to the COVID-19 restrictions. A dose-dependent decrease in cell viability was observed after using the IKKα inhibitor. However, AR antagonist alone was sufficiency in ER-negative/AR-positive cell lines.

In conclusion, the result in this thesis using three patients tissue cohorts suggest that IKKα, pIKKα-Ser176 and pIKKα-Thr23 may be associated with clinical outcome. However, the prognostic roles of these proteins are different depending on tumour subtypes and cellular locations, and no clear conclusion could be reached. There has been growing recognition of the crucial links between the NF-κB pathway and the critical components in the tumour microenvironment (TME). Future work investigating the complex linkage between the NF-κB pathway and the crucial components in TME in breast cancer, such as programmed death-ligand (PD-L1) and T-lymphocyte-associated protein 4 CTLA-4, is needed. This will serve as a therapeutic potential of inhibition either NF-κB or molecules involved in NF-κB activation such as IKKα inhibitors, combined with immune checkpoint blockade for patients with breast cancer, especially TNBC.

# Publications and presentations

## Publications relating to this thesis

Khongthong P, Roseweir AK, Edwards J. The NF-KB pathway and endocrine therapy resistance in breast cancer. Endocr Relat Cancer. 2019;26(6): R369-r80.

## Poster presentations

Phungern Khongthong, Jean Quinn, Lindsay Bennett, Antonia Roseweir, Joanne Edwards. (2018) Role of IKKα and IKKα phosphorylation in patients with ER positive early-stage invasive breast cancer.
NCRI, Glasgow, November 2018.

Phungern Khongthong, Jean Quinn, Lindsay Bennett, Antonia Roseweir, Joanne Edwards. (2019) High IKKα and phosphorylated IKKα (p-IKKα) associated with poor survival outcomes in patients with high tumour PD-L1 expression endocrine receptor (ER) positive breast cancer.
BACR, Newcastle, 2019.

Khongthong P, Bennett L, Quinn J, Mallon E, Roseweir A, Edwards J. (2019) The association between IKKα, immunologic markers, and clinical outcomes in patients with ER-positive tamoxifen-treated breast cancer.
AACR Special Conference on Tumour Immunology and Immunotherapy; Boston, November 2019.
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# Chapter 1 Introduction

# 1.1 Breast cancer epidemiology

## 1.1.1 Breast cancer incidence, mortality and survival

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women throughout the world (7). In the United Kingdom (UK), breast cancer remains the most frequent cancer in women, with an estimated 54,700 new cases in 2017 (8). Over the last decade, the death rate from breast cancer has decreased by 21% in the UK. This reduction has been attributed to increased screening and adjuvant therapy (9). The relative survival rates for women diagnosed between 2013-2017 after 1 year and 5 year by stage is shown in Table 1.1 (10).

| Stage | Survival (%) | |
|---|---|---|
| | 1-year | 5-year |
| 1 | 100.0 | 97.9 |
| 2 | 98.9 | 89.6 |
| 3 | 95.5 | 72.0 |
| 4 | 66.0 | 26.2 |

**Table 1.1 Survival rate of breast cancer in England by stage.** *The percentage survival at 1 and 5 years for patients diagnosed between 2013 and 2017.*

# 1.2 Pathology of invasive breast cancer

Most invasive breast cancers originate from the terminal duct lobular unit regardless of histological type. Invasive (infiltrating) ductal carcinoma is the most common histologic type of breast cancer, comprising 70% to 80% of all cases (11). Other types of invasive breast cancer comprise 20% to 30% of all cases. These include classic tubular carcinoma or classic mucinous carcinoma, which have a low incidence of lymph node metastasis (11).

**A** **B**

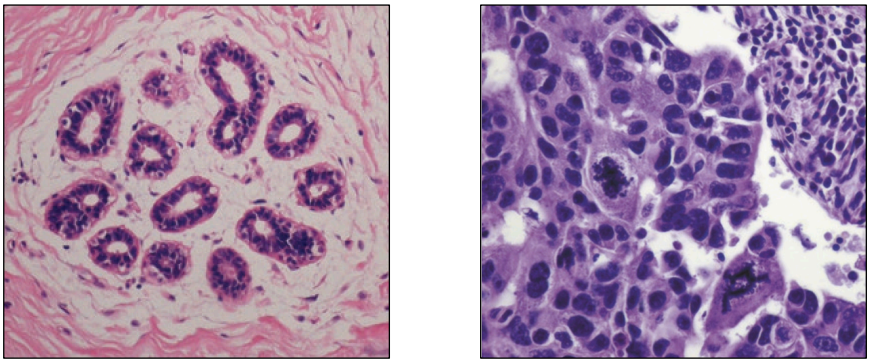

**Figure 1.1 Histopathology of normal breast and invasive breast cancer.** *Normal breast tissue shows multiple acini embedded in the stroma with uniform oval nuclei **(12)** (A). Invasive breast cancer tissue shows highly pleomorphic nuclei **(12)** (B).*

## 1.3 Staging

The Tumour, Node, Metastasis (TNM) staging system is the accepted system that is used to determine staging for solid tumour including breast cancer. T refers to the extent of tumour, N refers to the extent of regional lymph node involvement and M refers to tumour metastasis outside the primary breast and regional lymph nodes. T stage in breast cancer is assessed by tumour size that is defined as the largest diameter of the primary tumour. Large tumour size is associate with an increased number of lymph node involved (13). The N number or number of lymph node involved is a strong prognostic factor for subsequent metastasis. Patients with lymph node involvement have a worse clinical outcome when compared to those with absent lymph node involvement, with a five-year survival of 85% and 95%, respectively (14). Lastly, patient with M-stage 1 that present with distant metastasis at the time of diagnosis have poor prognosis and are unlikely to be cured (15).

The eighth edition of the American Joint Committee on Cancer (AJCC) staging system is based on the TNM system and integrates both anatomic extent and biologic phenotype of the tumour into the complete staging criteria shown in Table 1.2.

Tis: Ductal carcinoma in situ
T1: Tumour ≤20 mm in greatest dimension
T2: Tumour >20 mm but ≤50 mm in greatest dimension
T3: Tumour >50 mm in greatest dimension
T4: Tumour of any size with direct extension to the chest wall and/ or to the skin

pN0: No regional lymph node metastasis
pN1: Micrometastases; or metastases in 1–3 axillary lymph nodes
pN2: Metastases in 4–9 axillary lymph nodes
pN3: Metastases in 10 or more axillary lymph nodes

M0: No clinical or radiographic evidence of distant metastases
pM1: Any histologically proven metastases in distant organs; or if in non-regional nodes

| Stage | | T | N | M |
|---|---|---|---|---|
| 0 | | Tis | N0 | M0 |
| I | IA | T1 | N0 | M0 |
| | IB | T0-T1 | N1mi | M0 |
| II | IIA | T0-T1 | N1 | M0 |
| | | T2 | N0 | M0 |
| | IIB | T2 | N1 | M0 |
| | | T3 | N0 | M0 |
| III | IIIA | T0-T2 | N2 | M0 |
| | | T3 | N1-N2 | M0 |
| | IIIB | T4 | N0-N2 | M0 |
| | IIIC | Any T | N3 | M0 |
| IV | | Any T | Any N | M1 |

**Table 1.2 AJCC Anatomic Stage Groups**

## 1.4 Prognostic and Predictive Factors in Breast Cancer

TNM parameters are strong prognostic factors and reflect tumour burden (13-15). In addition, several factors that have been established as classical prognostic factors of breast cancer, including patients age, tumour grade and vascular invasion.

### 1.4.1 Patient age

Patient age is one of the classical prognostic factors for breast cancer (16, 17). Patients aged <35 years have a worse prognosis with 75% absolute five-year survival compared to 84-88% for patients aged 35-69 years (18). This group of patients frequently present at an-advance stage and have ER-negative disease (18). Some studies have demonstrated that age >65 was associated with increase breast cancer mortality (16, 17). This is attributed to a lower rate of nodal examination, they did not receive treatment and multiple comorbidity (16, 17).

### 1.4.2 Tumour grade

The Nottingham prognostic index (NPI) is a grading system that is used to classify patients with primary, operative breast cancer into three prognostic group (19). This prognostic index is based on tumour size, tumour grade and lymph node involvement (20). The components of tumour grade include nuclear pleomorphism, mitosis, and tubule formation (20). A grade equivalent “score” was derived from combining these variables (Table 1.3). Lymph node involvement was defined as follows.

| Feature | Score |
|---|---|
| **Tubular formation** | |
| Majority of tumour (>70%) | 1 |
| Moderate degree (10-75%) | 2 |
| Little or none (<10%) | 3 |
| **Nuclear pleomorphism** | |
| Small, regular uniform cells | 1 |
| Moderate increase in size and variability | 2 |
| Mark variation | 3 |
| **Mitotic counts** | |
| Dependent on microscope field are | 1-3 |
| 1 point | |
| 2 points | |
| 3 points | |

**Table 1.3 summary of method for assessing tumour grade in breast cancer**

Tumour grade is allocated as follows.

3-5 points: grade I well-differentiated
6-7 points: grade II moderately differentiated
8-9 points: grade III poorly differentiated

Lymph node involvement:
1 = no lymph node 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

NPI can be calculated by:

NPI = (Tumour size in cm x 0.2) + Stage (based on lymph node involvement) +tumour grade

Based on NPI, patients can be classified into three group. Patients with NPI <3.4 are classified as good prognostic group. Patients with NPI 3.4-5.4 are classified as moderate prognostic group. Patient with NPI>5.4 are classified as a poor prognostic group. The higher the NPI score, the worse the prognosis (19).

### 1.4.3 Tumour involvement of lymphovascular space

The presence of tumour invasion in either blood vessels (vascular invasion), lymphatic vessels (lymphatic invasion) or both is a prognostic factor in respect of local and distance metastasis in patients with node-negative disease (21). Lymphovascular invasion (LVI) is

assessed using Haematoxylin & Eosin stained full sections and recorded as present or absent (21).

### 1.4.4 Proliferation Markers

Ki-67 is a marker of proliferation that measure the percentage of cells in the G1 phase of the cell cycle (22). The relationship between Ki-67 and prognosis in early stage breast cancer has been comprehensively studied (22). A study has shown that high Ki-67 was associated with higher risk of disease relapse and worse breast cancer survival in both node-negative and node-positive disease (22). The use of Ki-67 as a prognostic marker in clinical practice is still controversial due to interobserver variability and lack of uniform thresholds for scoring. Currently, an expert panel from the American Society of Clinical Oncology (ASCO) does not recommend the use of Ki-67 to assess prognosis for early breast cancer (23). However, Ki-6 7is still used to classify breast cancer into four major subtypes (24).

### 1.4.5 Oestrogen receptor (ER) and progesterone receptor (PR)

ER and PR are members of the nuclear hormone receptor superfamily (25, 26). They are located in the cytosol and function as ligand-dependent transcription factors (27). Binding to their specific ligand results in nuclear translocation, binding to responsive elements and induction f target gene transcription (25, 27). There are two forms of ER, ER-alpha (ER-α) and ER-beta (ER-β) (25). Only ER-α has been established for use in clinical practice (25).

ER is expressed in approximately 70% of invasive breast cancers (28). Immunohistochemistry (IHC) staining of ER and PR is report using the Allred scoring method. This method combines both the proportion of positive cells and staining intensity (28) (Table 1.4). The cut-off point for a positive result is 1% of nuclei positive. Similarly, PR-positive is defined as ≥1% positive nuclei (28). Expression of ER and PR are a favourable prognostic factor and strongly predictive of response to endocrine therapy (29). A significantly reduced disease recurrence was observed in ER-positive breast cancer patients who received 5 years of adjuvant tamoxifen (30).

| Positive cells (%) | Proportion score | Intensity | Intensity score |
|---|---|---|---|
| 0 | 0 | None | 0 |
| < 1 | 1 | Weak | 1 |
| 1-10 | 2 | Intermediate | 2 |
| 11-33 | 3 | Strong | 3 |

| | | | |
|---|---|---|---|
| 34-66 | 4 | | |
| ≥ 67 | 5 | | |

**Table 1.4 Allred Score for ER and PR evaluation**

Allred score =Proportion score+Intensive score
0-2 = Negative
3-8 = Positive

### 1.4.5.1 Human epidermal growth factor receptor-2 (HER2)

HER2 is a member of the tyrosine kinase family and is an oncogene that is located in the 17q12 region. Overexpression or amplification of this gene is found in about 20% of invasive breast cancer and is associated with an aggressive phenotype of breast cancer. HER2 positive breast cancer is defined by protein overexpression 3+ using IHC stain or a ratio 2.0 on a dual probe fluorescent in situ hybridisation (FISH) that is expressed as the ratio of HER2 signal to chromosome 17 centromeric enumeration probe (CEP) signal and HER2 copy number signals/cell ≥4 (31) (Table 1.5) (Table 1.6) (32).

| Score | IHC | Result |
|---|---|---|
| 0 | No or incomplete membrane staining in <10% of invasive tumour cells | |
| 1+ | Faint or weak incomplete membrane staining in >10% of tumour cells | Negative |
| 2+ | A weak to moderate incomplete membrane staining in >10% of tumour cells or<br>Strong complete membrane staining in ≤ 10% of tumours | Borderline |
| 3+ | A strong (intense and uniform) complete membrane staining in >10% of tumour cells | Positive |

**Table 1.5 Immunohistochemistry (IHC) of HER2.**

| FISH | Result |
|---|---|
| Dual probe HER2/CEP17 ration <2.0 AND an average HER2 copy number <4.0 signals per cell | Negative |
| Dual probe HER2/CEP17 ration <2.0 with an average HER2 copy number 4.0-6.0 signals per cell OR a ration of 1.80-1.99 | Borderline |
| Dual probe HER2/CEP17 ration ≥ 2.0 OR an average HER2 copy number ≥ 6.0 signals per cell | Amplified |

**Table 1.6 HER2 in fluorescent situ hybridization testing (FISH).**

### 1.4.6 Intrinsic subtype

The intrinsic subtype is a molecular classification by integrated molecular analysis of breast cancer with diverse genetic and epigenetic alterations that converge phenotypically to major subtypes (33, 34). The pivotal studies demonstrated a distinctive gene expression profile among breast cancer subtypes and categorised tumours into five intrinsic subtypes: luminal A, luminal B, HER2 overexpression, and basal-like and normal-like tumours (33, 35). All five intrinsic subtypes were mapped to immunohistochemistry (IHC)-based subtype except for the normal-like tumours, accounting for up to 8% (36). Consistent with recommendations from the 13th International Breast Cancer Conference in 2013, breast cancers were categorised into four subtypes by using the IHC for surrogate markers. These included luminal A, luminal B, HER2 overexpression and basal-like (37).

Luminal A subtype accounts for 50%-60% of all breast cancers. Tumour in this subtype is the indolent, more endocrine sensitive and better prognosis (37). Luminal A subtype is defined as tumours with ER and PR positive, HER2 negative and low Ki-67 (proliferating cell nuclear antigen) (37) (Table 1.7). The cut-point value between “low” and “high” of K-67 is vary between the laboratories. A threshold of ≥20% is indicative of ‘high’ Ki-67, which was voted by a majority of the St Gallen international expert panel (37). The added value of PR with a cut-point of ≥20% is required to distinguish between luminal A and luminal B (37). Tumours in this subtype also have a recurrence risk ‘low’ based on multi-gene-expression assay (37). Tumour in this group have a higher level of ER related genes, including oestrogen receptor alpha 1 (ESR1), hepatocyte nuclear factor 3 alpha (FOXA1), X-box binding protein 1 (XBP1) and GATA binding protein 3 (GATA3). These tumours also have lower levels of proliferation-related genes (33).

| Intrinsic subtype | Luminal A | Luminal B | | HER2 overexpression | Basal-like |
|---|---|---|---|---|---|
| Clinicopathologic surrogate definition | Luminal A-like<br>All of:<br><br>ER and PR positive<br>HER2 negative<br>Ki-67 "low"<br>Recurrence risk "low" based on multi-gene-expression assay (if available) | 'Luminal B-like (HER2 negative)<br>ER positive<br>HER2 negative and at least one of<br>Ki "High"<br>PR "negative or low"<br><br>Recurrence risk "high" based on multi-gene-expression assay (if available) | 'Luminal B-like (HER2 positive)'<br>ER positive<br>HER 2 over-expression or amplified<br>Any Ki-67<br>Any PR | HER2 overexpression<br>ER and PR absent<br>HER 2 over-expression or amplified | Triple negative<br>ER and PR absent<br><br>HER2 negative |
| mRNA expression | High ER cluster | Lower ER cluster | | HER2 signature | Basal signature |
| | Low proliferation | High proliferation | | High proliferation | High proliferation |
| Protein expression | High oestrogen signalling | Less oestrogen signalling | | High protein and phospho-protein expression of EGFR and HER2 | High expression of DNA repair proteins |

**Table 1.7 Breast cancer intrinsic subtype classification (33, 37).**

Luminal B subtype represents 15%-20% of all breast cancer and is the more aggressive, less endocrine sensitive and worse prognosis (37). Luminal B is classified into two groups. A luminal B-like HER2 negative includes tumours that are ER-positive, HER2 negative and at least one of these features, including PR-negative or low, High Ki-67 and recurrence risk 'high' based on the multi-gene-expression assay. A luminal B-like HER2 positive includes tumours that are ER-positive, HER2 overexpression or amplification, any Ki-67 and any PR (37). The significant difference from the luminal A subtype is the lower ER cluster, but higher proliferation-related genes are found in the luminal B subtype. These include nuclease sensitive element-binding protein 1 (NSEP1) and cyclin E1 (CCNE1) (33).

HER2 overexpression subtype accounts for 15%-20% of all breast cancer and is the more aggressive and worse prognosis (33, 37). This subtype is defined by high expression of HER2 by IHC or HER2 gene amplification by Fluorescent in Situ Hybridisation (FISH) and absence of ER and PR (37). Tumours in this subtype have a high histological grade and are highly proliferative (33).

Basal-like subtype accounts for up to 10% of all breast cancers (33). Tumours in this subtype do not express ER, PR and HER2 but have high basal myoepithelial markers, including cytokeratin 5 (CK5), CK14 and CK17 (33). Basal-like subtypes are frequently referred to as triple-negative breast cancer (TNBC). However, they are not completely synonymous. The basal-like subtype is defined by gene expression analysis and is mainly used in the research setting, whereas the term TNBC refers to the IHC-based classification of breast cancer that absence of ER and PR and HER2 (33, 37). Tumour in this group is more often found in young women, has a higher risk of recurrence, and is more likely to metastasize to the brain and lungs (38).

TNBC has a diversity of gene expression patterns, subclassified into six distinct subtypes (Table 1.8). These include basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and a luminal androgen receptor (LAR) subtype (38). Tumours in both BL1 and BL2 subtypes display high expression of cell cycle and DNA damage response genes. While BL1 is characterised by elevated expression of Ki-67, BL2 shows high expression of growth factor signalling, gluconeogenesis, glycolysis and myoepithelial markers such as TP63 (39). Tumour in the IM subtype is defined by elevated expression of genes involved in immune signalling genes, including immune cells such as tumour-infiltrating lymphocytes (TILs) and CD8+T cell; cytokine signalling such as

JAK/STAT, TNF, and NF- κB (40). Tumours in M and MSL subtypes share high expression of genes involved in epithelial-mesenchymal-transition (EMT), cellular differentiation and extracellular matrix remodelling. While the M subtype shows elevated proliferation genes, the MSL subtype displays increased expression of genes involved in angiogenesis and growth factor pathways (38-40). Tumour in LAR subtype is characterised by elevated genes that affect steroid synthesis and hormone signalling, including androgen receptor and its coactivators such as FOXA1, GATA3 and XBP1 (39, 41).

| TNBC subtypes | Basal-like 1 | Basal-like 2 | Immunomodulatory | Mesenchymal | Mesenchymal stem-like | Luminal androgen receptor (LAR) |
|---|---|---|---|---|---|---|
| Signalling pathway | Cell cycle<br>Proliferation<br>DNA damage response pathway | Cell cycle<br>Proliferation<br>Growth factor signalling<br>Gluconeogenesis | Immune cell signalling | EMT<br>Proliferation | EMT<br>Growth factor signalling<br><br>Angiogenesis | Hormone signalling<br><br>Androgen receptor and its coactivators |
| Chemosensitivity | Very good | Very poor | Medium | Medium | Medium | Poor |
| Potential markers | *ATR, BRCA, MYC, NRAS, Ki-67* | *EGFR, MET, EPHA2, TP53* | *JAK1/2, STAT1/4, IRF1/7/8, TNF* | *Wnt, ALK, TGF-b* | *GFR, PDGFR, ERK1/2, VEGFR2* | *AR, FOXA1, KRT18, XBP1* |
| Potential treatment | Cisplatin, PARP inhibitors | Cisplatin; PARP and growth factor inhibitors | | PI3K/mTOR, Src inhibitors | PI3K/mTOR, Src inhibitors factor signalling, angiogenesis | AR antagonist; PI3K, Hsp90 inhibitor |

**Table 1.8 Subclassification of TNBC (38)**

### 1.4.7 Androgen receptor in TNBC

Androgen receptor (AR) has been indicated as a therapeutic target, and the current availability of selective AR inhibitors such as enzalutamide and bicalutamide are approved for the treatment of prostate cancer. It is emerging as a new marker and a potential therapeutic target for treatment for breast cancer patients whose tumours express AR.

Androgen receptor is a steroid hormone receptor that belongs to the nuclear receptors' family along with oestrogen, progesterone, glucocorticoid, and mineralocorticoid receptor. It has been demonstrated to link with other transcription factors that control specific genes that involve the stimulation or suppression of both cell proliferation and apoptosis (42). AR binding to its ligands and translocated into the nucleus to bind with its co-regulators to induce transcription of its target genes that involve in various biological processes, including cell proliferation such as *FOXO1A*; lipid metabolism such as *PMVK* and *SEC14L2*; and steroid metabolism such as *SOAT2* and *OSBPL11 (43)*. In addition to its transcription role, AR also has been demonstrated to have a non-transcriptional function that interacts with other signalling pathways, including PI3K and Src family (44).

Tumour in luminal AR-positive TNBC showed many genes involved in lipid synthesis, steroid synthesis, and androgen/oestrogen metabolism. These consisted of numerous downstream AR targets genes and AR co-activators, including *DHCR24, ALCAM, FASN, FKBP5, APOD, PIP, SPDEF and CLDN8*) and luminal genes such as *FOXA1, KRT18 and XBP1 (39)*.

Androgen receptor is expressed based on the cut-off value that is used to define its positivity. In a systemic review of 19 studies with a total of 7693 women with early-stage breast cancer, the cut-off values were 1%, 5% and 10%. AR expression was found more frequent in ER-positive tumours with accounted for 75% (42). AR expression was found approximately 30% in TNBC (42).

The prognostic role of AR in patients with TNBC is controversial. Several studies suggested a negative prognostic role of AR in TNBC. In detail, a study showed that high expression of AR was observed in 17.7% of patients, with the cut-off value for high expression was 10 % of tumour cells positive. Patients with AR expression TNBC tumours had poorer overall survival (45). In addition, patients with AR-positive TNBC had a shorter disease-free interval following neoadjuvant chemotherapy (46). In contrast to these results, there is

growing evidence suggest that AR was associated with a favourable prognosis in LAR TNBC. A recent meta-analysis demonstrated that expression of AR was found in 24.4% of patients with TNBC and was associated with post-menopausal status, low tumour grade, and nodal involvement (47). Another meta-analysis demonstrated that AR-positive tumours were associated with a lower risk of recurrence, longer disease-free survival, but no association was found between overall survival and AR status (48).

Using antiandrogen therapy in metastatic breast cancer has been investigated. The first clinical trial was conducted in 2013 to report activity for treating patients with metastatic AR-positive TNBC. A phase II study that demonstrated a clinical benefit rate (defined as having complete response, partial response or stable of disease) of 19% with a median progression free survival of 12 weeks, suggesting the potential role of targeting AR in TNC (49). A new generation AR antagonist, enzalutamide, was investigated in AR-positive TNBC. Patients with AR-positive tumours defined as having AR IHC $\geq$ 10% had a clinical benefit rate at 16 and 24 weeks of 35% and 29% with the progression free survival of 32 weeks (50).

Other pathways have been explored to enhance anti-AR therapy, such as the PI3K/mTOR pathway and cell cycle signalling (51). It has been shown that AR-positive TNBC cell lines with PI3K mutations had better sensitivity to PI3K/mTOR inhibition (51). Moreover, recent work from our lab demonstrated the prognostic role of cytoplasmic IKKα and its phosphorylation of threonine 23 in prostate cancer. High expression of IKKα was associated with low AR expression and higher cancer specific survival in hormone naïve prostate cancer. However, a high level of nuclear IKKα was associated with a lower time to death from recurrence in castration-resistant prostate cancer (52). Therefore, this thesis will also investigate the role of AR in combination with IKKα and its phosphorylation to demonstrate if they have an enhancing effect in the TNBC subgroup.

Breast cancer is a collection of heterogeneity disease. Each of breast cancer subtype has different in histology and clinical outcomes in terms of response to treatment, risk of disease recurrence and sites of metastasis. The major challenges of treatment in patient with luminal disease are remaining of recurrence risk after 5 years (53, 54). In addition, prognostic indicator to assess the risk of disease recurrence are still insufficient to determine appropriated treatment and minimise treatment toxicities (53).

Moreover, there is not currently available of molecular-based targeted therapy for early stage TNBC with only about 20% of patients that response to standard chemotherapy (54). Although advances in tumour subclassification can separated TNBC to six unique subtypes, this classification has not yet translated to specific choice of treatment except those patients with germline BRCA1/2 mutation who have higher response to neoadjuvant anthracycline and taxane-based chemotherapy, and response to platinum-based chemotherapy.

Therefore, studies that investigate molecular markers or prognostic indicators are needed in order to provide the specific choice of treatment and avoid treatment toxicities in breast cancer, especially in TNBC tumour with limited choice of treatment apart from standard chemotherapy.

### 1.4.8 Prognostic genetic platform

Several gene-based assays for breast cancer are used to define molecular subtypes, prognostic value and predictive response to systemic therapy, including endocrine and chemotherapy (28). Among these assays, only the 21-gene assay (Oncotype Dx®) has been clinically validated to predict the benefit of adding adjuvant chemotherapy in the low-risk group of patients to further reduce the risk of disease recurrence (55-58). The Oncotype DX test or a 21-gene assay is performed on formalin-fixed paraffin-embedded tissue (55-58). A panel of 16 cancer-related genes and 5 reference genes is used to compute a recurrence score (RS) with a range from 0 to 100 (55-58). The RS is used to determine the prognostic value in patients with ER-positive, HER2-positive tumour treated with endocrine therapy and also has been used to predict the benefit of giving adjuvant chemotherapy to patients with HR-positive, HER2-negative, node-negative breast cancer. Patients in this group with RS $\geq$ 31 (high risk) have a higher risk of distant recurrence, therefore they will have a clear benefit from receiving adjuvant chemotherapy (58).

MammaPrint is a 70-gene assay that is used to identify patients with a low risk of distant recurrence regardless of high-risk clinical features (based on tumour grade, size and nodal status) (59). In subgroup analysis of a study that used a combination of clinical criteria (using Adjuvant Online) and genomic analysis via the MammaPrint assay, it was demonstrated that patients with high clinical risk/low genomic risk only had a minimal benefit from receiving adjuvant chemotherapy (59).

A 50-gene assay (PAM50) is used to categorise patients with ER-positive disease into low, medium and high-risk groups (60). A study demonstrated that patients with lymph node negative tumours and low PAM-50 risk of recurrence score (ROR) had a risk of distant recurrence of 5% whereas patients with high ROR had a risk of distant recurrence of 17.8%.

Although multigene assays provide useful prognostic value in considering the addition of adjuvant chemotherapy to adjuvant endocrine therapy, these types of tests are still limited in low-income countries. A study that focuses on affordable genomic testing is essential for low-income countries to provide comparable treatments and reach comparable survival rates to that of high-income countries.

### 1.4.9 Integrating of multi-gene signatures into intrinsic breast cancer subtype

Intrinsic subtype classification based on combining the classical well-defined immunohistochemical markers recommended by the St Gallen international expert panel is an uncomplicated and practical approach that helps therapeutic decision-making for physicians. Although current treatments provide better survival in early breast cancer, the risk of distant recurrence after five years remains, especially in patients with ER-positive/HER2 negative cancers (53).

It was uncertain whether adding adjuvant chemotherapy is beneficial for all these patients to prevent disease relapse (54). Previous work by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) in 2012 demonstrated that only 10% of patients benefit from adjuvant chemotherapy (54). However, none of the classical clinicopathological parameters (age, tumour size, lymph node involvement, tumour grade, ER/PR and HER2) contributed to determining any of these patients (54). Therefore, in most patients with the luminal disease (ER-positive/HER2-negative), the traditional clinicopathological parameters are insufficient to distinguish patients who benefit from adjuvant hormonal treatment alone or a combination of hormone-chemotherapy (54). Multigene assays have demonstrated better efficacy and reproducibility, result in providing additional predictive power to predict the ten years risk of disease recurrence for these patients, resulting in helping the clinician to better classify patients who benefit from adjuvant hormonal treatment alone or in combination with chemotherapy therapy (54).

To date, different commercial multigene tests are available such as the 21-gene assay (Oncotype Dx®), MammaPrint and A 50-gene assay (PAM50). These assays were clinical validated and economic validated in the United States (US), the European Union (EU), and the UK (61-65). However, these tests are not included in reimbursement schemes in all countries (66). Among these assays, the Oncotype Dx® is the only validated multigene assay that has an IA level by ESMO guideline, a strong recommendation by ASCO, a level I recommendation from NCCN guideline and has been clinically used in the US and the European countries (61-65). In addition, the Oncotype Dx® is likely to be cost-effective in patients with LN-negative disease and an NPI of more than 3.4 and is recommended by NICE guidelines (63).

## 1.5 Treatment of invasive breast cancer

Therapeutic options for patients with invasive breast cancer are complex and varied. Clinical staging allows an efficient identification for local treatment and helping in determining systemic treatment options. These options classified for patients with early breast cancer and those with advance disease. This thesis covers only treatment options for patients with early breast cancer.

Treatment for patients with early breast cancer involves multimodalities, including local treatment (surgery and radiotherapy) and systemic treatment (chemotherapy, endocrine therapy and molecular targeted therapy), delivered in various sequences. The following section will describe local treatment first (Surgery and radiotherapy) follow by systemic treatment (chemotherapy, endocrine therapy and targeted therapy).

### 1.5.1 Surgery

Surgery is the initial treatment for patients with clinical stage I or II disease and is classified as breast-conserved therapy (BCT) and mastectomy. Currently, in western countries, BCT followed with radiotherapy (RT) is the preferred local treatment for women with newly diagnose breast cancer (67, 68). This type of surgery removes the tumour mass and leaves as much normal breast as possible with clear resection margins and an acceptable cosmetic result (69). The aim of these modalities is to preserve cosmetic appearance and to keep a low rate of recurrence. Randomise clinical trials were conducted to compare mastectomy and BCT and demonstrated an equivalent result in terms of distant-disease-free survival and overall survival (69).

Modified radical mastectomy (MRM) is another surgical option for those patients who are not candidates for BCT such as patients with large tumour size, multicentric tumours, prior radiation to the chest wall of the breast or contraindications for RT, and unable to achieve negative surgical margins (67, 68) The entire breast and axillary lymph node are removed (level I and level II) (67, 68).

Axillary lymph node dissection (ALND) is the standard treatment for patients with biopsy proven axillary metastasis. The level I and level II lymph nodes dissection with at least 10 lymph nodes are required for providing accurate axilla staging (67, 68) . However, ALND is associated with lymphedema of upper limbs especially in patients who had undergone axillar node dissection combined with RT (67, 68). Therefore, sentinel lymph node (SLN) biopsy is recommended for patients that are clinically node negative. SLNB allows identification of the first lymph node that is responsible for lymphatic drainage from the breast. The SLN biopsy is identified by injection of blue dye or radioactive colloid into the breast, which will then accumulate in the drainage lymph node (67, 68). After SNL biopsy, no further axillary surgery is required for patients with T1 or T2 tumours and 1 to 2 positive SLNs. Level I and II axillary dissection is required for patients who have not met any of these criteria (67, 68) .

Level of lymph node:

Level I = Inferolateral to the pectoralis minor muscle
Level II = Posterior to the pectoralis minor muscle and below the axillary vein
Level III = Medial to the pectoralis minor muscle and against the anterior chest wall

### 1.5.2 Radiation therapy

The aim of adjuvant radiotherapy is to eradicate some microscopic tumour foci that might remain after breast-conserving surgery (70). The report from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) indicates that adjuvant radiotherapy after breast-conserving surgery halves the 10-years risk of locoregional recurrence and reduced the rate of annual breast cancer death by a sixth (70). Whole-breast RT (WBRT) is a standard of care for most patients treated with breast-conserving surgery and those patients treated with mastectomy with high risk of recurrence (71). There are two types of WBRT, which have been shown to have an equivalent result in tumour control. Firstly, conventional schedules delivered to the whole breast in 1.8 to 2 Gy daily fractions for 4.5 to 5 weeks with a total

dose of 45 to 50 Gy. Secondly, Hypofractionated schedules deliver a higher radiation dose at each interval but for a shorter duration (40-42.5 Gy for 3 to 5 weeks).

### 1.5.3 Adjuvant systemic treatment

Decisions on the use of systemic adjuvant treatment are based on recurrence risk and prediction of sensitivity to particular types of treatments. The decision also includes short- or long-term treatment related toxicity, the patients' health status and comorbidities. Standard criteria that are used to estimate responsiveness to treatment include tumour size, grade, lymph node involvement, ER/PR status, HER2 status and intrinsic subtypes.

Adjuvant systemic treatment for patients with invasive breast cancer can be applied with regards to their intrinsic subtype as shown in Figure 1.2.

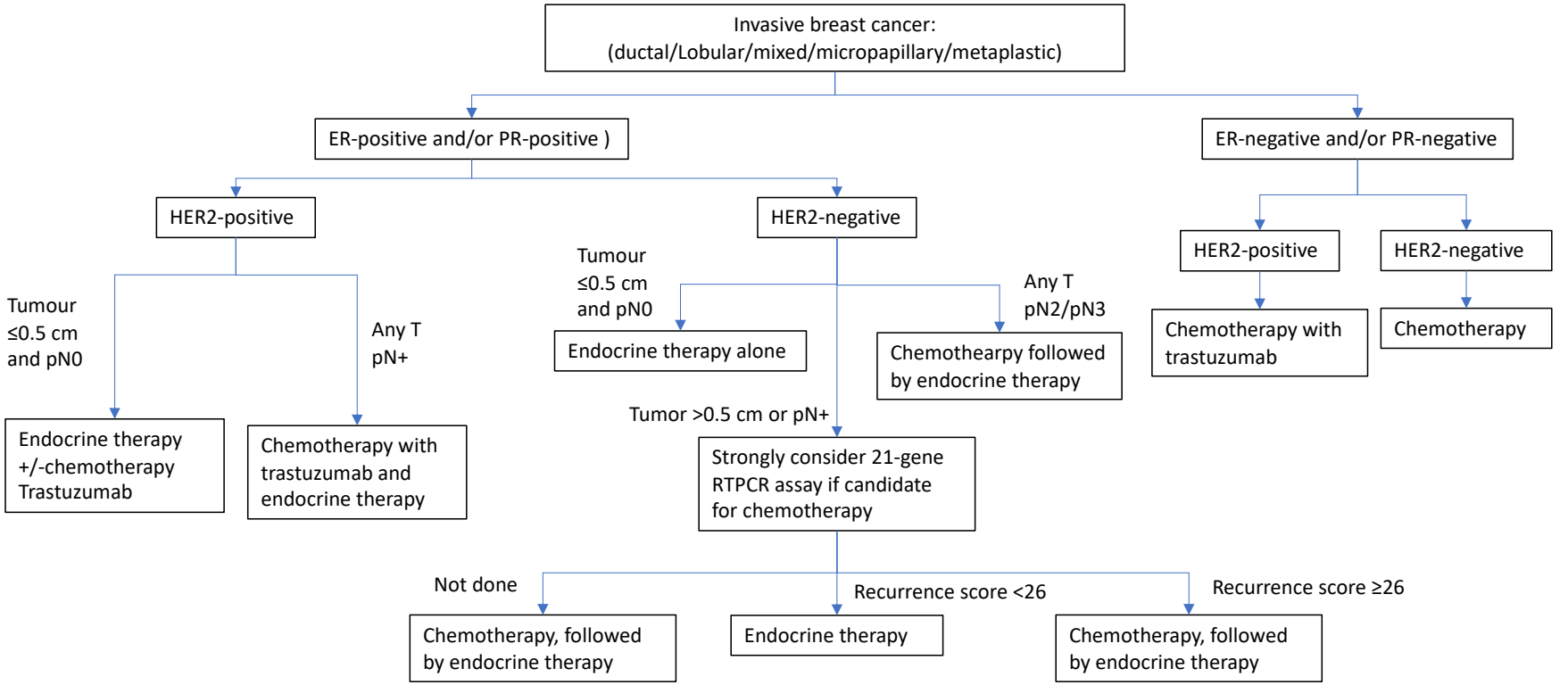


**Figure 1.2 Flowchart represent adjuvant systemic treatment recommendations for early-stage breast cancer.** *Pathological node (pN0), Tumour size (T)*

## 1.5.4 Chemotherapy

Adjuvant chemotherapy is an essential strategy to lower risk of recurrence and improve survival, regardless of patient age, ER status, or whether patients received adjuvant endocrine treatment (37, 72, 73). This strategy is recommended for patients with node-positive and high-risk node-negative tumours such as grade 3 tumours, high Ki-67, ER-negative tumours, HER2-positive tumours, TNBC tumours, high Oncotype DX RS, high-risk Mammaprint signature (37). Taxane-based and anthracycline-based chemotherapy is superiority over cyclophosphamide, methotrexate and 5-fluorouracil-based or non-anthracycline regimens (54). For adjuvant anthracycline-based regimens versus no chemotherapy, the risk reduction for breast cancer death is 0.84 and the risk reduction for any recurrence was 0.79 (54). For adjuvant taxane-based regimens, the risk reduction was 0.87 and 0.86 for breast cancer death and any recurrence, respectively (Figure 1.3).

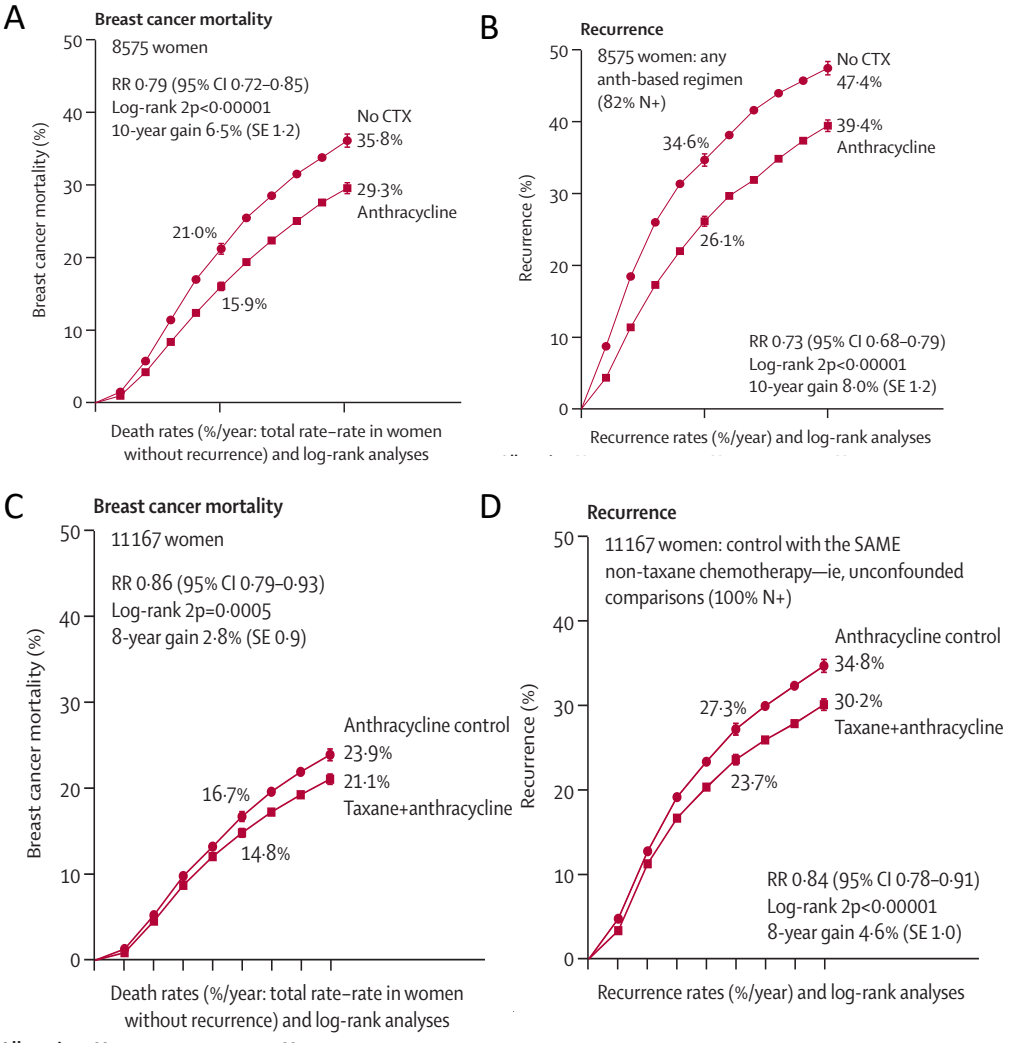


**Figure 1.3 Breast cancer mortality and time to recurrence for adjuvant chemotherapy (54).**

*Time to breast cancer death (A) and time to recurrence (B) for anthracycline-based chemotherapy versus no chemotherapy. Time to breast cancer death (C) and time to recurrence (D) for taxane-based chemotherapy versus anthracycline-based chemotherapy.*

The luminal A subtype is less responsive to chemotherapy (37, 68). Thus, the majority of these cancers do not receive chemotherapy, except for those patients with aggressive tumour features including high grade, proliferation and vascular involvement (37, 68). There are several tests that can be considered if there was uncertainty regarding the use of adjuvant chemotherapy, including urokinase plasminogen activator– plasminogen activator inhibitor 1 (uPA-PAI1) and gene expression assays such as MammaPrint and Oncotype DX (37, 68). These tests are recommended for patients with low-risk tumours (pT1a-b, G1, ER high, pN0), patients with comorbid disease who are not candidates for adjuvant chemotherapy or special types of cancer that have excellent prognosis such as solid papillary carcinoma and low-grade encapsulated papillary carcinoma (37, 68).

Preoperative or neoadjuvant chemotherapy is considered for patients with locally advanced breast cancer (stage III disease, T3 or T4 lesions) and patients with stage I or stage II disease who plan to have breast-conserving surgery but a high tumour-to-breast ratio that would have suboptimal cosmetic outcomes. Meta-analysis demonstrated no increase or decrease in survival with preoperative versus postoperative chemotherapy (74). Patients with unicentric, higher grade, HER2-positive tumour or TNBC tumour often respond dramatically to chemotherapy, therefore, they will be most likely converted to BCT after neoadjuvant chemotherapy. It has been reported that patients with TNBC tumours and HER2 positive tumours have pathological complete response (pCR) rate that exceeds 50% (75, 76).

### 1.5.5 Biological therapy

Over expression or amplified HER2 is present in almost 15% of primary breast cancers and is an adverse prognostic factor, which is associated with a high risk of recurrence, low response to tamoxifen and CMF (cyclophosphamide, methotrexate, fluorouracil)-based regimen (77, 78). Evidence has demonstrated that addition of trastuzumab, the humanized monoclonal antibody against the HER2 protein, to chemotherapy improved disease-free survival and overall survival for patients with HER2 overexpressing breast cancer. Patients treated with adjuvant trastuzumab had a 48% reduction in risk of recurrence and a 39% reduction in the risk of death from any cause (Figure 1.4) (79).

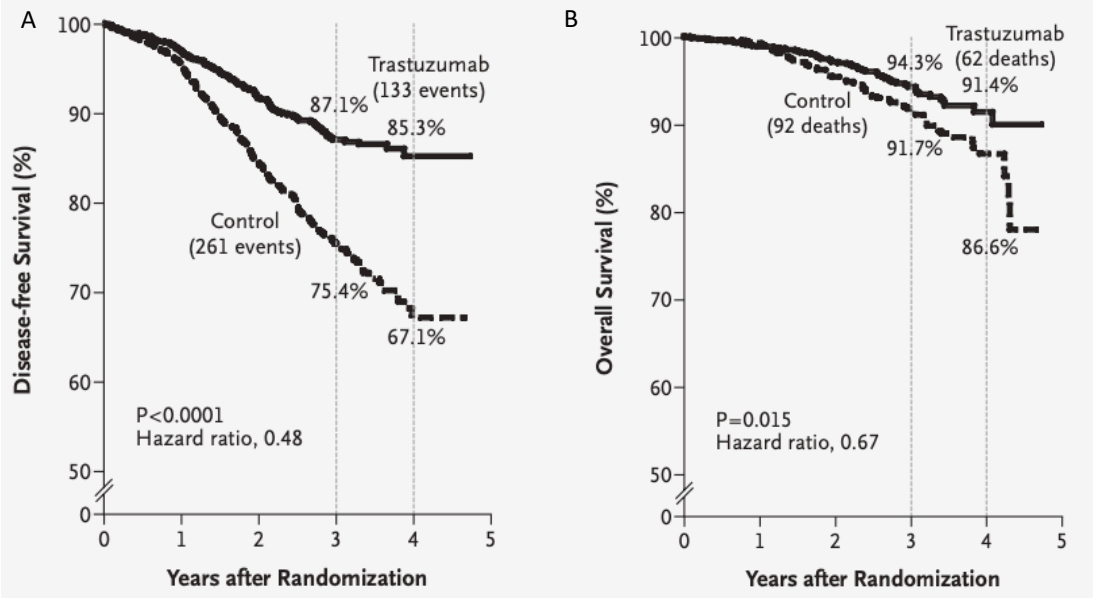


**Figure 1.4 Kaplan-Meier curve for adjuvant trastuzumab (79).** *Kaplan-Meier curve for disease free survival (A) and overall survival (B)*

## 1.5.6 Endocrine therapy

Endocrine therapy is one of the three systemic treatment modalities that are widely used as adjuvant treatment for early-stage breast cancer and is a standard treatment for patients with ER or PR positive tumour (>1%). There are three types of endocrine therapy in breast cancer: selective oestrogen receptor modulators (SERMs), aromatase inhibitors and selective oestrogen receptor down regulators (SERDs) (Figure 1.5).

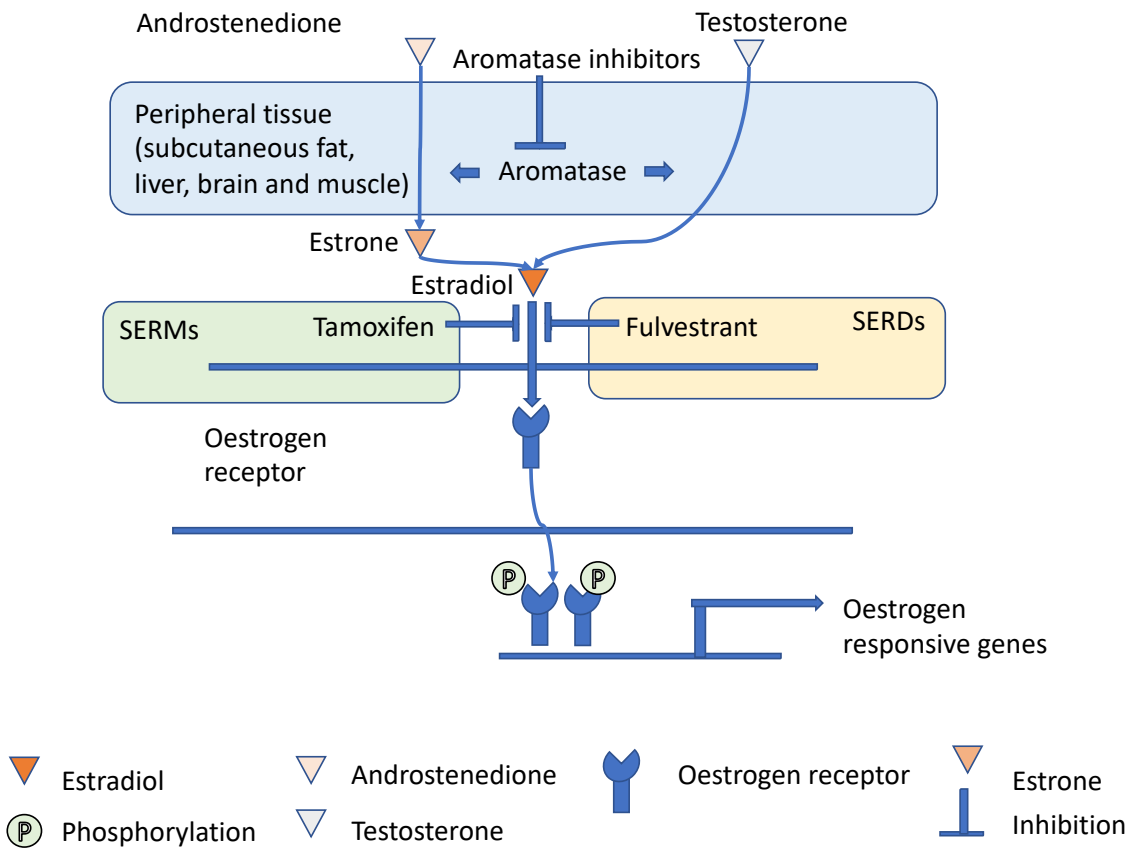


**Figure 1.5 Mechanism of action of endocrine therapies in breast cancer**

### 1.5.6.1 Selective oestrogen receptor modulators (SERMs)

SERMs are competitive inhibitors of oestrogen binding to oestrogen receptor (ERs) in breast tissue. There are three agents in this group, tamoxifen, raloxifene and toremifene. All of these agents have a mixed agonistic and antagonistic effect, depending on their target tissue. Tamoxifen is the most strongly established adjuvant endocrine therapy for both premenopausal and postmenopausal women. 5 year therapy of tamoxifen reduces the annual rate of death by 31% and the annual rate of recurrence by 39% (72). A study of 10 years extended therapy with tamoxifen demonstrated that, the recurrence risk during years 5 to 14 was 21.4% in patients receiving tamoxifen versus 25.1% in the control group. Although extended adjuvant treatment provided an interesting outcome, several side effects were reported including increased risk for pulmonary embolism and endometrial cancer (80).

### 1.5.6.2 Aromatase inhibitors

Aromatase inhibitors (AIs) inhibit function of the aromatase enzyme that converts androgens (androstenedione and testosterone) to oestrogen (estrone and oestradiol) leading to oestrogen

depletion (81). They are classified into type 1 and type 2 inhibitors. Type 1 inhibitors are steroidal analogues of androstenedione that irreversibly bind to aromatase enzyme, these agents include formestane and exemestane (81). Type 2 inhibitors are nonsteroidal and reversibly bind to aromatase enzyme, these agents include anastrozole and letrozole (81). AIs reduce oestrogen production by 90% to almost undetectable levels (81). Adjuvant AI is a gold standard and highly recommendation for postmenopausal women (68). Adjuvant AI has been investigated as upfront therapy, as a sequential therapy following 2 or 3 years of tamoxifen and as extended therapy following 5 years of tamoxifen (82-86). A modest improvement in disease free survival (DFS) has been observed for each strategy (82-86). Current clinical practice guidelines recommend that adjuvant AIs is considered at some point as either upfront therapy or as a sequential therapy after tamoxifen (68). There are several side effects of AI treatment, including associations with accelerated osteoporosis and a high incidence of bone fracture (82-86). Therefore, monitoring of bone mineral density is required in patients receiving AIs treatment (82-86). Furthermore, both tamoxifen and AIs are associated with menopausal symptoms, including sexual dysfunction and hot flashes (82-86).

#### 1.5.6.3 Selective oestrogen receptor down regulators (SERDs)

Fulvestrant is the only approved agent that acts as a competitive antagonist of oestrogen binding to ER, resulting in an inhibition of ER dimerization and reduction of nuclear translocation leading to inhibition of oestrogen dependent gene transcription (87). Fulvestrant is used in the metastatic setting and demonstrated significantly improved progression free survival (16.6 vs 13.8 months) when compared with anastrozole (68).

### 1.5.7 Neoadjuvant systemic treatment or preoperative treatment

Neoadjuvant systemic treatment is a treatment given before surgery for non-metastatic breast cancer. The goal of this option is to induce tumour response before surgery and facilitate breast-conserving therapy (68). This option also provides prognostic information based on response to treatment. Pathological complete response (pCR) is a strong prognostic factor and is associated with better disease free and overall survival (88). This associated is strongest in patients with TNBC when the pCR rate exceeds 50% (75, 76, 89).

Preoperative or neoadjuvant chemotherapy is considered for patients with locally advanced breast cancer (stage III disease, T3 or T4 lesions). This option is also recommended for

patients with stage I or stage II disease who plan to have breast-conserving surgery but have a high tumour-to-breast ratio that would have suboptimal cosmetic outcomes (68).

The same regimens are used for neoadjuvant chemotherapy (NAC) as for adjuvant chemotherapy. However, the addition of taxanes to an anthracycline-containing regimen has been shown to have significant rates of pCR (74). HER2 targeted therapy and chemotherapy are candidates for patients with HER2-positive tumours. Pertuzumab is a monoclonal antibody that also binds to the HER2 protein. Dual Anti-HER2 blockade with trastuzumab and pertuzumab has demonstrated significant improvements in pCR range from 57% to 66% (90).

Neoadjuvant endocrine therapy alone can be considered for patients with strongly ER-positive tumour (ER≥10%) (91). Aromatase inhibitors or tamoxifen are the options for neoadjuvant treatment in premenopausal women (59, 92). However, AI is the preferable option for postmenopausal women (91). A study has demonstrated that patients treated with anastrozole have a higher objective response when compared with tamoxifen (39.5% vs 35.4%) (91).

## 1.6 NF-κB pathway

The molecular mechanisms that promote cancer have been characterised as the hallmarks of cancer, including sustaining proliferative signalling, evading growth suppressors, resisting cell death, replicative immortality, angiogenesis, invasion and metastasis, genomic instability, reprogramming of energy metabolism and avoiding immune destruction (93, 94). Underlying these hallmarks are genetic and epigenetic abnormalities that promote their abnormal levels, functions and interactions of proteins and signalling pathways. In addition to ER and HER2 pathway, numerous alterations have emerged crucial drivers of breast cancer progression and resistance to treatment. An alteration in the phosphatidylinositol 3-kinase (PI3K) pathway and mechanistic targeting of the rapamycin (mTOR) pathway plays a critical role in endocrine resistance (95). Cyclin-dependent kinases (CDKs), a large family of serine–threonine kinases, have also been indicated as key factor in breast cancer cell cycle regulation (96). A highly diverse collection of tumours from TNBC also demonstrated key drivers that promote tumour heterogeneity and aggressiveness, they include, androgen receptor (AR) and PD1/PD-L1 pathway (97),

There is also growing evidence that the nuclear factor kappa B (NF-κB) pathway plays a pivotal role in cancer development and progression. In breast cancer, NF-κB has been demonstrated to be involved in tumour progression and resistance to treatment. Therefore, the NF-κB pathway represents an attractive target for translational research to investigate the potential prognostic and therapeutic role of its key members.

NF-kB was first identified as a specific transcription factor that binds to the kappa immunoglobulin gene enhancer in the nuclei of mature B cells (98). It is now known that NF-κB activity is found in all cell types and regulates transcription of various genes that are involved in immune and inflammatory responses. With the appreciation that infection and chronic inflammation are associated with carcinogenesis, the NF-κB pathway has been demonstrated to be key mechanistic link between inflammation and cancer. There are two major pathways that have been identified, the canonical and non-canonical NF-κB pathways (99).

### 1.6.1 The NF-κB family members

The NF-κB family consists of five members: NF-kB1 (p50 or its precursor p105), NF-kB2 (p52 or its precursor p100), RelA (p65), RelB and c-Rel (Figure 1.6). All NF-κB proteins

carry a Rel-homology domain (RHD), which contains a nuclear localisation sequence (NLS) and is responsible for dimerization, DNA-binding and interplay with the inhibitor of nuclear factor kappa B (IκB). A transcription activation domain is also present in p65, RelB and c-Rel and is responsible for transcriptional activity. The NF-κB family members form homo- and hetero-dimers that regulate specific target gene transcription. In the resting state, NF-kB dimers are retained in the cytoplasm by IkBs that interaction with the RHD and prevent its NLS from functioning. The IκBs proteins consist of IκBα, IκBβ, BCL-3, IκBϵ, and IκBγ, which contain ankyrin repeat motifs that are responsible for binding to the RHD (99, 100). These repeat motifs are also found in the C-terminal of p100 and p105, which also serve as IkBs to inhibit their nuclear translocation and retain the heterodimers in the cytoplasm (101, 102). Upon phosphorylation by an IκB kinase (IKK), the IκBs protein is ubiquitinated and subsequently degraded by proteasomes. Following this step, the NFκ-B dimer is release and translocates into the nucleus to interaction with co-activators or co-suppressors in order to regulate the transcription of its target genes. There are two recognised NF-κB pathways, the 'canonical' and 'non-canonical', which are activated by distinct stimuli and regulate distinct downstream cascades.

### 1.6.2 Canonical NF-κB pathway

The canonical NF-κB pathway is mediated by kinase complexes in response to various stimuli, including pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin-1 (IL-1); and pathogen-associated molecular patterns (PAMPs) such as the bacterial endotoxin lipopolysaccharide (LPS). Binding of these ligands to their receptor results in recruitment and activation of a kinase complex, which consists of the regulatory subunit NF-κB essential modulator (NEMO or IKKγ) and the two catalytic subunits IKKβ and IKKα (Figure 1.6). IKKβ has been demonstrated as a key kinase in the canonical NF-κB pathway and is responsible for phosphorylation of the IκB (103). Generally, the IκB binds to p65/p50 dimers that cause their cytoplasmic retention. After phosphorylation, the IκB is ubiquitinated and subsequently degraded by the proteasome, resulting in release of the p65/p50 dimer allowing them to translocate to the nucleus to activate transcription of their target genes, which are mainly required for suppression of apoptosis in response to TNF-α (103, 104).

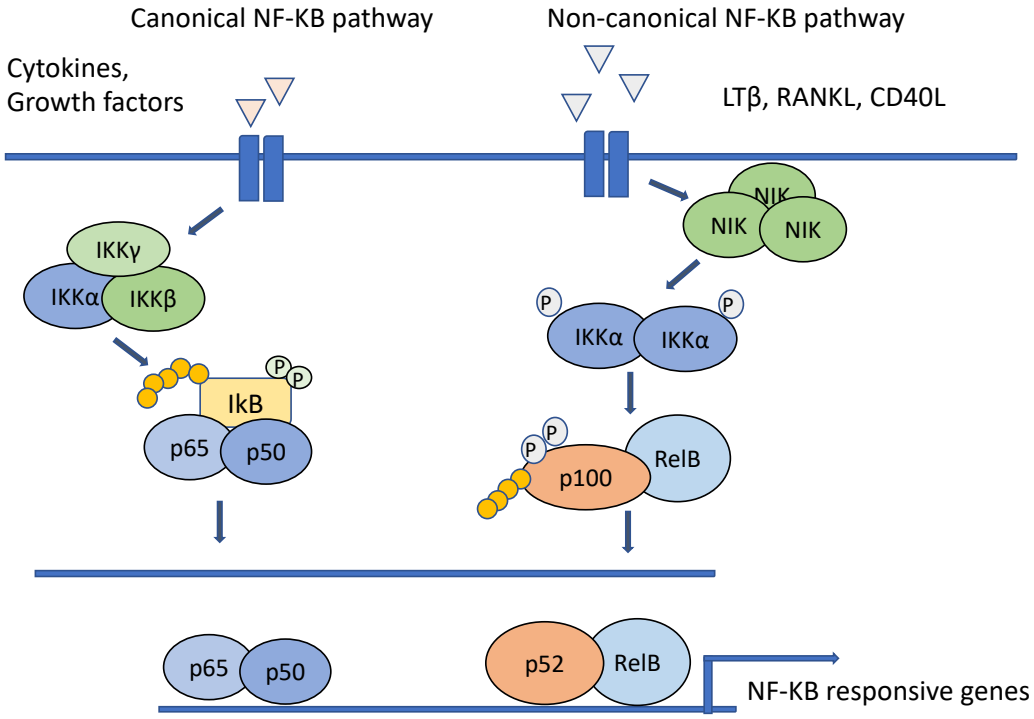


**Figure 1.6 Schematic represent canonical and non-canonical NF-κB pathway.**

## 1.6.3 Noncanonical NF-κB pathway

The canonical and non-canonical NF-κB pathways typically function as a coordinative system. Based on initial studies, the function of the non-canonical pathway is thought to be more selective and is activated by a specific group of stimuli including TNFR family members, such as BAFF-R (B cell–activating factor receptor) and CD40L (on B cells), receptor activator of nuclear factor kappa-B ligand (RANKL) and lymphotoxin-beta (LTβ) (105-107). Activation through their receptors enables accumulation and stabilisation of NIK, which is a member of the MAP kinase kinase kinase (MAP3K) family. Upon stabilisation, NIK phosphorylates and supports recruitment of IKKα to further phosphorylate p100, which typically exists in a heterodimer complex with RelB, resulting in proteasome-dependent processing of p100 to p52. A heterodimer of p52-RelB is then enabled to translocate into the nucleus in order to stimulate transcription of their target genes, which are mainly involve in lymphoid organ development and the adaptive immune response (Figure 1.6) (99).

## 1.6.4 The IκB kinase (IKK) subunits

IKKα and IKKβ are crucial kinases of the IKK complex. They have been purified and cloned to investigate their ability to phosphorylate the inhibitory IκB (108).These two kinases contain a similar structure (50% sequence identity), including a N-terminal protein kinase

domain, a helix-loop-helix (HLH) that controls IKK kinase activity, and a C-terminal leucine zipper (LZ), which has role in homo- or heterodimerization (109). However, significant difference in biological function between these two kinases have been identified (110).

### 1.6.5 IKKβ

IKKβ deficient mice die within several weeks of gestation due to massive liver apoptosis, which has resulted from increased sensitivity to TNF-α-induce apoptosis (111, 112). Similar results have been demonstrated in mice that are deficient for p65, they died during fetal development due to a severe deficiency of fetal liver-derived lymphocytes (113). These results suggest a major role of IKKβ in the canonical NF-κB pathway is activation of the innate immune response. Activation of IKKβ is dependent on phosphorylation at serine 177 and serine 181 within the activation loop of the kinase domain (114).

### 1.6.6 IKKα

Although IKKα and IKKβ are similar in structure, different phenotypes between their single knockout mice indicate distinct physiological roles for them (115). IKK-α deficient mice ~~can~~ survive after birth, but suffer from multiple morphological defects, including skeletal development and severe defects in epidermal differentiation, which are related to perinatal death (115). While IKKβ is detected predominantly in the cytoplasm, IKKα can be detected in both the cytoplasm and the nucleus (116-118). The discovery of nucleo-cytoplasmic shuttling of IKKα led to the first identification of a nuclear role of KKα in NF-κB mediated gene expression, including IkBα and IL-8 (117, 119). Nuclear IKKα has also been demonstrated to be involved in cell cycle, apoptosis and tumour progression in various type of tumour including breast, colon, prostate, pancreatic and gastric cancer (120-124).

Several potential mechanisms of IKKα nuclear translocation have been identified. The kinase domain of IKKα has been shown to contain a nuclear localization sequence (NLS), which is not found in IKKβ, and is believed to be involved in nuclear transportation (125). Mutation of this residue led to impaired nuclear translocation of IKKα (109). As IKKα can translocate into the nucleus in response to various stimuli, including TNF-α, oestrogen, EGF, and cisplatin, it is reasonable that these signalling pathways are also important for regulation of nucleo-cytoplasmic shuttling of IKKα. In contrast to the wild type form, a kinase mutant of IKKα has been demonstrated to have decreased nuclear accumulation, indicating that kinase activity is also needed for IKKα nuclear translocation.

## 1.6.7 IKKα activation and its phosphorylation

Activation of IKKα requires phosphorylation of Serine 176 and Threonine 23, resulting in a conformational change in the activation loop, which provides the kinase domain catalytically active (126). It has been shown that each phosphorylation site is responsible for specific upstream activator results in controlling their particular downstream pathway (114, 126, 127).

### 1.6.7.1 Phosphorylation of IKKα on serine 176

Phosphorylation of IKKα on serine 176 (Ser176) has been demonstrated to be a classical activation site that responds to activation from NIK in the non-canonical NF-κB pathway. Activation through this site leads to a conformational change in the activation loop of IKKα, which activates p100 and induces its ubiquitination and proteolytical processing to p52. Replacement of serine 176 with alanine prevents IKKα activation resulting in inhibition of the processing of p100 to p52 in the non-canonical NF-κB pathway (114, 126). Activation through serine 176 on IKKα has been known as NF-κB dependent function activity of IKKα and response to activating a transcriptional process of genes involved in a proinflammatory process such as IL-6, cell cycle gene such as cyclin D1, and extracellular protease such as MMP3 (128).

### 1.6.7.2 Phosphorylation of IKKα on threonine-23

The phosphorylation of IKKα on threonine-23 (Thr23) is strongly dependent on AKTT (127). In response to TNF activation, AKT increased IKKα phosphorylation significantly, whereas minor phosphorylation was observed on the IKKα mutant (T23A), which was threonine 23 was mutated to alanine (T23A) (127). These finding was confirmed by several subsequent studies and demonstrated that only IKKα was phosphorylated by AKT but not IKKβ (129). In the hepatocellular carcinoma cell line, PI3K/AKT pathway has been shown to stimulate the IKK complex by phosphorylation of IKKα on threonine 23, which subsequently activate the transcription activity of NF-κB (129). The activation of NF-κB by AKT was based on phosphorylation of p65 at serine 534, induced by the IKK complex that consists of IKKα, IKKβ and NEMO. In response to TNF stimulation, AKT stimulates IKK complex activity by phosphorylation on threonine 23 in the IKKα subunit. The IKK complex then phosphorylates both the p65 at Serine 534 and the IκB protein, resulting in enhanced transcriptional activity of NF-κB (130). There is growing evidence that the nuclear function of IKKα is dependent on activation at threonine 23, which contributes to tumour progression

via both NF-κB dependent and NF-κB independent activity. Moreover, activation at threonine 23 on IKKα bridges the NF-κB pathway with several parallel pathways, including the signalling cascades initiated by phosphatidylinositol 3-kinase (PI3K) and Akt (129, 130).

## 1.7 NF-κB/IKKα and cancer

NF-κB has been demonstrated to be a key transcription factor that links inflammation and cancer. Activation of NF-κB has been reported in hematologic malignancies including acute myelogenous leukaemia (AML), lymphomas and multiple myeloma (131-133). NF-κB is also involved in tumour progression in various types of solid tumours, including prostate cancer, breast cancer, melanoma, pancreatic and colon cancer (134, 135). NF-κB contributes to cancer development through multiple mechanisms, including enhancing or maintaining a chronic inflammatory microenvironment, inducing tumour cell proliferation and preventing apoptosis, regulation of tumour angiogenesis and promoting tumour metastasis and resistance to treatment.

NF-κB promotes an accumulation of proinflammatory cytokines, including TNF-α, IL-1, and further induce a pro-tumorigenic microenvironment in patients with inflammatory bowel disease (IBD) (136). The tumour microenvironment in turn enhances tumour immune cell suppression and induces tumour escape from immunosurveillance. NF-κB promotes transcription of regulatory T cells (Treg) development by regulating the forkhead transcription factor family member (FOXP3) expression (137). NF-κB also induces expression of genes that are involved in anti-apoptosis, including c-IAP1/2 and XIAP (138). NF-κB induces tumour angiogenesis by regulating transcription of fibroblast growth factor (FGF), IL-8, and metalloproteinase-9 (MMP-9). In breast cancer, NF-κB has been extensively studied and has been demonstrated to be involved in multiple mechanisms.

## 1.8 NF-κB/IKKα and breast cancer

NF-κB activity has been shown to be involved in mammary gland development, and is elevated during pregnancy, suppressed during lactation and reactivated during involution (139, 140). IKKα has also been demonstrated as a key factor in the control of mammary epithelial proliferation in response to RANKL signalling. This activation occurs through a linear cascade that includes RANKL, RANK, IKKα, IκBα, NF-κB and cyclin D1 (141). As a crucial driver in this cascade, it is not surprising that aberrant activation of NF-κB/IKKα has been reported in development and progression of breast cancer.

Elevated NF-κB DNA binding activity has been observed in breast cancer cell lines, in rat mammary tumours when compared to normal rat mammary gland and in human primary breast tumour (142). Several members of the NF-κB family have been investigated and were reported to be overexpressed in breast cancer cell lines and in breast cancer tissue. These include p100, p65, p50, p52 and c-Rel. Over expression of growth factors, cytokines and the hypoxic condition that is common in the tumour microenvironment have been reported in contributing to constitutive NF-κB activation in breast cancer. These factors can induce expression of different genes that are involved in tumour invasion and metastasis though the NF-κB pathway. Although a number of genes are induced by NF-κB pathway, not all of them are overexpressed in breast cancer or indicated to be involved in breast cancer initiation, progression, metastasis and resistance to treatment.

### 1.8.1 NF-κB/IKKα in breast cancer initiation and progression

Tumour progression requires multiple processes, including, proliferation, angiogenesis and protection against apoptosis signals from host immune systems. Several genes that are involved in these processes are regulated by NF-κB/IKKα (143). It has been reported that IL-1 that is secreted from breast cancer cells can induce NF-κB activation in stromal fibroblast to enhance expression of IL-6 and urokinase plasminogen activator (uPA), both of which are potent inducers of angiogenesis (144).

Activation of p50/p65 heterodimer was observed before malignant transformation in MBA- or benzo[a]pyrene-induced mammary tumours in rats and human mammary epithelial cells (HMECs) in culture (145). The *c-myc* oncogene, which is known to be overexpressed in breast cancer is the primary target gene for this dimer (145, 146). Expression of p50/p65 was also observed to stimulate transcription of cyclin D1, a key player of $G_1$ checkpoint control (147). Upon stimulation by growth factors, cyclin D1 forms an active complex with cyclin dependent kinase 4/6 (CDK4/6) which phosphorylates retinoblastoma proteins (RB) and separates them from the E2F1 transcription factors, which will further stimulate cell cycle progression (148). Aberrant expression of cyclin D1 leads to uncontrolled cell proliferation. NF-κB binding sites were identified in the cyclin D1 promoter and are responsible for NF-κB induce transcription of the cyclin D1 (147). Inhibition of p50/p65 caused a reduction of growth factor-induced cyclin D1-associated kinase activity and further impaired cell cycle progression in breast cancer cells lines (147).

A study has also demonstrated that IKKα in conjunction with ERα and AIB1/SRC-3 can induce oestrogen-dependent gene transcription to promote cell proliferation in breast cancer cell lines (149). Oestradiol treatment induced phosphorylation of ERα at serine residue 118 by IKKα, and this result was prevented by silencing IKKα. AIB1/SRC-3 is a crucial activator that is required for oestradiol-induced activation of cyclin D1 and c-*myc* expression (149). The study also showed that upon treatment with oestradiol, AIB1/SRC-3 was phosphorylated at serine residue 857 by IKKα, again this response was reduced by IKKα siRNA (149).

### 1.8.2 NF-κB/IKKα in breast cancer metastasis

Tumour metastasis is the outcome of interactions between host cells and tumour cells and consists of a multistep process, including degradation of extracellular matrix; detachment from the primary tumour; invasion to surrounding tissue; intravasation to blood or lymphatic vessels; extravasation to interstitial space and colonisation in the target organ (150). Extracellular matrix degradation and intravasation requires two protease systems, including the matrix metalloproteinase such as MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13 and urokinase plasminogen activator (uPA)/uPA receptor (uPAR)/plasminogen system (151). Overexpression of uPA/uPAR and MMP-9 is essential for tumour migration and colonisation of various cancer cell lines, including breast cancer cell lines (152). NF-κB can promote extracellular matrix degradation, migration and colonisation to tumour cell by inducing expression of uPA and MMP-9 (153).

### 1.8.3 Crosstalk between NF-κB/IKKα and other pathways in breast cancer

Interactions between NF-κB/IKKα and other transcription factors or signalling pathways in breast cancer progression has been extensive studied, including ER, androgen receptor (AR) and EGFR.

#### 1.8.3.1 Crosstalk between NF-κB/IKKα pathway and ER signalling

Multiple mechanisms are involved in the crosstalk between NF-κB pathway and ER signalling. NF-κB directly interacts with the DNA-binding function of ER through collaboration with FOXA1 to enhance latent ER-binding sites and induce translation of their target genes (154). NF-κB interacts with ER co-activator or co-repressor, which will then alter ER transcriptional activity (149). NF-κB also suppresses ER activity through inducing

expression of the zinc finger repressor B-lymphocyte-induced maturation protein (BLIMP1), which can bind to the ER promoter region and repress ER transcription (155). Oestrogen induces the formation of the IKKα, ER and steroid receptor co-activator (SRC) 3 complex on the promoter region of cyclin D1 gene (149, 156). IKKα is in turn able to phosphorylate both ER and SRC3 to further increase their transcription activity (149, 156).

#### 1.8.3.2 Crosstalk between NF-κB/IKKα pathway and AR signalling

Androgen receptor (AR) is a member of the nuclear receptors family together with ER and PR. Overexpression of AR characterises a specific molecular subset of TNBC, named the luminal androgen receptor (LAR). Although, the mechanism by which AR contributes to the development of breast cancer is not clearly understood, it has been shown that androgens can induce proliferation in breast tissue and promote tumour formation in animal models (3). Several studied have demonstrated crosstalk between the NF-κB pathway and AR to promote tumour progression in prostate cancer and induce tumour resistance to treatment (11, 12). However, there is limited evidence on crosstalk between NF-κB and AR in breast cancer.

SRC3 is a steroid receptor coactivator that belongs to the nuclear receptors (NR) gene superfamily (157). It has been shown that SRC3 expression enhances initiation and progression of ER-positive breast cancer. SRC3 also functions as a co-activators for other transcription factors, including NF-κB and AR (157). It has been identified that SRC3 has a powerful interaction with AR. Binding potency between AR and SRC3 is correlated with AR mutations in prostate cancer (158). As a steroid receptor coactivator, it is possible that SRCs provide a key link between NF-κB and AR. However, evidence supporting this hypothesis is limited.

#### 1.8.3.3 Crosstalk between NF-κB/IKKα and EGFR pathway

A study has demonstrated that, upon treated with ionizing radiation, MCF-7/HER-2 cells that overexpress ER2 can activate the PI3K/AKT pathway, resulting in enhanced NF-κB activation. Following this activation, upregulation of several pro-survival genes was observed. These genes include a mitochondrial antioxidant enzyme manganese superoxide dismutase, cyclin B1 and HER-2 itself. Consequently, MCF-7/HER-2 cells become both radio- and chemo-resistant (159).

## 1.9 NF-κB-independent function of IKKα in breast cancer

### 1.9.1 Cell cycle regulation

D-type cyclins (cyclin D1, D2, and D3) are essential for G1 to S phase progression (160). Upon phosphorylation, the retinoblastoma tumour suppressor protein (pRb), cyclin D1, and its partner cyclin-dependent kinases (CDKs) liberates E2F to activate the expression of cyclin E and another gene that involve in cell cycle progression (160). Evidence has demonstrated that IKKα plays a critical role during cell cycle progression through several processes. IKKα induces transcription of cyclin D1 by phosphorylating ERα, AIB1/SRC-3, and histone H3 lead to enhance proliferation of breast cancer cells (149) (Figure 1.7).

IKKα is also demonstrated to regulate the M phase of the cell cycle by directly phosphorylating Aurora A at Thr288, increasing Aurora A activation and enhancing centrosome maturation (161). IKKα controls cell cycle progression by regulating E2F1-dependent transcription. Silencing of IKKα reduced transcription of E2F1 gene and other E2F1-responsive genes, including cyclin E, thymidine kinase 1 and proliferating cell nuclear antigen (PCNA) in breast cancer cell lines (162).

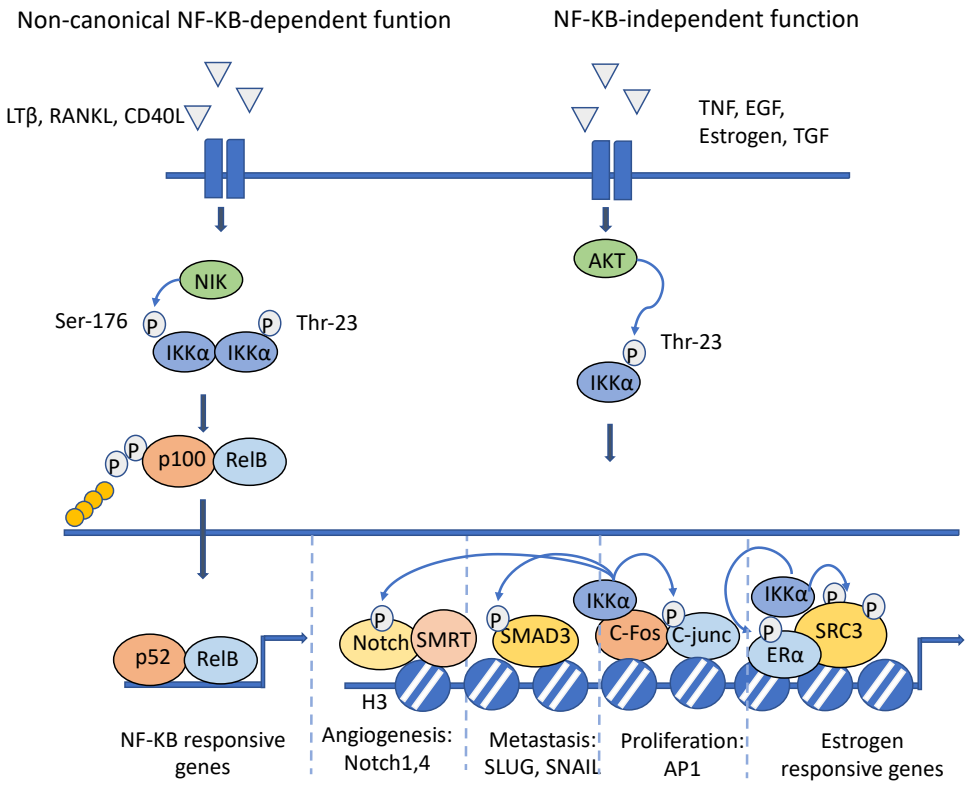


**Figure 1.7 Schematic represents the function of IKKα in non-canonical NF-κB-dependent and NF-κB-independent pathways.**

### 1.9.2 Tumour progression and metastasis

IKKα has been demonstrated to regulate transcription of the various gene that control tumour progression and metastasis. In response to EGF stimulation, IKKα phosphorylates c-Fos at Ser32 results in increasing stability and promoter recruitment of c-Fos to increase activating protein-1 (AP-1) gene transcription, which leads to enhance proliferation and migration of breast cancer cell line (163, 164) (Figure 1.7).

IKKα involves in Notch-dependent transcription to enhance angiogenesis and tumour invasion (165). IKKα derepress Notch activity by reducing the expression of FOXA2 transcriptional activity through phosphorylation on it at Ser107 and Ser111, which represses NUMB expression, a transcriptional product of FOXA2 (165). Without suppressing by NUMB, Notch will free and gain transcriptional activity to enhance the transcription of its targeted genes, including Notch-1 and Notch-4, responsible for tumorigenesis (165, 166).

IKKα has been involved in epithelial to mesenchymal transition (EMT) by inducing gene expression of SLUG and SNAIL transcription factors, reducing the adherent's junction protein E-cadherin (167). In response to TGFβ stimulation, IKKα translocases into the nucleus, interact with SMADs and regulates SMAD complex formation on SLUG and SNAIL promoter region, resulting in metastasis of breast cancer cells (167) (Figure 1.7).

### 1.9.3 Crosstalk with tumour microenvironment in inducing tumour progression

IKKα have been found to bridge the link between tumour and their microenvironment in castration-resistant prostate cancer (CRPC) (168). Tumour infiltrating B cells (TIBC) expressing lymphotoxin-β have been reported to induce activation and nuclear translocation of IKKα in prostate cancer cells. After activation by lymphotoxin-β, IKKα, in conjunction with STAT3, induces ligand-independent androgen receptor transcriptional activity in prostate cancer, promoting castration resistance and metastasis in prostate cancer (168). In prostate cancer, IKKα also has been reported to phosphorylate histone H3 at Ser10 on the promoter of maspin, a crucial suppressor of metastasis, through an unidentified mechanism (169).

IKKα have been found to bridge the link between tumour and their microenvironment in promoting metastasis of breast cancer cells (170). Tumour-infiltrating $FOXP3^+$ Treg cells

are a major source of RANKL during metastatic spread (170). Upon binding with RANKL, RANK phosphorylate and induce nuclear translocation of IKKα results in repress an expression of the metastasis inhibitor maspin, leading to induce metastasis of TNBC cell lines (170).

### 1.9.4 IKKα and non-canonical NF-κB pathway as biomarkers

Clinical studies have demonstrated the prognostic role of IKKα and other members of the non-canonical NF-κB pathway in breast cancer (171). A study of NF-κB-related gene expression showed that CHUK, which encode IKKα, was associated with poor relapse-free survival in patients with ER-positive tumours. In contrast, the expression of MAP4K4 (NIK) and RelB were associated with better relapse-free survival (172). Activation of RelB and p52 was associated with worse disease free-survival (DFS) in ER-positive breast cancer (171). Co-activation of both RelB and p52 demonstrated a stronger association with overall survival and early relapse (171). High expression of myoglobin, the non-canonical NF-κB target gene, was associated with shorter DFS (171). Cytoplasmic IKKα was associated with cancer specific survival and recurrence-free survival on tamoxifen in luminal A tumours (173).

### 1.9.5 Clinical-translational advance in targeting IKKα

The potential role of IKKα as a target of breast cancer has recently been highlighted in the development of inhibitors to target this kinase. The first-in-class selective IKKα inhibitors have recently emerged and demonstrated effective target engagement in a preclinical study (174). This compound inhibited IKKα without affecting IKKβ in the canonical NF-κB pathway (174).

In conclusion, this chapter summarises the evidence that demonstrated the non-canonical NF-κB pathway and the development and progression of breast cancer. It also reveals a crucial role of IKKα in controlling breast cancer cell progression and metastasis through NF-κB-dependent and NF-κB-dependent regulation. It also points out that IKKα plays a critical link between the NF-κB pathway and other crosstalk pathways, including ER, AR, and Akt pathways. Several clinical studies have demonstrated the prognostic role of IKKα breast cancer. However, evidence that focuses on the phosphorylation site of IKKα at Ser-176 and Thr-23 in breast cancer is limited. Therefore, investigating the prognostic role of IKKα and its phosphorylation is needed.

## 1.10 Research aims and hypothesis

This thesis hypothesis is that IKKα and its phosphorylation are associated with breast cancer development, resistance to treatment, and potentially a novel therapeutic target for breast cancer treatment. This thesis investigates the role of IKKα and phosphorylation at serine 176 (pIKKα-Ser176) and threonine 23 (pIKKα-Thr23) in breast cancer progression. Archival tissue will be used to investigate an association between clinical outcomes and the expression of IKKα, pIKKα-Ser176, and pIKKα-Thr23. Other proteins, including their upstream kinase, NIK, pAKT and another potential crosstalk protein such as androgen receptor (AR), will also be investigated in the discovery cohort of mixed breast cancer subtype and will validate in ER-positive tamoxifen-treated cohort and the TNBC cohort. The archival samples of TNBC with available gene expression profiles will be used to establish independent gene expression profiles associated with low and high expression of IKKα pIKKα-Ser176, and pIKKα-Thr23. Furthermore, our proprietary preclinical selective IKKα inhibitors, androgen blockade and a combination of both will be investigated in breast cancer cell lines to establish if IKKα represents a clinical target for breast cancer treatment alone or in combination therapy. In order to address the hypothesis, the main objectives were to:

- Establish if expression of IKKα, pIKKα-Ser176, or pIKKα-Thr23 could be employed as prognostic markers in breast cancer.

- Establish which downstream markers are associated with high IKKα-expression to serve as predictive biomarkers.

- Investigate whether inhibition of IKKα alone or in combination with AR offers a new therapeutic option for treatment of breast cancer.

# Chapter 2 Materials and methods

## 2.1 Tissue studies

Expression of IKKα, pIKKα-Ser176, pIKKα-Thr23, pAKT, and NIK was assessed using IHC on a tissue microarray (TMA) of breast cancer patients in order to determine whether these members of the pathway and their upstream kinases associated with clinical outcome in patients with invasive breast cancer. A dataset of AR expression was available for the 1800-cohort and the Belfast cohort. All antibodies were validated before use in the patient cohort to confirm the specificity of the antibody.

### 2.1.1 Antibody validation

The antibody validation was demonstrated by a single band of the correct size on a western blot, followed by blocking peptide experiments and/or appropriate expression in silenced or stimulated cell pellets (Table 2.1). Antibodies for phospho-IKKα Ser176, phospho-IKKα Thr23, were validated as part of this thesis. The remaining antibodies had previously been used and validated by our lab members as previously published (173, 175).

| **Protein** | **Antibody** | **Validation method** |
|---|---|---|
| IKKα | Genway, GWB-662250 | • Validated in prostate cancer cell lines<br>• Single band on Western blot – 85kDa<br>• Western blot of IKKα silenced lysates<br>• IHC on IKKα silenced cell pellets |
| IKKα (Ser176) | Abcam, ab138426 | • Validate in breast cancer cell line<br>• Single band on Western blot- 85kDa<br>• Cell pellets of breast cancer cell lines |
| IKKα (Thr23) | Abcam, ab 38515 | • Validate in breast cancer cell line<br>• Single band on Western blot- 85kDa<br>• Cell pellets of breast cancer cell lines |
| pAKT | Cell Signalling, 12694S | • Validate in prostate cancer cell lines<br>• Single band on Western blot- 60kDa<br>• Western blot of pAKT treated with the PI3K inhibitor LY294002 |
| NIK | Bio-techne, MAB6888 | • Validated in colon cancer cell line |

| | | |
|---|---|---|
| | | • Single band on Western blot – 150kDa<br>• Breast cancer blocking peptide |

**Table 2.1 Antibody validation.** *Protein of interest, antibody used, and validation method are detailed, as well as tumour types that antibodies were validated and described in chapter 3. Methods associated with this are described in section 2.2.*

### 2.1.2 Patient tissue microarray (TMA)

Three different cohorts were used in this thesis. The 1800-Bre-TMA (the 1800 cohort) was used as a discovery cohort that included mixed breast cancer subtypes and used to identify markers and the cut-off values. The ST-Bre-TMA (the STB cohort) was used as a validated cohort that included ER-positive tamoxifen-treated patients. There was no crossover between the 1800 cohort and the STB cohort. The Northern Ireland cohort was used as a validated cohort that included ER-negative tumours.

For the 1800-Bre-TMA and the ST-Bre-TMA cohorts, the TMAs were constructed from formalin-fixed paraffin-embedded tissue (FFPE) blocks from breast cancer patients, which were retrieved from archives. Tumour rich areas were identified on the full-face section of haematoxylin and eosin-stained slides and marked by Dr Elizabeth Mallon (Consultant Pathologist at the Department of Pathology, Southern General Hospital). The marked slides were matched to the corresponding area on the donor block. Three different 0.6mm$^2$ cores were punched in order to achieve a high degree of concordance between result from the full sections and to minimise the issue of intra-tumour heterogeneity. Each core was subsequently inserted into the recipient paraffin blocks.

There are several advantages of using TMAs as a research tool for the investigation of putative prognostic molecular targets. Using TMAs significantly reduces experimental handling time compared to individual full sections (176). Due to the small amount of tissue required for construction, the remaining valuable tissue can be preserved for other experiments (176). TMAs are cost-effective and time saving as the amount of reagent was reduced and a large amount of tumour core can be stained in one run. For example, there are 456 patients in the STB-Bre-TMA with triplicate samples of each patient consisting of 15 slides. TMAs allow the standardised for the entire cohort as they are stained in one run with the same conditions. However, due to the small size of TMA cores, the tissue cores can be vulnerable to loss while performing experiments when compared to the full sections (176).

Ethical approval was granted by the Research Ethics Committee of the North Glasgow University Hospitals NHS Trust (NHS GG&C rec no 16WS0207).

The present study was conducted and reported in accordance with the Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria, with the exception of checklist item 9 (177). As the study was performed using pre-existing tissue collections and available clinic-pathological data sets, there was no power calculation used to determine the number of sample size. However, as the number of events for each end point were about 100, these observed events should allow effective sample size for statistical significance.

#### 2.1.2.1 Cohort 1: 1800-Bre-TMA (Discovery cohort)

This retrospective TMA of patients with breast cancer was constructed by Clare Orange (TMA and Image Analysis Unit manager, University Department of Pathology, Queen Elizabeth University Hospital). The cohort included 850 patients presenting with invasive breast cancer in the West of Scotland (at Glasgow Royal Infirmary, Glasgow Western Infirmary and Stobhill Hospital), between 1995 and 1998. Clinico-pathological data was available, including patient age, tumour size, grade and treatment the patient received. ER, PR, HER2 status and Ki67 were determined by Dr Zahra Mohammed.

The established classical prognostic markers, including tumour size, tumour grade, lymph node status, ER status, PR status and HER2 status, were associated with clinical outcomes in terms of cancer specific survival and recurrence free survival (Table 2.2). Age was not associated with cancer specific survival or recurrence free survival (Table 2.2).

| Clinicopathological characteristics | Patients number (%) | Cancer specific survival | | Recurrence free survival | |
|---|---|---|---|---|---|
| | | HR | P | HR | P |
| Age<br>≤50<br>>50 | <br>248 (29.2)<br>602 (70.8) | 1.05 (0.77-1.45) | 0.748 | 1.32 (0.98-1.79) | 0.073 |
| Size (mm)<br>≤20<br>21-50<br>>50 | <br>496 (58.4)<br>309 (36.4)<br>44 (5.2) | 2.14 (1.71-2.68) | **<0.001** | 1.88 (1.51-2.35) | **<0.001** |
| Grade | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| I<br>II<br>III | 161 (18.9)<br>382 (44.9)<br>305 (35.9) | 1.98 (1.57-2.49) | **<0.001** | 2.01 (1.60-2.53) | **<0.001** |
| Lymph node status<br>Negative<br>Positive | <br>480 (57.6)<br>348 (40.9) | 3.25 (2.37-4.45) | **<0.001** | 2.70 (1.99-3.66) | **<0.001** |
| ER status<br>Negative<br>Positive | <br>276 (32.5)<br>570 (67.1) | 0.57 (0.42-0.77) | **<0.001** | 0.48 (0.36-0.65) | **<0.001** |
| PR status<br>Negative<br>Positive | <br>448 (52.7)<br>396 (46.6) | 0.54 (0.39-0.74) | **<0.001** | 0.43 (0.31-0.69) | **<0.001** |
| Her-2 status<br>Negative<br>Positive | <br>699 (82.2)<br>128 (15.1) | 1.84 (1.28-2.62) | **0.001** | 2.19 (1.56-3.08) | **<0.001** |

**Table 2.2 Established classical prognostic markers and survival outcomes in the 1800 cohort.** *Cox regression analysis for cancer specific survival and recurrence free survival of established classical prognostic markers in the 1800 cohort.*

Clinical information of the patients was held by Professor Joanne Edwards. Patients with insufficient tumour tissue or incomplete follow up were excluded. Clinical endpoints included cancer specific survival (CSS) and recurrence free survival (RFS). CSS is the length of time from curative surgery to the date of death from breast cancer (178). RFS is the length of time from curative surgery to the date of the first recurrence, which includes locoregional and distant recurrence (179).

The survival curves were estimated for each group using the Kaplan-Meier method and compared statistically using the log-rank test. Patients who died from other causes when the data were analysed had censored observations for the cancer specific survival.

#### 2.1.2.2 Breast cancer subtype in the 1800-Bre-TMA

The 1800-Bre-TMA was a cohort that includes ER-positive and ER-negative patients with an available PR status, HER2 status and Ki67 (173, 180). The value of 14% of positive cells was used as a cut-off for Ki67 (37, 180).

Therefore, the cohort was categorised into four subtypes based on four IHC markers, including ER, PR, HER2 and Ki67, in accordance with the recommendation by the St Gallen international expert panel (37) and the previous publications (173). The IHC criteria for breast cancer subtype was defined as follow:

1. Luminal A: ER or PR positive, HER2 negative and low Ki67
2. Luminal B:
   - Luminal B (Her2-negative): ER or PR positive, HER2 negative and high Ki67
   - Luminal B (Her2-positive): ER or PR positive, HER2 positive and any Ki67
3. HER 2 enriched: ER and PR negative but HER2 positive
4. Triple negative: ER negative, PR negative and HER2 negative

The majority of patients were luminal A (43.6%), following by luminal B (21%), TNBC (20.7%) and HER2 enriched (8.6%) (Table 2.3). Among 178 patients in luminal B, 55 (6.5%) patients had HER2-positive (Table 2.3). 52 (6.2%) patients with missing HER2 or Ki67 status were excluded from the analysis.

| Subtype | | IHC markers | Number of patients (%) | Average breast cancer specific survival (years) | Average recurrence free survival (years) |
|---|---|---|---|---|---|
| Luminal A | | ER or PR positive<br>HER2 negative<br>Low Ki67 (<14%) | 371 (43.6) | 16.6 | 13.7 |
| Luminal B | Luminal B (HER2 negative) | ER or PR positive<br>HER2 negative<br>High Ki67 (≥14%) | 123 (14.5) | 14.7 | 11.99 |
| | Luminal B (HER2 positive) | ER or PR positive<br>HER2 positive<br>any Ki67 | 55 (6.5) | | |
| HER2 enriched | | ER and PR negative<br>HER2 positive | 73 (8.6) | 13.1 | 9.97 |
| Triple negative | | ER and PR negative<br>HER2 negative | 176 (20.7) | 13.9 | 12.54 |

**Table 2.3 subtyping of the 1800 TMA cohort using four IHC markers.** *Four marker that included ER, PR, HER2 and Ki67 were used to subtype the cohort into four groups*.

Kaplan-Meier curves were plotted, and the log-rank was used to compare each subtype. Patients with luminal A tumour had a longer breast cancer specific survival (16.6 years, HR 0.33 95% CI 0.20-0.54, P<0.001) and recurrence free survival (13.7 years, HR 0.26 95% CI 0.16-0.42, P<0.001) compared to all other subtypes (Figure 2.1). This finding was in line

with previous reports that demonstrated luminal A tumours had the greatest survival (181). Patients with luminal B tumours had an average cancer specific survival of 14.7 years and recurrence free survival of 11.99 years (Table 2.3). Patients with HER2 enriched had an average cancer specific survival of 13.1 years and recurrence free survival of 9.97 (Table 2.3). Patients with triple-negative breast cancer had an average cancer specific survival of 13.9 years and recurrence free survival of 12.54 (Table 2.3).

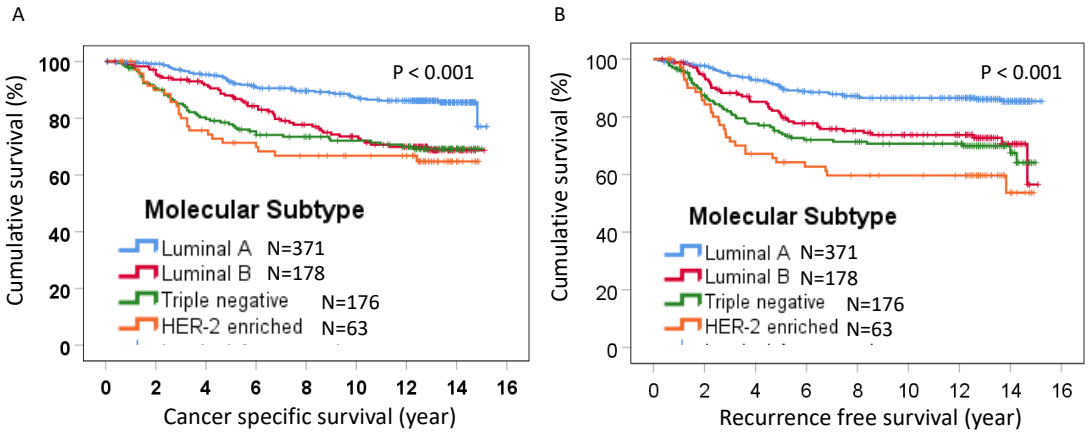


**Figure 2.1 Breast cancer subtypes and clinical outcomes in the 1800 cohort.** *Kaplan-Meier curves show cancer specific survival (A) and recurrence free survival (B) for each subtype.*

### 2.1.2.3 Cohort 2: ST-Bre-TMA (ER-positive cohort)

In order to confirm the results from the discovery cohort, this validation cohort was stained for NIK, pIKKα-Ser176 and pIKKα-Thr23. This retrospective TMA of breast cancer and clinical database was retrospectively constructed by Dr Sian Tovey. The TMA included 456 patients presenting with invasive breast cancer and undergoing curative surgery between 1980 and 1999 within Glasgow Royal Infirmary. Patients were included in this cohort base on initial ER-positive pathological reports. 20 patients who had ER-negative and 44 patients those ER unknown status were excluded. This exclusion left 392 patients for analysis. All patients in the study were treated with adjuvant tamoxifen treatment. A database of corresponding clinic-pathologic characteristics was available as well as recurrence and survival follow up time. The staining and scoring of IKKα and pAKT was performed by Dr Lindsay Bennett and by Dr Tove Kirkegaard, respectively.

The established classical prognostic markers, including tumour size, tumour grade, lymph node status and PR status, were associated with clinical outcomes in terms of cancer specific

survival, recurrence free survival and recurrence on tamoxifen (Table 2.4). HER2 status was not associated with cancer specific survival and recurrence free survival but was associated with recurrence on tamoxifen (Table 2.4). Age was not associated with any clinical outcomes.

| Clinicopathological characteristics | Patients number (%) | Cancer specific survival | | Recurrence free survival | | Recurrence fon tamoxifen | |
|---|---|---|---|---|---|---|---|
| | | HR | P | HR | P | HR | P |
| Age<br>≤50<br>>50 | <br>72(18.4)<br>319(81.4) | 1.90 (0.99-3.70) | 0.055 | 1.13 (0.67-1.91) | 0.644 | 1.35 (0.71-2.56) | 0.358 |
| Size (mm)<br>≤20<br>21-50<br>>50 | <br>193(49.2)<br>155(39.5)<br>22(5.6) | 2.60 (1.88-3.61) | **<0.001** | 2.29 (1.67-3.14) | **<0.001** | 2.51 (1.76-3.58) | **<0.001** |
| Grade<br>I<br>II<br>III | <br>96(24.5)<br>188(48)<br>97(24.7) | 1.87 (1.39-2.53) | **<0.001** | 1.72 (1.28-2.29) | **<0.001** | 2.10 (1.49-2.95) | **<0.001** |
| Lymph node status<br>Negative<br>Positive | <br>188(48)<br>171(43.6) | 3.47 (2.15-5.60) | **<0.001** | 3.52 (2.18-5.69) | **<0.001** | 3.76 (2.15-6.58) | **<0.001** |
| PR status<br>Negative<br>Positive | <br>145(37)<br>234(59.7) | 0.64 (0.44-0.97) | **0.033** | 0.59 (0.39-0.87) | **<0.008** | 0.50 (0.32-0.79) | **0.003** |
| Her-2 status<br>Negative<br>Positive | <br>362(92.3)<br>27(6.9) | 1.70 (0.82-3.51) | 0.147 | 1.73 (0.87-3.44) | 0.118 | 2.43 (1.21-4.91) | **0.013** |

**Table 2.4 Established classical prognostic markers and survival outcomes in the STB cohort.** *Cox regression analysis for cancer specific survival, recurrence free survival and recurrence on tamoxifen of established classical prognostic markers in the STB cohort.*

The outcome of interests included cancer specific survival (CSS), recurrence free survival (RFS) and recurrence on tamoxifen (RoT). CSS is the length of time from curative surgery to the date of death from breast cancer (178). RFS is the length of time from curative surgery to the date of first recurrence, which includes locoregional and distant recurrence. RoT is the length of time from curative surgery to the date of any recurrence while patients are on tamoxifen.

#### 2.1.2.4 Breast cancer subtype in the STB TMA

The STB TMA cohort included ER-positive patients with an available PR status, HER2 status and Ki67 (173, 180). The cohort was categorised into luminal A and luminal B using ER, PR, HER2 status and Ki67. The value of 14% of positive cells was used as a cut-off for Ki67 (37, 180).

81 (20.7%) patients were excluded from analysis due to lack of Ki67 and HER2 status. This exclusion left 311 patients, of which 221 were categorised as luminal A (56.4%), and 90 were categorised as luminal B (23%). Among the luminal B subtype, only 27 (6.8%) patients had HER2 positive (Table 2.5).

| Subtype | IHC markers | Number of patients (%) | Average breast cancer specific survival (years) | Average recurrence free survival (years) | Average recurrence on tamoxifen (years) |
|---|---|---|---|---|---|
| Luminal A | ER or PR positive<br>HER2 negative<br>Low Ki67 (<15%) | 221 (56.4) | 16.60 | 12.97 | 12.63 |
| Luminal B<br>Luminal B (HER2 negative) | ER or PR positive<br>HER2 negative<br>High Ki67 (≥15%) | 63 (16.10) | 14.16 | 10.61 | 6.73 |
| Luminal B (HER2 positive) | ER or PR positive<br>HER2 positive<br>any Ki67 | 27 (6.89) | | | |

**Table 2.5 subtyping of the STB TMA cohort using four IHC markers.** *Four markers that included ER, PR, HER2 and Ki67 were used to subtype the cohort into luminal A and luminal B subtype.*

Kaplan-Meier curves were plotted, and the log-rank was used to compare luminal A and luminal B subtypes. Patients with luminal A tumour had a longer breast cancer specific survival (16.60 years, HR 0.50 95% CI 0.32-0.79, P=0.003), recurrence free survival (12.97 years, HR 0.44 95% CI 0.28-0.68, P<0.001) and recurrence on tamoxifen (12.97 years, HR 0.32 95% CI 0.19-0.52, P<0.001) compared to luminal B tumour (Figure 2.2). This finding was in line with previous reports that demonstrated luminal A tumours had better survival when compared to luminal B tumours (181).

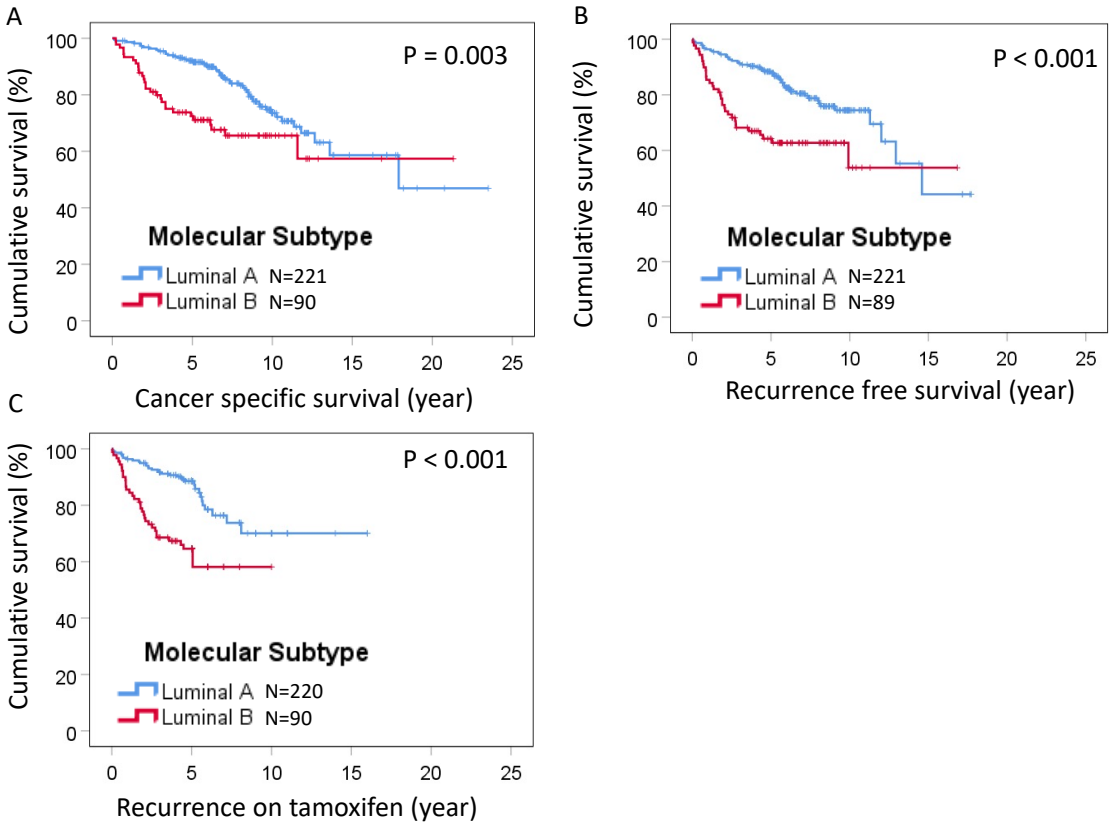


**Figure 2.2 Breast cancer subtypes and clinical outcomes in the STB cohort.** *Kaplan Meier curves showing cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C) for each subtype.*

### 2.1.2.5 The Northern Ireland or the Belfast cohort (ER-negative cohort)

In order to confirm the results from the discovery cohort, this cohort was stained for IKKα, pIKKα-Ser176 and pIKKα-Thr23. This retrospective TMA of breast cancer and clinical database was constructed by Dr Niamh Buckley (Queen's University Belfast). The TMA included 109 patients presenting with invasive breast cancer between 2000 and 2013. A data base of clinicopathological features was available, including patient age, tumour size, grade,

lymph node status and AR status. Patients with TMA cores missing or if inadequate quality for scoring were excluded.

The established classical prognostic markers, including tumour size and lymph node status, were associated with clinical outcomes in terms of overall survival and recurrence free (Table 2.6). Age and tumour grade were not associated with the outcomes (Table 2.6).

| Clinicopathological characteristics | Patients number (%) | Overall survival | | Recurrence free survival | |
|---|---|---|---|---|---|
| | | HR | P | HR | P |
| Age<br>≤50<br>>50 | <br>47 (43.1)<br>62 (56.9) | 2.72 (0.36-20.87) | 0.336 | 2.00 (0.27-15.00) | 0.501 |
| Size (mm)<br>≤20<br>21-50<br>>50 | <br>31 (28.4)<br>53 (48.6)<br>25 (22.9) | 1.99 (1.27-3.10) | **0.003** | 1.89 (1.28-2.79) | **0.001** |
| Grade<br>I<br>II<br>III | <br>0/(0)<br>13 (11.9)<br>96 (88.1) | 1.59 (0.56-4.53) | 0.383 | 1.73 (0.68-4.37) | 0.25 |
| Lymph node status<br>Negative<br>Positive | <br>63 (57.8)<br>45 (41.3) | 4.10 (2.11-7.96) | **<0.001** | 3.39 (1.95-5.88) | **<0.001** |

**Table 2.6 Established classical prognostic markers and survival outcomes in the Belfast cohort.** *Cox regression analysis for overall survival and recurrence free survival of established classical prognostic markers in the Belfast cohort.*

The outcome of interests included overall survival (OS) and RFS. OS is the length of time from curative surgery to the date of death from any cause (178). RFS is the length of time from curative surgery to the date of first recurrence, which includes locoregional and distant recurrence.

#### 2.1.2.6 Control tissue

Full tissue sections and TMAs of breast cancer patients without linked clinical data were available for antibodies optimisation. These were used as positive and negative controls in IHC experiments. Negative controls were included in every step of the IHC process with the exception of primary antibody and these slides were instead incubated with antibody diluent.

Positive controls were included in every step and incubated in identical solutions as to the full sections and TMAs of the patients.

## 2.1.3 Immunohistochemistry

IHC was performed on the patient tissue to assess the expression of members of the non-canonical NF-κB pathway. The IHC technique uses the primary antibody to detect the specific antigenic site followed by incubation with EnVision™ (K5007, Dako, Glostrup, Denmark). The EnVision™ reagent consists of a dextran backbone to which a large number of peroxidase (HRP) molecules and secondary antibody molecules have been coupled. The secondary antibody reacts equally with rabbit and mouse immunoglobulins. After incubation with secondary antibody, the tissue is incubated with the substrate system, which consists of 3,3'-diaminobenzidine (DAB) chromogen and hydrogen peroxide. Following interaction with the HRP molecules on the EnVision™, the substrate system produces a brown end product at the target antigen, which can be visualized by a light microscope. IHC consists of several step as following described.

### 2.1.3.1 Preparation of slides

Once antibodies had been validated and optimised, tissue slides were requested from NHS Research Scotland GGC Biorepository. The slides were stored at 4ºC and were baked for 15 minutes at 56ºC to minimise risk of tissue lost before performing IHC.

### 2.1.3.2 Dewaxing and rehydration

Slides were dewaxed by immersing in xylene for 5 minutes (x2) and rehydrated through a series of graded alcohols (2x 100% for 3 minutes, 1x 90% alcohol for 2 minutes and finally 1x 70% for 2 minutes) and then washed in running water.

### 2.1.3.3 Antigen (or epitope) retrieval

In formaldehyde-based fixation, epitope recognition by antibody can be masked due to cross-linking of formaldehyde and amino groups. Antigen retrieval is required to break the protein crosslinks and allow antibody binding and is performed by the heat induced epitope retrieval (HIER). The HIER was performed in a solution of either Citrate Buffer pH6 or TE Buffer pH9 (see table 2.3). 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. Tris/EDTA Buffer pH9 consisted of 1mM Ethylenediaminetetraacetic acid (EDTA) (27285, Sigma-Aldrich, St Louis, USA) and 5mM Tris Base (BP152-1, Fisher Scientific, Loughborough, UK) in 1L distilled water.

The buffer solutions were preheated in a microwave for 13.5 minutes in a pressure cooker without the lid. The tissue slides were then placed into the solutions and the lid was secured. The slides were heated for around 2 minutes until under pressure, following with a further 5 minutes of heating under pressure. Slides were left in the antigen retrieval buffer for 30 minutes for cooling, then were rinsed in running water for 10 minutes.

#### 2.1.3.4 Blocking endogenous peroxidase activity

Endogenous peroxidase activity can be found in the cell and tissue and can react with the substrate solution when using horseradish peroxidase (HRP) system in detection step, leading to falsely positive IHC results. To avoid this, the slides were incubation with in 3% hydrogen peroxide (23619.297 30% Hydrogen peroxide VWR Chemicals) for 10 minutes and then were rinsed in running water.

#### 2.1.3.5 Blocking non-specific binding

Tissue sections of TMAs were circled with the Dako delimiting pen (S2002, Dako, Copenhagen, Denmark) to create a hydrophobic barrier and ensure solutions covered all the tissue. This technique allows a uniform staining and reduces the amount of solution required (Table 2.7).

Hydrophobic interactions between other tissue proteins and the antibody can occur during performing IHC, leading to non-specific background staining. This interaction can be prevented by blocking with 1x Caesin (SP-5030, Vector Laboratories, Burlingame, CA, USA) at 25°C for 30 minutes (diluted in antibody diluent).

| **Protein of interest** | **Buffer solution** | **Blocking conditions** | **Antibody dilution & incubation conditions** |
|---|---|---|---|
| IKKα | Citrate pH6 | 1 x caesin 30 mins | 1:3000 dilution, overnight 4 ºC (Genway, GWB-662250, rabbit) |
| IKKα Ser176 | Tris-EDTA pH 9 | 1 x caesin 30 mins | 1:100 diultion, overnight 4 ºC (Abcam, ab138426, rabbit) |
| IKKα Thr23 | Citrate pH6 | 1 x caesin 30 mins | 1:100 dilution, overnight 4 ºC (Abcam ab38515, rabbit) |

| | | | |
|---|---|---|---|
| pAKT (Ser473) | Tris-EDTA pH 9 | 1 x caesin 30 mins | 1:600 dilution, overnight 4 ºC (Cell Signalling, 12694S, mouse) |
| NIK | Citrate pH6 | 1 x caesin 30 mins | 1:600 dilution, overnight 4 ºC (Biotechne, MAB6888, mouse) |

**Table 2.7 Optimal antibody conditions for immunohistochemistry.** *The antigen retrieval buffer, blocking solution and antibody incubation conditions for each protein*

#### 2.1.3.6 Incubation with primary antibody

After incubation with the blocking solution, the slides were immediately incubated in primary antibody. Antibodies were diluted in antibody diluent (Dako, Copenhagen, Denmark) and added to slides. Optimal dilution was optimised on practice tissue before being applied to the cohort. Only antibody diluent was applied to the negative control slide. Slides were incubated at 4 ºC overnight.

#### 2.1.3.7 Incubation with secondary antibody

After incubation with the primary antibody, the slides were washed twice in TBS for 5 minutes, following with incubation in ImmPRESS ((MP-7401, Vector Laboratories, Burlingame, CA, USA) which detects both mouse and rabbit primary antibodies for 30 mins at 25 ºC. The slides were washed twice again with TBS for 5 minutes.

#### 2.1.3.8 Detection and visualisation

A 3,3'-diaminobenzidine (DAB) kit was used as a chromogen to visualise the protein of interest. A DAB peroxidase kit was used (SK-4100, Vector Laboratories, Burlingame, CA, USA). In order to make the solution, 1 drop of DAB buffer solution, 2 drops of DAB substrate solution and 1 drop of hydrogen peroxide solution was added to 5ml of distilled water. This solution was added to the tissue slides and incubated for 5 minutes, following with washing in running water for 10 minutes.

#### 2.1.3.9 Counterstaining

Counterstaining was performed by immersing slides in Harris Haematoxylin (LAMB/230-D, Raymond A Lamb Ltd, Eastbourne, UK) for 1 minute followed by a quick dip in 1% acid alcohol (396ml 70% ethanol and 4ml hydrochloric acid) to remove excess haematoxylin. Slides were blued in Scott's Tap Water Substitute (STWS) (80mM Magnesium sulphate

(230391, Sigma, St Louis, USA) and 40Mm sodium hydrogen carbonate (102475W, VWR Poole, UK) in 2L of distilled water)

#### 2.1.3.10 Dehydration and mounting of slides

After counterstaining, slides were 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).

#### 2.1.3.11 Scanning and visualisation of slides

Slides were scanned with a 20x objective lens by Dr. Jennifer Hay (Pathology Department Queen Elizabeth University Hospital). Slides were then visualised on a NanoZoomer Digital Pathology (Hamamatsu Photonics K.K., Shizuoka, Japan).

### 2.1.4 Scoring of IHC

#### 2.1.4.1 Weighted histoscore

Evaluation of protein expression for each core was assessed by observers blinded to clinicopathological information. The expression of protein was scored using the weighted histoscore method as previous described by Kirkegaard *et al* (182). This method 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. A second observer then independently scored 10% of cores, blinded to the previous observer score as well as clinicopathological information. Statistical analysis was performed using the interclass correlation coefficient (ICCC) to measure inter-observer reliability. An ICCC of >0.7 was classified as having good correlation.

Examples of IHC represent staining intensity were shown in Figure 2.3.

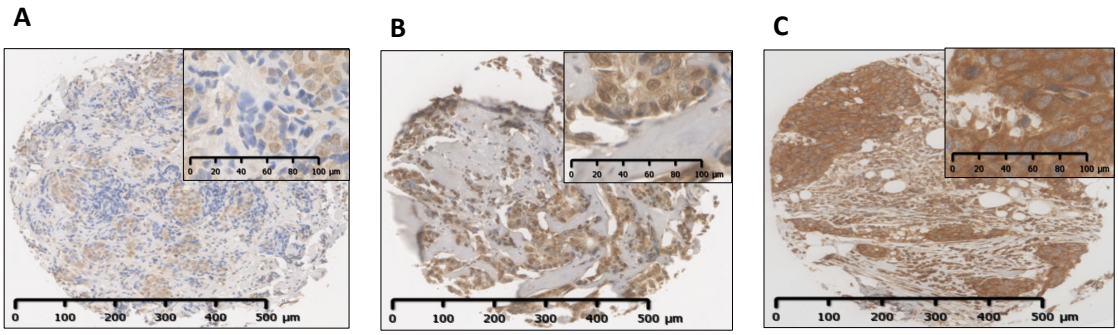


**Figure 2.3 Examples of immunohistochemistry results represents staining intensity.** *(A) represents the weak intensity of cytoplasm and nuclear. (B) represents the strong intensity of nuclear. (C) represents the strong intensity of cytoplasm.*

### 2.1.5 Statistical Analysis

Thresholds for protein expression were firstly classified using receiver operating characteristic curve (ROC) analysis. Since the values from ROC curve were not able to be used to define an optimal cut-off, the median histoscore was therefore used as the optimal cut-off.

Kaplan-Meier curves were plotted to assess breast cancer specific survival, recurrence free survival and recurrence on tamoxifen. Chi squared tests were conducted to assess the relationship between each protein of interest and clinicopathological characteristics. Hazard ratios were calculated using Cox regression with 95% confidence intervals. Values of $P<0.05$ were reported as statistically significant. All statistical analysis was performed by myself and repeated by a second (and senior) investigator (Joanne Edwards) in order to validate methods and results.

## 2.2 *In vitro* studies

### 2.2.1 Culturing of breast cancer cell lines

MCF7 (ER positive) and MDA-MB-231 (ER negative) and MDA-MB-453 (AR-positive triple-negative) breast cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM+ GlutaMAX™) (31966-021, Life Technologies, Paisley, UK) with 10% Fetal Bovine Serum (FBS) (F9665, Sigma-Aldrich, Gillingham, UK), 10 Units/ml Penicillin/Streptomycin (15140-122, Life Technologies, Paisley, UK).

Cells were grown in T-75 flasks and maintained in 5% CO2 at 37°C and media was changed twice per week. Once a confluency of around 70% was reached, cells were passaged. To passage cells, flask was washed with 5 ml of PBS to remove traced of serum and 1 ml of trypsin (25300-054, Life Technologies, Paisley, UK) was added to detach cells from the flask and then incubated at 37°C in 5% for 5 minutes. Microscopy was used to confirm the cells were detached from the flasks. DMEM containing serum was added to inactivate the trypsin. After trypsinisation, cells were transferred to conical tube and centrifuged at 1200 rpm for 5 minutes and media was removed. DMEM was added and cells were gently pipetted before being passage 1:3 into new-T-75 flasks with fresh DMEM. Cells were routinely tested for mycoplasma every 3 months.

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 (D8418, Sigma-Aldrich, Gillingham, UK) which is used as a cryoprotectant. Cells were then immediately placed in a freezing container 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 P8-14 and passages used in experiments were P11-16. MDA-MB-231 cell stocks were P14-18 and experiments used P16-20.

The characteristic of these cell lines has been demonstrated in the literature, which analyses gene expression profiles of 51 human breast cancer cell lines (183, 184). It has shown that MCF7 has gene profiles that classify the luminal cluster. MDA-MB-231 has gene profiles that classify the basal-like, and the MDA-MB-453 has gene profiles that classify the luminal group. The characteristic of each cell line was shown in Table 2.8 (183, 184). In addition to the literature telling us the ER, PR and HER2 status of the cell lines, this was confirmed in our own lab by a previous PhD student.

There was a potential risk of cross-contamination of cell cultures in this study. The Short Tandem Repeat (STR) DNA profiling has been indicated as a standard for cell line authentication (185). It identifies short tandem repeat (STR) markers found in a specific

locus of a DNA fingerprint of every human cell line (185). Performing the STR before starting cell line work should be done in a future study to avoid cross-contaminated or misidentification.

| Cell lines | Gene cluster | IHC profiles | Culture media |
|---|---|---|---|
| MCF7 | Luminal | ER+, PR+, HER2- | DMEM, 10% FBS |
| MDA-MB-231 | Basal like | ER-, PR-, HER2- | DMEM, 10% FBS |
| MDA-MB-453 | Luminal | ER-, PR-, HER2-, AR+ | DMEM, 10% FBS |

**Table 2.8 Cell lines.** *Cell line name and characteristics.*

### 2.2.2 Stimulation of the non-canonical NF-κB pathway in breast cancer cells

Various ligands were applied to the cell lines in order to activate the non- canonical NF-κB pathway. Proteins were then extracted to investigate expression of the pathway members. As previous studies from our lab have demonstrated the maximum fold change was observed after 24 hours lymphotoxin stimulation for an increase in expression of IKKα, phosphor-p100 (serine 866/870) and p100/p52, a 24 hour incubation period was used in the present study (173) . Studies have demonstrated that EGF can induce NF-κB expression in breast cancer cell lines with different time points (164, 186). Thus 30 minutes incubation with 100ng/mL of EGF was applied to the cells.

- Lymphotoxin $\alpha_1\beta_2$: 20ng/ml Lymphotoxin $\alpha_1\beta_2$ (LTx) (8884-LY, R&D Systems, MN, USA) was used for 24-hours.

- EGF: 100 ng/ml epidermal receptor growth factor (EGF) (E2520, Sigma-Aldrich) was used for 30 minutes.

For western blots, cells were seeded in 6 well plates at $3x10^5$ cells/well, grown to 70% confluency before being serum starved for 24 hours followed by stimulation with ligand at the desired concentration and incubation period before protein was lysed.

### 2.2.3 Pharmacological Inhibition of the non-canonical NF-κB pathway in breast cancer cells

In order to observe the effect of pharmacological inhibition of IKKα on breast cancer cell lines, MDA-MB-231 and MDA-MB-453 were treated with IKKα inhibitor (SU 1433 and SU 1644).

Cells were seeded in 6 well plates at $3x10^5$ cells/well, grown to 70% confluency before being serum starved for 24 hours. Cells were then pre-treated with IKKα inhibitor with serial dilutions of 10mM stock for 30 minutes before addition of stimulating ligand (lymphotoxin $\alpha_1\beta_2$ 20ng/ml) for 24 hours.

## 2.3 Western blotting

### 2.3.1 Lysis of protein

Cells were exposed to the appropriate agonist/inhibitor for the relevant length of time and then placed on ice to stop the reactions and prevent protein degradation or dephosphorylation. Cells were washed twice with 750µl ice cold PBS, followed by the addition of 250µl (for a 12 well plate) or 500µl (for a 6 well plate) 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 scraped and pushed through a 21-gauge needle to shear chromosomal DNA. The samples were then transferred to Eppendorf tubes and centrifuged at 13000rpm for 15 mins at 4°C then the supernatant transferred to a fresh Eppendorf and stored at -20°C until needed. The samples were heated at 95 °C before used.

### 2.3.2 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

Precast gels (were used in a Bio-Rad Mini-PROTEAN electrophoresis tank filled with electrophoresis running buffer (25mM Tris, 192mM glycine 0.1% (w/v) SDS pH8.3). 30-40 uL of sample was loaded per well using a Hamilton microsyringe. Electrophoresis was performed at a constant voltage of 120V for usually 90 minutes, until bromophenol blue dye had reached the bottom of the gel.

### 2.3.3 Protein transfer

Transferring proteins from the gel to PVDF membrane (0.45µM pore size Millipore IPVH00010 Cork, Ireland) were performed by electrophoretic blotting in wet conditions. A sandwich of sponge, filter paper, gel, membrane, filter paper and sponge were immersed in transferred buffer (25M Tris, 19mM glycine, 20% methanol pH 8.3). The sandwich was stacked on the Bio-Rad cassette as in Figure 2.4. The stack was slotted into a Bio-Rad Mini-PROTEAN electrophoresis tank and a constant current of 300mA was applied for 90

minutes. An ice pack also included to cool the tank. The presence of SDS gives the protein a negative charge, so the cassette was orientated with the membrane towards the positive electrode to allow the negatively charged proteins migrate from the gel and bind to the nitrocellulose membrane.

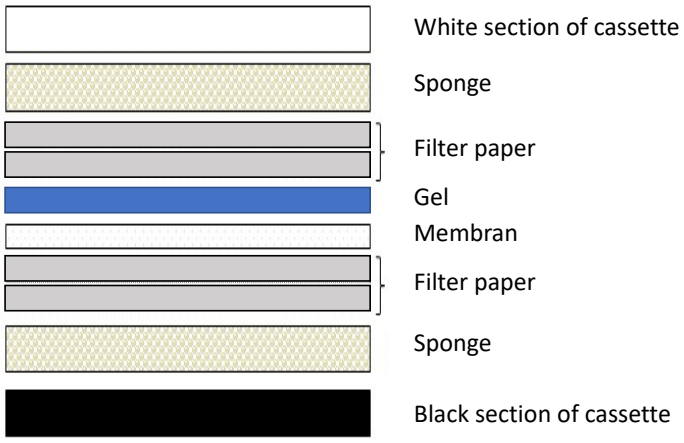


**Figure 2.4 Assembly of sandwich for western blot transfer.** *Diagram shows how the sandwich of sponge, filter paper, nitrocellulose membrane and gel were stacked for transfer.*

## 2.3.4 Blocking, staining and visualisation

After transfer of proteins, the membrane was removed, and non-specific binding was blocked by incubation in a solution of 3% BSA (bovine serum albumin) in ~~a~~ 0.1% TBST (Tris buffer saline;TBS with Tween®) for 90 minutes on a platform shaker. Membranes were incubated overnight in optimal concentration of the primary antibody at $4^0$C. Conditions for each antibody are listed in Table 2.9. The following day membranes were washed three times in 0.3% TBST at 10 minutes intervals on a platform shaker. Secondary HRP-conjugated antibody (either rabbit or mouse, depending on primary antibody used) was diluted to 1:10,000 in 0.3% BSA, and membranes and incubated at room temperature for 90 minutes with gentle shaking. Membranes were washed three times again in TBST at 15 minutes intervals. The proteins of interest were detected by chemiluminescence. Membranes were incubated in 2 mls of enhanced chemiluminescence (Pierce™ ECL Western Blotting Substrate 32106, ThermoScientific Rockford USA) for 5 minutes and visualised using Syngene: G:BOX blot imaging systems.

| Protein of interest | Antibody | Species | Blocking conditions | Primary antibody conditions |
|---|---|---|---|---|
| p100/p52 | 3054599, Millipore | Mouse | 3% BSA in TBST | 1:1000 (3% BSA 0.1% TBST), overnight room temperature |
| phospho-p100 | 4810, Cell Signalling | Rabbit | 3% BSA in TBST | 1:3000 (3% BSA 0.1% TBST), overnight room temperature |

**Table 2.9 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

The subsequent detection of other proteins was performed on the membranes. The membranes were incubated in 10ml of a re-blot solution (2504, Millipore, USA) for 15 minutes on a shaker. The membrane was washed in TBST 3 times for 10 minutes to remove all stripping buffer. Membranes were then ready for reproving, following the protocol previously described. Lysates were assessed for levels of β tubulin as a loading control (1:10000, ab21058, Abcam).

### 2.3.6 Quantification of expression levels

Level of expression of proteins via western blots were measurement using ImageJ (NIH). Levels were normalised to a housekeeping protein β tubulin, and fold change to control calculated using a t-test analysis. 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 20ng/ml of lymphotoxin-β (LTX) for 24 hours and 100 ng/ml EGF for 30 minutes. Flask were washed with PBS and cells were detached with trypsin before transfer to 15ml conical tubes and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, cells were resuspended in 1ml PBS and transferred to 1.5ml eppendorfs and

spun down again. PBS was discarded and cells resuspended in 4% formalin and incubated at room temperature for 15 minutes to fix the cells.

Pellets were formed by centrifugation for 5 minutes and supernatant was discarded. Then washed with PBS to remove all traces of formalin. Heated 1% agarose was then pipetted to the bottom of the eppendorfs to lift the pellet. Followed by refrigeration for 30 minutes or until agarose solidified. Pellets in agarose were removed from eppendorfs and placed into a cassette and dehydrated through a series of graded alcohols, then submersed in xylene twice for 15 minutes and then placed in melted wax for an hour. Pellets were then carefully removed from cassettes and placed into a mould, which was filled with wax and then cooled.

### 2.4.2 Cutting cell pellets

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

### 2.4.3 IHC of cell pellets

Slide of the pellets were used in IHC to assess expression level of the protein of interest. The same protocol as IHC on the TMAs was applied with the following exception.

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

## 2.5 Gene expression profiling

### 2.5.1 TempO-Seq assay

The TempO-Seq assay for FFPE tissues was performed as a service by BioClavis with reference to the standard TempO-Seq chemistry (187-189). The assay was carried out following the protocol from the User Manual that was provided with the kits. The section was scraped from the slide and directly placed into BioSpyder lysis buffer and overlaid then incubated at 95oC for 5 min with mineral oil, following wit incubation at 95℃ for 5 minutes in order to dissolve and separate the paraffin from the lysate. FFPE protease was added, the sample incubated and manually homogenised by pipetting. A 2 uL aliquot of the processed

lysate was then added to a microplate well that contained a mix of Detector Oligos (DOs) and annealing buffer in order to measure each targeted gene. DO panels included in the current study were designed against the independent gene expression profiles associated with low and high IKKα and its phosphorylation. The sample was facilitated to complete annealing of DOs to their target RNAs by exposing it to a ramp in temperature from 70℃ to 45℃ and then overnight incubation at 45℃. As the DOs anneal to RNA sequences f <100 nucleotides, the hybridisation process is highly resistance to RNA fragmentation, thus making it a robust assay for use with FFPE tissue. Unbound and incorrectly bound DOs were degraded by the addition of a nuclease mix. A ligase mixture was then added to allow ligation of DOs into full-length probes. After incubation at 80℃ for 15 minutes, the ligase enzymes were inactivated followed by amplification of ligated probes in a PCR step. The amplification products contain sequence tags that separate the result in one sample from every other sample. The PCR products from each well were pooled into a single sequencing library. Sequencing readouts were then demultiplexed to generate FASTQ files, which were sent to BioCalvis (the manufacturer of BioSpyder) for initial analysis.

The normal files of the human whole transcriptome were sent back to our lab. Differentially expressed genes (DEGs) were dentified using RStudio Team (2020) (RStudio: Integrated Development for R. RStudio, PBC, Boston, MA). As cytoplasmic IKKα, cytoplasmic pIKKα-Ser176 and nuclear pIKK-Thr23 demonstrated prognostic significance when analysis on patients' tissue, therefore DEGs were assessed between tumours with high and low expression of cytoplasmic IKKα, pIKKα-Ser176 and nuclear pIKK-Thr23

Genes with a statistically significant fold change in expression were identified. A log2 fold change of 0.5 in both directions and an adjusted P value (padj) of <0.1 were considered to determine the final DEGs. "Top" genes were determined by ranking using padj. Cluster analysis was then used to group the top 20 DEGs into clusters based on the similarity in their expression profiles. The Gene Ontology (GO) was used for unification of biology, including cellular component, biological process and molecular function (190). The search tool for the retrieval of interacting Genes (STRING) database 10.0 was utilised to identify the DEGs regarding their GO and relating pathway (191-193).

Protein-Protein Interaction (PPI) network was constructed and visualised using the STRING online database with a required interaction score of > 0.4 (medium confidence), a false discovery rate (FDR) < 0.05 and A PPI enrichment P-value <0.05 (191). Maximum number

of interactors to show the first and the second shell were limited at no more than 10 interactions.

## 2.6 Phenotypic assay:

### 2.6.1 The Luminescent Cell Viability Assay

Cell viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (CatG7571, Lot 0000354138, Promega, USA). This luminescent assay is a method that recognises the number of viable cells based on quantitation of the ATP present, which indicates the presence of metabolically active cells. After adding the CellTiter-Glo® reagent, mono-oxygenation of luciferin is catalysed by recombinant luciferase in the presence of Mg2+, ATP and oxygen, resulting in the generation of a luminescent signal proportional to the amount of ATP present (Figure 2.5). Luminescent signal was measured by The GloMax®-Multi Detection System.

Cells were seeded in 96 well plates at a density $15x10^3$ cell/well with 100uL of media. This density ensures that cells are confluent 96 hours after plating. DMSO (D8418, Sigma-Aldrich) and Staurosporine (S1250, Selleck) were sed as negative and positive controls, respectively. Cells were then treated with single agent IKKα inhibitor (SU1433 and SU 1644, university of Strathclyde), androgen receptor (AR) inhibitor (Enzalutamide; S125013, Selleck, USA). and a combination of IKKα inhibitor and AR inhibitor with a serial dilution of 10mM stock, cell plates were then incubated for 24, for 24, 48 and 72 hours. The assay was performed by adding 100µl CellTiter-Glo reagent to each cell well, covering the plate with a black lid and shaking at 450rpm for 2 minutes, followed by incubation at room temperature for 10 minutes. Luminescence was measured using Glomax multidetection system. Each treatment was repeated at least in triplicate on the cell plate and repeated to an N=3.

**Figure 2.5 The luciferase reaction.** *Mono-oxygenation of luciferin is catalysed by luciferase in the presence of ATP, Mg2+ and molecular oxygen.*

### 2.6.2 Statistical analysis

Statistical analysis for cell viability was performed using GraphPad Prism version 8. Experiments were performed three times. A one-way ANOVA with Turkey post-hoc test was performed to determine whether there was any statistically significant difference between the means of three independent groups.

# Chapter 3 : Expression of members of the non-canonical NF-κB pathways in the cohort of mixed breast cancer subtypes

# 3.1 Introduction

In the current chapter, expression of IKKα, phosphorylation of IKKα, at serine 176 and threonine 23 and the upstream kinases NIK and pAKT were assessed on tissue microarrays (TMAs) of breast cancer patients using IHC. Antibody validation was performed to confirm the specificity of each antibody utilised, and these were then optimised for IHC in breast cancer tissue prior to use in the 1800-cohort, which is the cohort of mixed breast cancer subtypes.

# 3.2 Antibody validation of members of the non-canonical NF-κB pathway

Antibody validation is an integral part of translational research, particularly for prognostic and predictive cancer-related biomarker studies. In order to produce reliable results, validation is essential to demonstrated antibody specificity and reproducibility. All antibodies that were used in this study were confirmed to be specific as shown below.

## 3.2.1 Validation of anti-NIK antibody

Specificity of anti-NIK antibody was confirmed as part of a previous study in our lab by Mr Lewis MacKenzie (194). Western blotting and IHC on cell pellets were performed to confirm specificity of the antibody. A single band of 125 kDa was observed on a western blot ( Figure 3.1) IHC using anti-NIK antibody was then performed on cell pellets of human umbilical vein endothelial cells (HUVECs), which were infected with B-galactosidase (β-gal) or NIK adenovirus. Increased expression was found only in the cells that were infected with NIK adenovirus, with low expression found in the cells that were infected with β-gal ( Figure 3.1) (194).

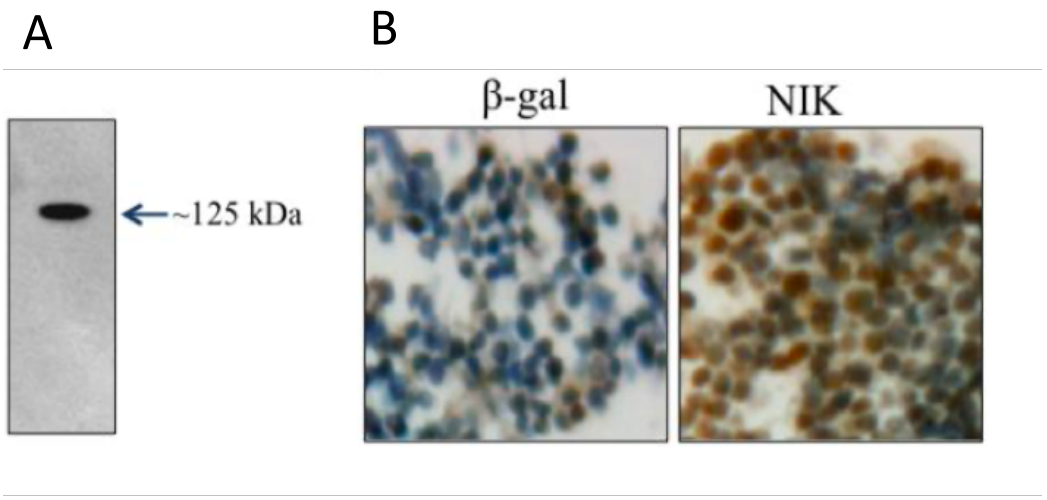


**Figure 3.1 Validation of the anti-NIK antibody.** *(A) Western blot showing a single band of appropriate size (125 kDa). (B) IHC was performed on cell pellets of HUVEC cells infected with β-galactosidase (B-gal) or NIK adenoviruses and showed an increase in staining in the NIK adenovirus infected cells.*

### 3.2.2 Validation of anti-IKKα antibody

Specificity of anti-IKKα antibody was previously validated in our laboratory by Mr Lewis MacKenzie (194). A single band of the predicted molecular weight (85 kDal) was observed by Western blotting in LNCAP prostate cancer cells (Figure 3.2). LNCaP cells were also pre-treated with 200nM siRNA against either IKKα or IKKβ. A reduction in expression was observed in lysates from the IKKα-silenced but not IKKβ-silenced cells (Figure 3.2). Then, IKKα-silenced and IKKβ-silenced cells pellets were made and IHC was performed. A decrease in expression was observed in IKKα-silenced cells but was not seen in IKKβ-silenced or control cells (Figure 3.2).

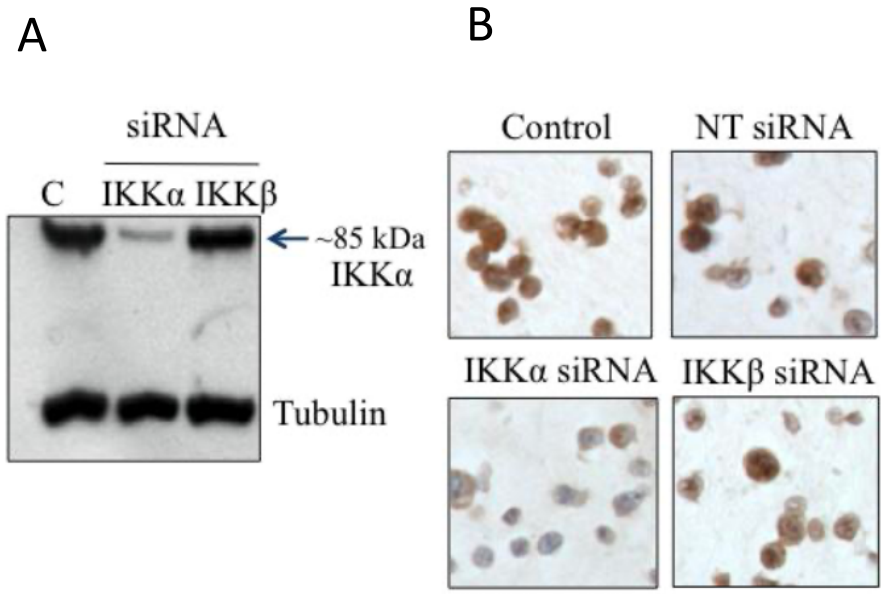


**Figure 3.2 Validation of the anti-IKKα antibody.** *(A) Western blot represented a single band of appropriate size (85 KDa) and a reduction of expression was observed in lysates from IKKα-silenced LNCap Cells. (B) Cell pellets of LNCap cells treated with siRNA showed a decrease in expression in IKK-α-silenced but not in IKKβ-silenced cells.*

### 3.2.3 Validation of anti-pIKKα-Ser176 antibody

The non-canonical NF-κB pathway is activated by a specific group of stimuli, including the TNF family members lymphotoxin-α/β, BAFF or CD40 ligand. These stimuli activate NIK to further phosphorylate IKKα at serine 176, which seems to be a classical phosphorylation site in response to the activation of the non-canonical NF-κB pathway (114). Other stimuli have been shown to be involved in activation of the non-canonical NF-κB pathway, including EGF and TNFα (195, 196). Thus, lymphotoxin and EGF were used in order to stimulate an expression of IKKα phosphorylation on cell pellets of MCF-7 breast cancer cell lines.

Two steps were done in order to confirm antibody specificity of anti-pIKKα-Ser176 antibody. First, a single band of the predicted size (85 kDa) was observed on a western blot of MDA-BM231 cell lysates for anti-pIKKα Ser176 by Dr Melania Montes Pérez (Figure 3.3). Then IHC on cell pellets of MCF7 breast cancer cell lines were performed, and increase cytoplasmic expression was observed when cells were exposed to 20ng/ml of lymphotoxin-β (LTX) compared to untreated and epidermal growth factor (EGF)-treated cells ( Figure 3.3). As expression of pIKKα Ser176 was predominant found in cytoplasm, indicating that phosphorylation at serine 176 is preferable response for cytoplasmic function of IKKα. This

result confirms that the antibody is specific as this site is predominantly activated by the non-canon NF-κB pathway.

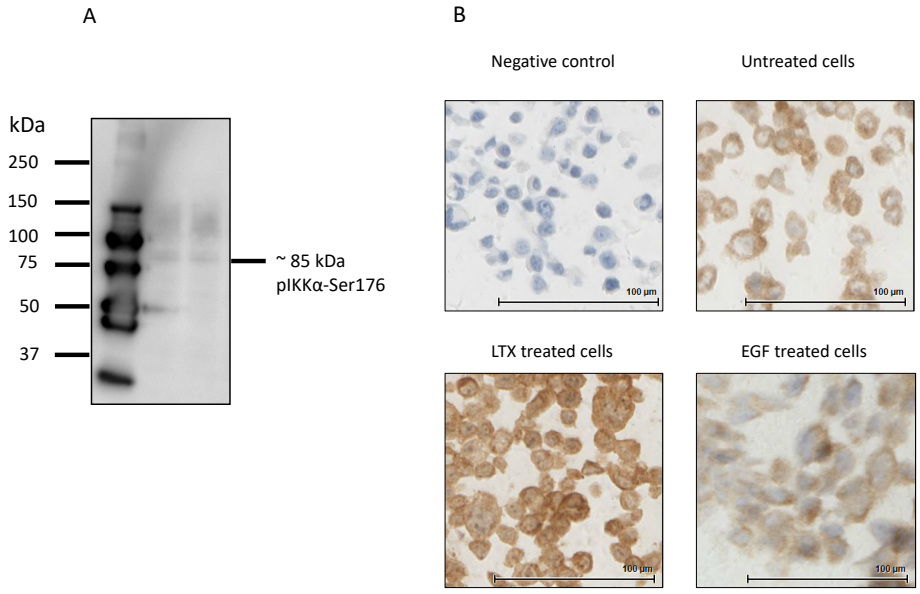


**Figure 3.3 Validation of the anti-pIKKα-Ser176 antibody.** *(A) Western blot showing a single band of appropriated size (85 kDa). (B) An increase in expression was observed in lysates from LTX treated cells and was predominantly found in cytoplasmic.*

## 3.2.4 Validation of anti-pIKKα-Thr23 antibody

As IKKα has been demonstrated to be a critical link between NF-κB pathway and other oncogenic pathway such as EGFR and PI3K/AKT, various factors have been shown to activate phosphorylation of IKKα as described in the previous section. While serine Ser176 is seen be the classical phosphorylation site on IKKα in response to NIK activation in the non-canonical NF-κB pathway, threonine 23 is indicated to be the critical phosphorylation site that allows signalling crosstalk between NF-κB and other pathways (127, 197). Thus, again lymphotoxin and EGF were used in order to stimulate an expression of IKKα phosphorylation on cell pellets of MCF-7 breast cancer cell lines.

A single band of the predicted size (85 kDa) was observed on a western blot of MDA-MB231 cell lysate for anti-pIKKα Thr23 by Dr Melania Montes Pérez (Figure 3.4). Then IHC on cell pellets of MCF7 breast cancer cell lines were performed, and increase nuclear expression was observed when exposed to both 20ng/ml LTX and 100 ng/ml EGF compared to untreated cells. As expression of pIKKα Thr23 was predominant found in the nucleus, indicating phosphorylation at threonine 23 is mainly response for nuclear function of IKKα.

This result confirms that the antibody is specific as this site is predominantly activated by both the non-canon NF-κB pathway and AKT.

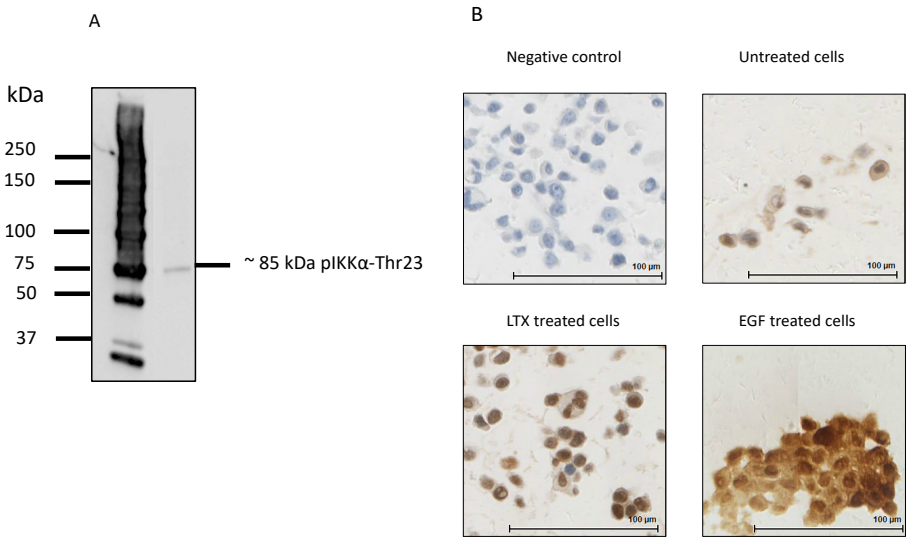


**Figure 3.4 Validation of the anti-pIKKα-Thr23 antibody.** *(A) Western blot showing a single band of appropriated size (85 kDa). (B) An increase in expression was observed in lysates from LTX and EGF treated cells and was predominantly found in the nucleus.*

## 3.2.5 Validation of anti-pAKT antibody

Specificity of anti-pAKT antibody was previously validated in the previous study from our lab (198). A single band of the predicted size (60 kDa) was observed on a western blot of LNCaP prostate cancer cells Figure 3.5. LNCaP cells were unstimulated (vehicles, acetic acid and ethanol), treated with the PI3K inhibitor (20 uM LY29002), treated with 10ng IGF-1, treated with LY29002 then stimulated with IGF-1. A reduction in expression was observed in lysates from the PI3K inhibitor cell but an increased expression was observed in lysate from IGF-1 stimulation.

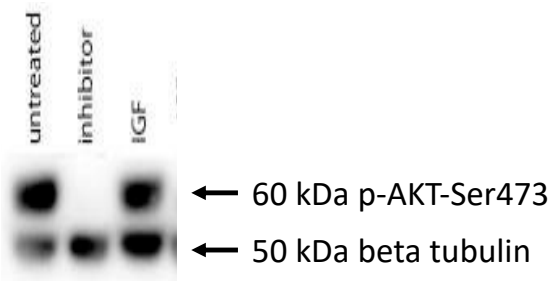


**Figure 3.5 Validation of the anti-pAKT (Ser473) antibody.** *Western blot represented a single band of appropriate size (60 KDa) and a reduction of expression was observed in lysates from the PI3K inhibitor cell, but an increased expression was observed in lysate from IGF-1 treated cell.*

## 3.3 Expression and clinical outcome of members of non-canonical NF-κB pathway in a cohort of patients with mixed breast cancer subtypes (the 1800-cohort)

The 1800-cohort included 850 patients presenting with invasive breast cancer between 1980 and 1998. A database of clinicopathological features was available, including patient age, tumour size, grade, ER status, PR status, HER2 status, AR status and lymph node status. Patients with TMA core missing or that had inadequate quality for scoring were excluded (Figure 3.6).

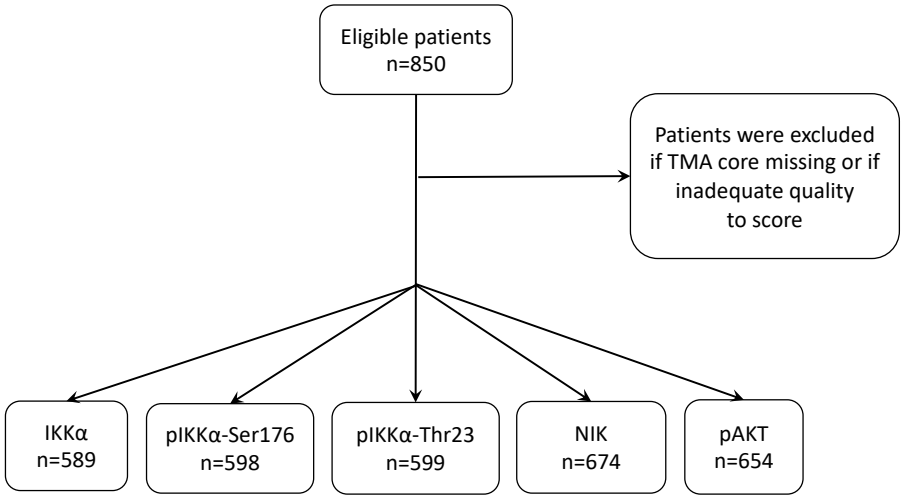


**Figure 3.6 Flow diagram demonstrating patient exclusions.** *Patients with missing cores or insufficient tumour for analysis were excluded*

The majority of patients were over 50 years old (70.8%). 496 (54.8%) patients had tumour size less than 20mm and 309 (36.4%) patients had tumour size between 21-50mm. Tumour grade II and grade III were predominant in this cohort with 382 (44.9%) and 305 (35.9%) patients, respectively. 480 (57.6%) patients had lymph node negative disease whereas 348 (40.9%) patients had lymph node involvement. Almost two third of the patients had ER positive tumour, however, only half of the patients were PR positive. Above 80% of the patients had HER2 negative tumour and 567 (66.7%) patients had low level of ki67. 371 (43.6%) patients had luminal A tumours, 178 (20.9%) patients had luminal B tumours, 176 (20.7%) patients had TNBC and 73 (8.6%) patients had HER2 positive tumours. Information on adjuvant treatment was available in 847 patients; of these 331 (38.9%) patients received adjuvant chemotherapy and 401 (47.2%) received adjuvant radiotherapy. Information on adjuvant endocrine therapy was available in 850 patients, of these 528 (62.1%) patients

received tamoxifen and 32 (3.8%) patients received anastrozole. The median follow-up for survival was 12.53 years (range 0.08-15.25 years) (Table 3.1).

Among 850 patients, 13 patients develop new primary breast cancer. However, the data on ipsilateral or contralateral breast cancer development was not available. The information on treatment that patients receive after they have disease recurrence was not available.

| Patient characteristics | Number, N=850 (%) |
|---|---|
| Age | |
| ≤50 | 248 (29.2) |
| >50 | 602 (70.8) |
| Histological type | |
| Other | 46 (5.4) |
| Lobular | 68 (8.0) |
| Ductal | 736 (86.6) |
| Size (mm) | |
| ≤20 | 496 (58.4) |
| 21-50 | 309 (36.4) |
| >50 | 44 (5.2) |
| Missing | 1 (0.1) |
| Grade | |
| I | 161 (18.9) |
| II | 382 (44.9) |
| III | 305 (35.9) |
| Missing | 2 (0.2) |
| Lymph node status | |
| Negative | 480 (57.6) |
| Positive | 348 (40.9) |
| Missing | 22 (1.4) |
| ER status | |
| Negative | 276 (32.5) |
| Positive | 570 (67.1) |
| Missing | 4 (1.5) |
| PR status | |
| Negative | 448 (52.7) |
| Positive | 396 (46.6) |
| Missing | 6 (0.07) |
| Her-2 status | |
| Negative | 699 (82.2) |
| Positive | 128 (15.1) |
| Missing | 23 (2.7) |
| Proliferation (Ki67) | |

| | |
|---|---|
| Low | 567 (66.7) |
| High | 228 (26.8) |
| Missing | 55 (6.5) |
| Molecular subtype | |
| Luminal A | 371 (43.6) |
| Luminal B/ | 178 (20.9) |
| TNBC | 176 (20.7) |
| HER2 | 73 (8.6) |
| Missing | 52 (6.1) |
| Adjuvant endocrine treatment | |
| No | 141 (16.6) |
| Yes | 528 (62.1) |
| ATAC trial | 32 (3.8) |
| Missing | 149 (17.5) |
| Adjuvant Chemotherapy | |
| No | 516 (60.7) |
| Yes | 331 (38.9) |
| Missing | 3 (0.4) |
| Adjuvant radiotherapy | |
| No | 446 (52.5) |
| Yes | 401 (47.2) |
| Missing | 3 (0.4) |
| Survival status | |
| Alive | 482 (56.7) |
| Cancer death | 174 (20.5) |
| Non-cancer death | 157 (18.5) |
| Missing | 15 (1.8) |
| Recurrence status | |
| No recurrence | 648 (76.2) |
| Recurrence | 181 (21.3) |
| Missing | 21 (2.5) |

**Table 3.1 Clinicopathological characteristic of the 1800 cohort**

An expression of protein of interest was assessed and the results were shown as a median histoscore and the interquartile range (Table 3.2)

| Proteins | Median histoscore (Interquartile range) |
|---|---|
| NIK | |
| Cytoplasmic | 140 (112.3-182.1) |
| Nuclear | 80 (51.7-93.3) |
| IKKα | |
| Cytoplasmic | 120 (95.0-160.0) |
| Nuclear | 100 (67.5-197.5) |
| pIKKα-Ser176 | |
| Cytoplasmic | 100 (66.3-115.5) |
| Nuclear | 70 (45.0.3-98.3) |
| pIKKα-Thr23 | |
| Cytoplasmic | 80 (48.3-98.0 |
| Nuclear | 90 (48.3-148.3) |
| pAKT | |
| Cytoplasmic | 90 (36.7-115.0) |
| Nuclear | 50 (11.7-90.0) |

**Table 3.2 A median histoscore and the interquartile range of the proteins of interest in the 1800-cohort.**

## 3.3.1 Expression of NIK in the 1800-cohort

### 3.3.1.1 Association of NIK with clinical outcome in the 1800-cohort

To assess expression of members of the pathway and its upstream regulators in the cohort of mixed breast cancer subtypes, expression of NIK, the kinase upstream of IKKα, was assessed. The expression of NIK was analysed in 674 (71.68%) of the patients from the 1800-cohort (N=850). The histoscore for cytoplasmic expression of NIK ranged from 0 to 300 with a median histoscore of 140 (IQR=112.3-182.1). The histoscore for nuclear expression of NIK ranged from 0-235 with a median histoscore of 80 (IQR=51.7-93.3). An example of immunostaining of a tumour with low and high NIK expression and histograms showing the distribution of histoscore for cytoplasmic and nuclear expression of NIK are shown in Figure 3.7. There was good and moderate correlation between observers with ICCC score of 0.87 for cytoplasmic and 0.72 for nuclear expression, respectively.

The Receiver Operating Characteristics (ROC) curve was plotted to determine the cut-off in order to classify low and high expression of cytoplasmic and nuclear expression of NIK. Since the area under the ROC curve (AUC) was 0.48 (P=0.460) for cytoplasmic and 0.42 (P=0.003) for nuclear expression of NIK, the values from ROC curves were unable to be used to define the optimal cut-off (Figure 3.7). The median histoscore was therefore used as the optimal cut-off.

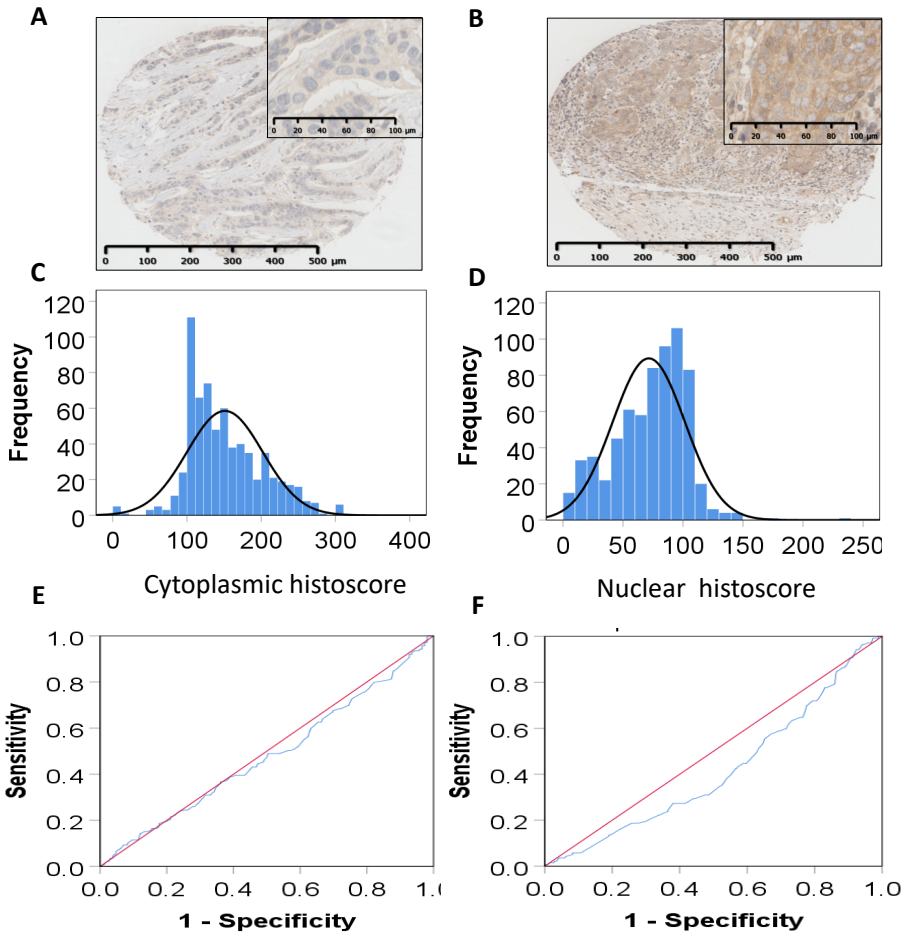


**Figure 3.7 Expression of NIK.** *(A) Example of low expression and (B) of high expression of NIK (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of NIK. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of NIK.*

Tumours with a cytoplasmic histoscore over 140 and tumour with a nuclear histoscore over 80 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-offs, 335 patients had high cytoplasmic expression and 339 patients had low cytoplasmic expression. 299 patients had high nuclear expression and 375 patients had low nuclear expression.

In order to determine whether NIK expression was associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of NIK were plotted as shown in Figure 3.8. Low and high expression were compared by using the log-rank test. Cytoplasmic expression of NIK was not associated with cancer specific survival (HR 0.93 95% CI 0.67-1.30, P=0.663 by the log-rank test, Figure 3.8) or recurrence free survival (HR 0.99 95% CI 0.71-1.38, P=0.955, Figure 3.8). However, high nuclear expression was associated with increased cancer specific survival (HR 0.53 95% CI 0.37-0.76, P<0.001, Figure 3.8) and increased recurrence free survival (HR 0.63 95% CI 0.44-0.89, P=0.009, Figure 3.8.)

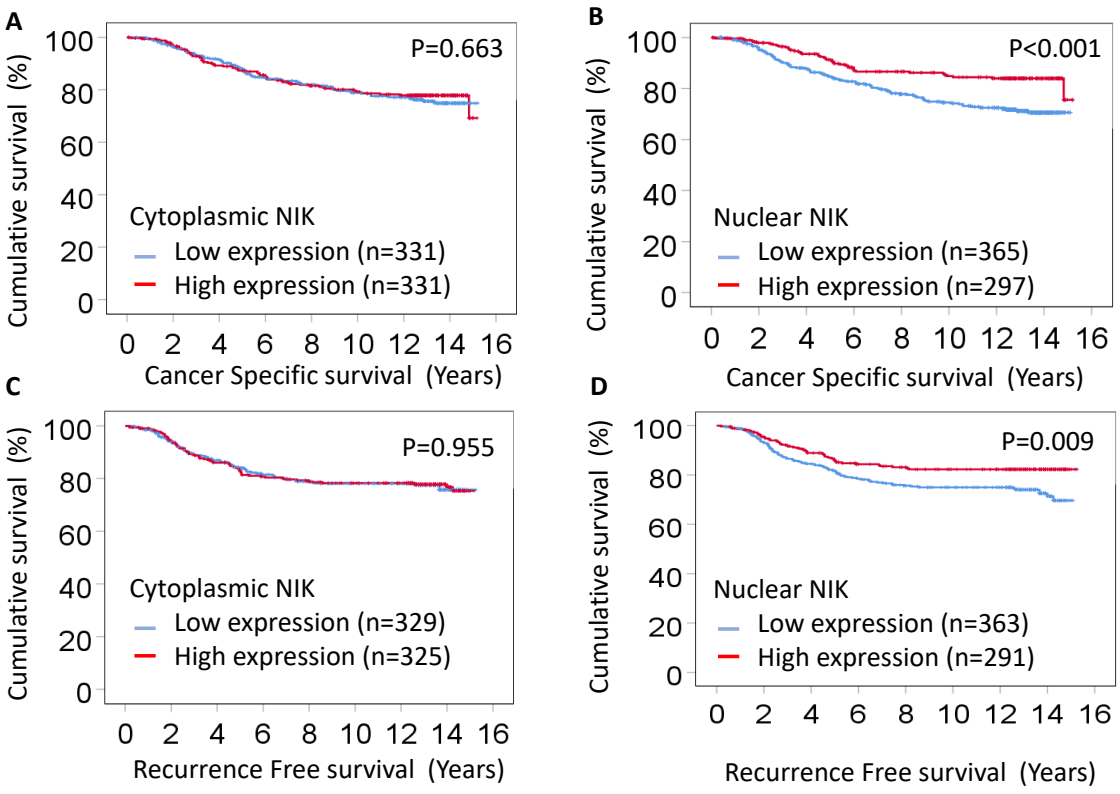


**Figure 3.8 Expression of NIK and clinical outcome in the 1800-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear NIK with cancer specific survival (A and B) and recurrence free survival (C and D).*

Multivariate analysis suggested that nuclear expression of NIK was an independent prognostic factor for cancer specific survival when combined with histological type, tumour size, tumour grade, lymph node status, ER status, PR status, HER2-status, Ki67 and molecular subtypes (HR 0.59 95% CI 0.40-0.86, P=0.007, Table 3.3).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>≤ 50/>50 | 1.05 (0.77-1.45) | 0.748 | | |
| Histological type<br>Other/ Lobular/Ductal | 1.87 (1.21-2.89) | **0.005** | 1.29 (0.77-2.18) | 0.339 |
| Size (mm)<br>≤ 20/ 21-50/ 21-50/>50 | 2.14 (1.71-2.68) | **<0.001** | 1.65 (1.24-2.20) | **0.001** |
| Grade<br>I/II/III | 1.98 (1.57-2.49) | **<0.001** | 1.55 (1.14-2.12) | **0.005** |
| Lymph node status<br>Negative/ Positive | 3.25 (2.37-4.45) | **<0.001** | 3.04 (2.06-4.49) | **<0.001** |
| ER status (<10WHS/>10WHS)<br>Negative/ Positive | 0.57 (0.42-0.77) | **<0.001** | 0.69 (0.34-1.44) | 0.327 |
| PR status<br>Negative/ Positive | 0.54 (0.39-0.74) | **<0.001** | 0.88 (0.56-1.37) | 0.559 |
| Her-2 status<br>Negative/ Positive | 1.84 (1.28-2.62) | **0.001** | 1.15 (0.64-2.07) | 0.637 |
| Proliferation (Ki67)<br>Low/ High | 1.64 (1.20-2.25) | **0.002** | 1.24 (0.78-1.96) | 0.366 |
| Molecular subtype<br>Luminal A/Luminal B /TNBC/HER2 | 1.46 (1.27-1.67) | **<0.001** | 1.26 (1.04-1.53) | **0.017** |
| Cytoplasmic NIK<br>Low/High | 0.93 (0.67-1.30) | 0.663 | | |
| Nuclear NIK<br>Low/High | 0.53 (0.37-0.76) | **0.001** | 0.59 (0.40-0.86) | **0.007** |

**Table 3.3 Univariate and multivariate analysis of NIK and clinicopathological characteristic in the 1800-cohort**

### 3.3.1.2 Associations between NIK and clinicopathological characteristics

Chi-squared analysis was performed in order to determine whether expression of NIK was associated with any clinicopathological characteristics of the patients in the 1800-cohort as shown in Table 3.4. Expression of cytoplasmic NIK was not associated with any of clinicopathological characteristics. However, expression of nuclear NIK was associated with histological type (P=0.004), tumour grade (P=0.007), ER status (P=0.007), HER2 status (P=0.007), Ki67 (P<0.001) and molecular subtype (P<0.001).

| Clinicopathological characteristics | Cytoplasmic NIK | | | Nuclear NIK | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 95 (28.3) | 103 (30.7) | 0.490 | 113 (30.1) | 86 (28.8) | 0.698 |
| >50 years) | 243 (71.7) | 232 (69.3) | | 262 (69.9) | 213 (71.2) | |
| Histological type | | | | | | |
| Other | 17 (5.0) | 19 (5.7) | 0.823 | 19 (5.1) | 17 (5.7) | **0.004** |
| Lobular | 26 (7.7) | 29 (8.7) | | 19 (5.1) | 36 (12.0) | |
| Ductal | 296 (87.3) | 287 (85.7) | | 337 (89.9) | 246 (82.3) | |
| Size (mm) | | | | | | |
| ≤20 | 203 (60.1) | 190 (56.7) | 0.379 | 210 (56.0) | 183 (61.4) | 0.234 |
| 21-50 | 122 (36.1) | 125 (37.3) | | 143 (38.1) | 104 (34.9) | |
| >50 | 13 (3.8) | 20 (6.0) | | 22 (5.9) | 11 (3.7) | |
| Grade | | | | | | |
| I | 65 (19.2) | 73 (21.8) | 0.701 | 73 (19.5) | 65 (21.7) | **0.007** |
| II | 166 (49.0) | 159 (47.5) | | 166 (44.3) | 159 (53.2) | |
| III | 108 (31.9) | 103 (30.7) | | 136 (36.3) | 75 (25.1) | |
| Lymph node status | | | | | | |
| Negative | 207 (61.8) | 181 (55.2) | 0.084 | 215 (57.8) | 173 (59.5) | 0.668 |
| Positive | 128 (38.2) | 147 (44.8) | | 157 (42.2) | 118 (40.5) | |
| ER status | | | | | | |
| Negative | 79 (23.3) | 78 (23.3) | 0.995 | 102 (27.2) | 55 (18.4) | **0.007** |
| Positive | 260 (76.7) | 257 (76.7) | | 273 (72.8) | 244 (81.6) | |
| PR status | | | | | | |
| Negative | 164 (48.4) | 156 (46.6) | 0.638 | 189 (50.4) | 131 (43.8) | 0.089 |
| Positive | 175 (51.6) | 179 (53.4) | | 186 (49.6) | 168 (56.2) | |
| Her-2 status | | | | | | |
| Negative | 287 (85.7) | 280 (84.8) | 0.765 | 304 (81.9) | 263 (89.5) | **0.007** |
| Positive | 48 (14.3) | 50 (15.2) | | 67 (18.1) | 31 (10.5) | |
| Proliferation (Ki67) | | | | | | |
| Low | 208 (65.6) | 230 (71.4) | 0.114 | 211 (60.1) | 227 (78.8) | **<0.001** |
| High | 109 (34.4) | 92 (28.6) | | 140 (39.9) | 61 (21.2) | |
| Molecular subtype | | | | | | |
| Luminal A | 165 (51.1) | 168 (52.0) | 0.878 | 158 (43.6) | 175 (61.6) | **<0.001** |
| Luminal B | 84 (26.0) | 83 (25.7) | | 108 (29.8) | 59 (20.8) | |
| TNBC | 49 (15.2) | 52 (16.1) | | 64 (17.7) | 37 (13.0) | |
| HER2 | 25 (7.7) | 20 (6.2) | | 32 (8.8) | 13 (4.6) | |

**Table 3.4 Association of NIK with clinicopathological characteristics of the 1800-cohort.** *Chi-squared analysis exploring association between NIK with clinicopathological characteristics of the cohort.*

### 3.3.1.3 Expression of NIK and clinical outcome in different subtypes

In order to determine whether expression of NIK was associated with clinical outcome in specific breast cancer subtype, the cohort was subdivided into luminal A, luminal B, TNBC and HER2-positive disease. Cytoplasmic expression of NIK was not associated with cancer specific survival in any of four breast cancer subtypes (luminal A, HR 1.03 95% CI 0.56-1.89, P=0.916; luminal B, HR 0.86 95% CI 0.47-1.55, P=0.605; TNBC HR 1.19 95% CI 0.60-2.37, P=0.627; HER2-positive HR 0.58 95% CI 0.20-1.67, P=0.302) (Figure 3.9).

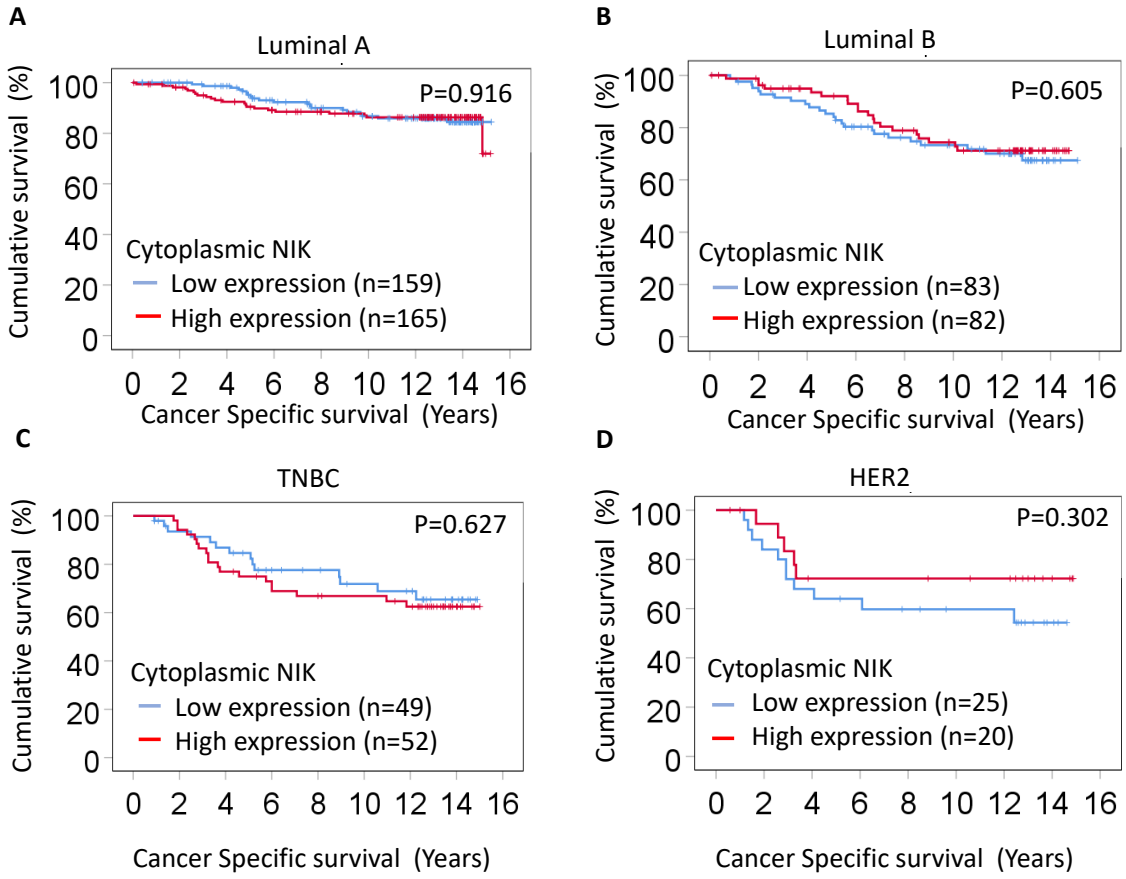


**Figure 3.9 Expression of cytoplasmic NIK and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic NIK and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumour (D)*

High expression of nuclear NIK was associated with improved cancer specific survival in luminal B tumours (HR 0.36 95% CI 0.16-0.80, P=0.009), with a trend towards improved survival in luminal A tumours (HR 0.56 95% CI 0.30-1.04, P=0.062). However, the association was not observed in other subtypes (TNBC, HR 1.29 95% CI 0.65-2.58, P=0.463: HER2-positive, HR 0.59 95% CI 0.17-20.7, P=0.401) (Figure 3.10).

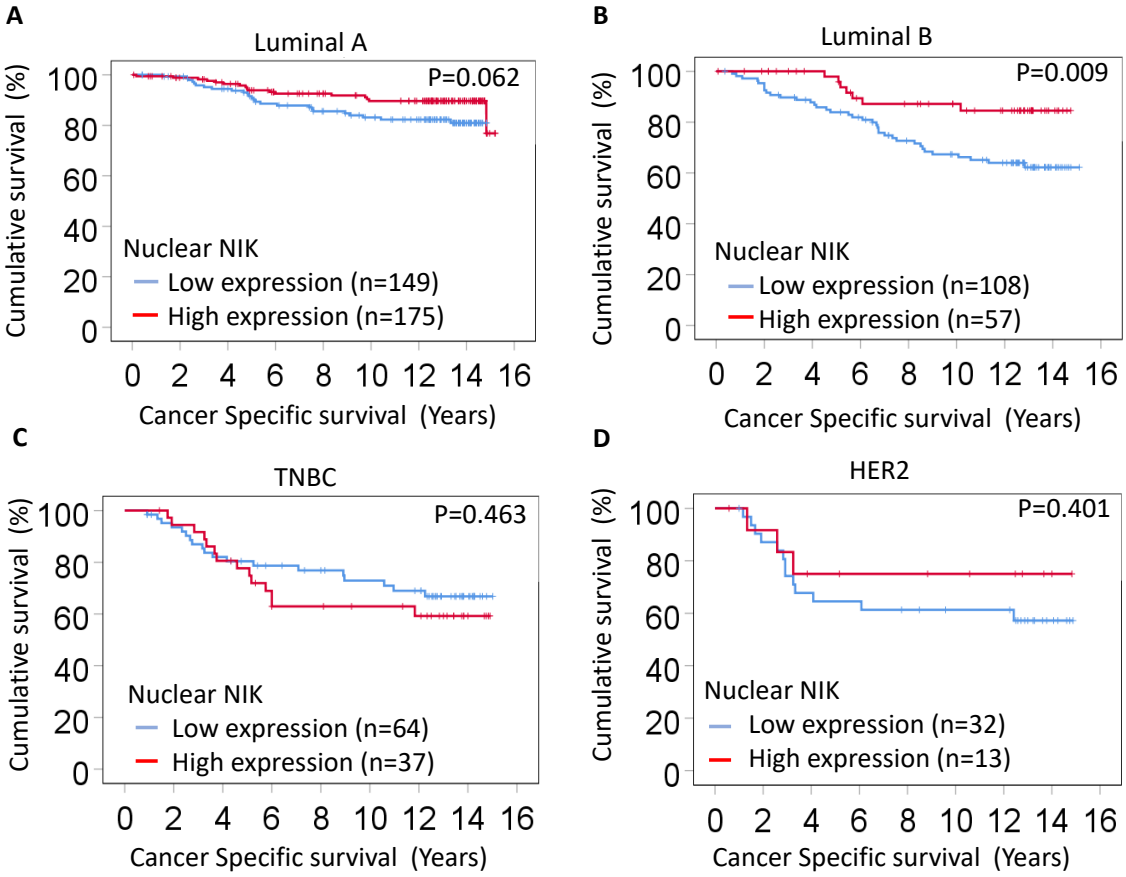


**Figure 3.10 Expression of nuclear NIK and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic NIK and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumour (D).*

Multivariate analysis suggested that nuclear expression of NIK was an independent prognostic factor for cancer specific survival in luminal B tumour, when combined with tumour size and lymph node status (HR 0.36 95% CI 0.16-0.81, P=0.013, Table 3.5).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 1.02 (0.55-1.91) | 0.945 | | |
| Histological type<br>Other/ Lobular/Ductal | 1.59 (0.68-3.69) | 0.285 | | |
| Size (mm)<br>20/ 21-50/ 21-50/>50 | 1.77 (0.14-2.76) | **0.012** | 1.45 (0.93-2.28) | **0.104** |
| Grade<br>I/II/III | 1.55 (0.99-2.42) | 0.051 | | |
| Lymph node status<br>Negative/ Positive | 2.62 (1.46-4.71) | **0.001** | 2.79 (1.51-5.17) | **0.001** |
| ER status<br>Negative/ Positive | 0.73 (0.10-5.29) | 0.754 | | |
| PR status<br>Negative/ Positive | 1.07 (0.59-1.93) | 0.831 | | |
| Her-2 status<br>Negative/ Positive | 1.30 (0.70-2.39) | 0.408 | | |
| Proliferation (Ki67)<br>Low/ High | 0.84 (0.38-1.87) | 0.669 | | |
| Cytoplasmic NIK<br>Low/High | 0.86 (0.47-1.55) | 0.605 | | |
| Nuclear NIK<br>Low/High | 0.36 (0.16-0.80) | **0.013** | 0.36 (0.16-0.81) | **0.013** |

**Table 3.5 Univariate and multivariate analysis for cancer specific survival of NIK and clinicopathological characteristic in luminal B tumours**

No association was observed between cytoplasmic expression of NIK and recurrence free survival in any of four subtypes (luminal A, HR 1.23 95% CI 0.67-2.24, P=0.509; luminal B, HR 0.80 95% CI 0.44-1.45, P=0.457: TNBC HR 1.42 95% CI 0.69-2.93, P=0.340; HER2-positive HR 0.73 95% CI 0.27-1.98, P=0.536) (Figure 3.11).

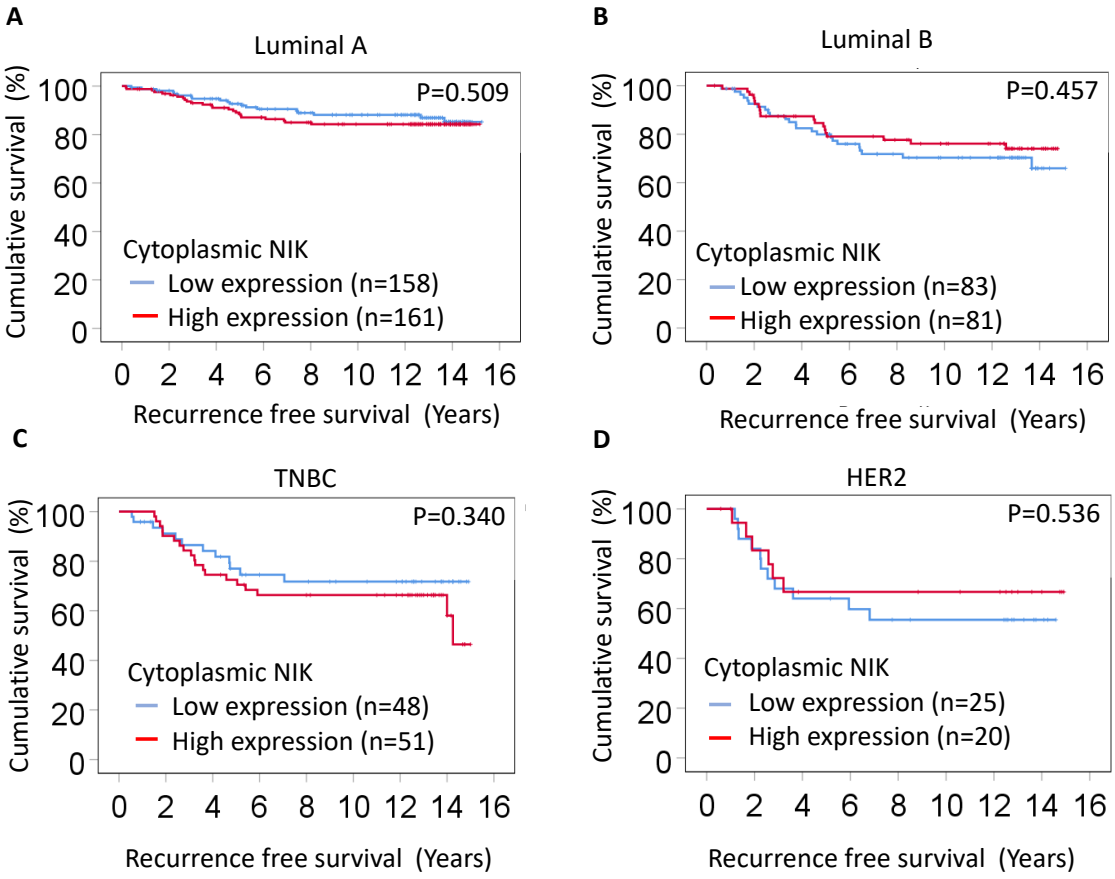


**Figure 3.11 Expression of cytoplasmic NIK and recurrence free survival in different subtypes.** *Kaplan Meier curves showing associations between cytoplasmic NIK and recurrence free survival in luminal A tumours (A), luminal B tumours, TNBC (C) and HER-2 positive tumours (D).*

Similarly, nuclear expression of NIK was not associated with recurrence free survival in any four breast cancer subtypes (luminal A, HR 0.74 95% CI 0.41-1.35, P=0.324; luminal B, HR 0.56 95% CI 0.28-1.14), P=0.108: TNBC, HR 1.33 95% CI 0.65-2.72, P=0.430; HER2-positive HR 0.53 95% CI 0.15-1.85, P=0.312) (Figure 3.12).

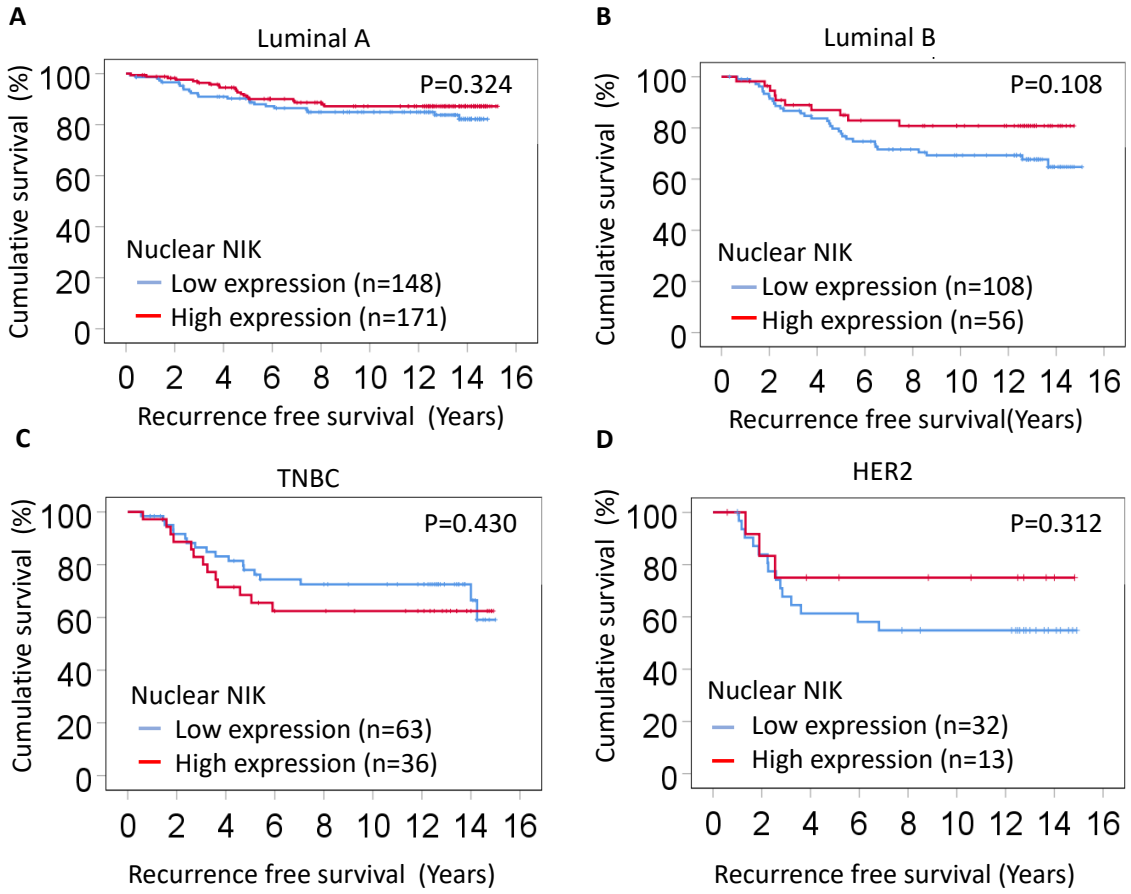


**Figure 3.12 Expression of nuclear NIK and recurrence free survival in different subtypes.**
*Kaplan Meier curves showing associations between cytoplasmic NIK and recurrence free survival in luminal A tumours (A), luminal B tumours, TNBC (C) and HER-2 positive tumours (D).*

## 3.3.2 Expression of IKKα

### 3.3.2.1 Association of IKKα with clinical outcome

Expression of IKKα was assessed in 589 patients from the 1800-cohort. The histoscore for cytoplasmic expression of IKKα ranged from 0 to 300 with a median histoscore of 120 (IQR=95.0-160.0). The histoscore for nuclear expression of IKKα ranged from 0 to 279 with a median histoscore of 100 (IQR=67.5-97.5). An example of immunostaining of low and high expression of IKKα and Histograms showing the distribution of histoscores for

cytoplasmic and nuclear expression of IKKα are shown in Figure 3.13. There was moderate correlation between observers with ICCC score of 0.73 for cytoplasmic and 0.73 for nuclear expression.

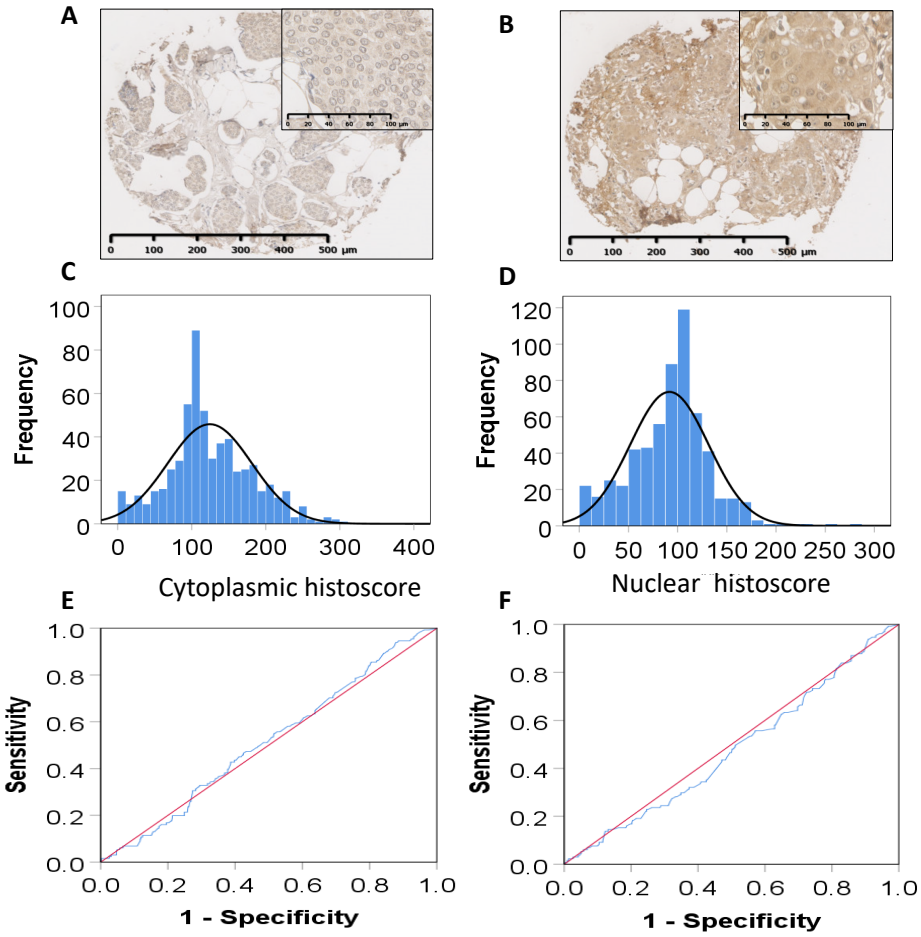


**Figure 3.13 Expression of IKKα.** *(A) Example of low expression and (B) of high expression of IKKα (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of IKKα. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of IKKα*

The ROC curve was plotted to determine the cut-off in order to classify low and high expression of cytoplasmic IKKα and nuclear IKKα (Figure 4.7). Since the AUC was 0.51 (P=0.648) and 0.48 (P=0.415) for cytoplasmic and nuclear IKKα, the values from ROC curves were unable to be used to determine optimal cut-off. Therefore, the median histoscore was used as an optimal cut-off to subdivide the cohort into low and high expression. Tumours with a cytoplasmic histoscore over 120 and tumour with a nuclear histoscore over 100 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-offs, 264 patients had high cytoplasmic expression and 325

patients had low cytoplasmic expression. 243 patients had high nuclear expression and 346 patients had low nuclear expression.

To determine whether IKKα expression was associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of IKKα were plotted as shown in Figure 3.14. Low and high expression were compared by using the log-rank test. Expression of IKKα was not associated with cancer specific survival (cytoplasmic IKKα, HR 1.14 95% CI 0.81-1.60, P=0.468: nuclear IKKα, HR 0.80 95% CI 0.56-1.14, P=0.215, Figure 3.14). Similarly, expression of IKKα was not associated with recurrence free survival (cytoplasmic IKKα, HR 1.16 95% CI 0.81-1.67, P=0.412: nuclear IKKα, HR 0.87 95% CI 0.60-1.26, P=0.455, Figure 3.14).

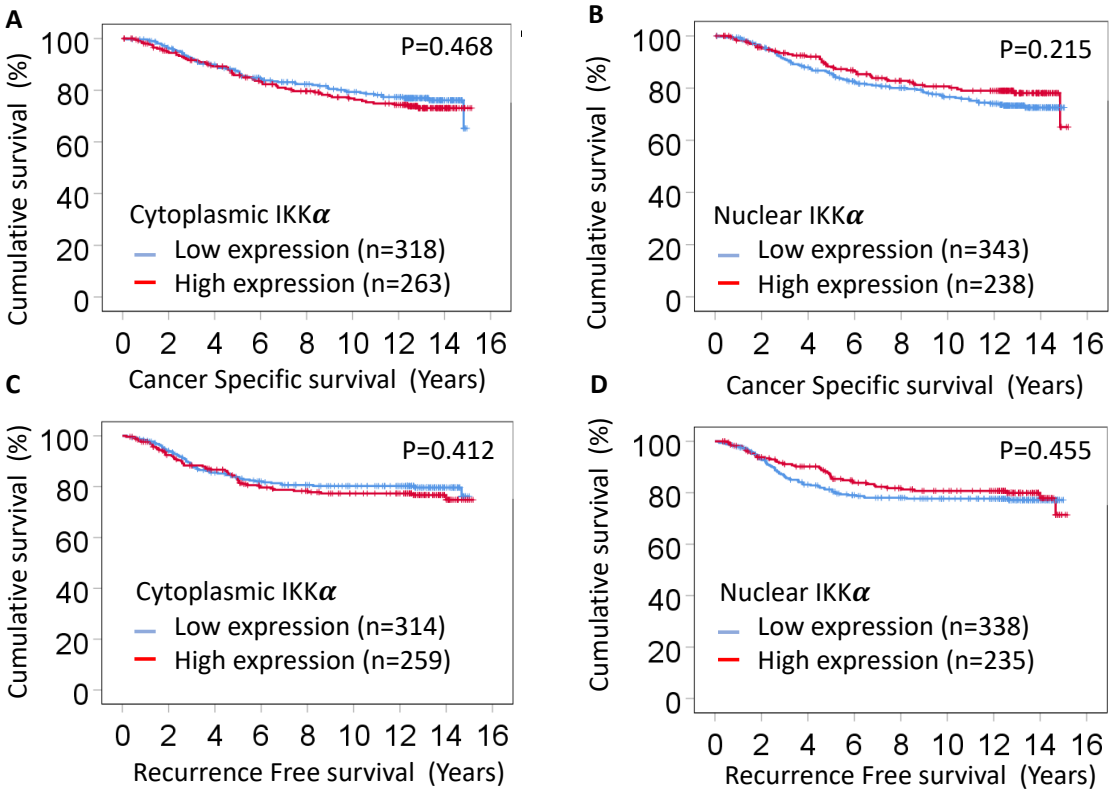


**Figure 3.14 Expression of IKKα and clinical outcome in the 1800-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear IKKα with cancer specific survival (A and B) and recurrence free survival (C and D)*

### 3.3.2.2 Associations between IKKα and clinicopathological characteristics

Chi-squared analysis was performed in order to determine whether expression of IKKα was associated with any clinicopathological characteristic of the patients in the 1800-cohort, as shown in Table 3.6. Expression of cytoplasmic IKKα was associated with HER2 status (P=0.006), proliferation (Ki67) (P=0.006) and molecular subtype (P=002). Expression of nuclear IKKα was associated with patient age (P=0.016), tumour grade (P=0.001), ER status (P<0.001), PR status (P<0.001) and molecular subtype (P=0.001).

| Clinicopathological characteristics | Cytoplasmic IKKα | | | Nuclear IKKα | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 104 (32.0) | 76 (28.8) | 0.400 | 119 (34.4) | 61 (25.1) | **0.016** |
| >50 years) | 221 (68.0) | 188 (71.2) | | 227 (65.6) | 182 (74.9) | |
| Histological type | | | | | | |
| Other | 16 (4.9) | 14 (5.3) | 0.074 | 15 (4.3) | 15 (6.2) | **0.003** |
| Lobular | 29 (8.9) | 11 (4.2) | | 14 (4.0) | 26 (10.7) | |
| Ductal | 280 (86.2) | 239 (90.5) | | 317 (91.6) | 202 (83.1) | |
| Size (mm) | | | | | | |
| ≤20 | 187 (57.7) | 129 (48.9) | 0.063 | 190 (55.1) | 126 (51.9) | 0.589 |
| 21-50 | 123 (38.0) | 116 (43.9) | | 138 (40.0) | 101 (41.6) | |
| >50 | 14 (4.3) | 19 (7.2) | | 17 (4.9) | 16 (6.6) | |
| Grade | | | | | | |
| I | 68 (20.9) | 47 (17.8) | 0.456 | 61 (17.6) | 54 (22.2) | **0.001** |
| II | 147 (45.2) | 116 (43.9) | | 140 (40.5) | 123 (50.6) | |
| III | 110 (33.8) | 101 (38.3) | | 145 (41.9) | 66 (27.2) | |
| Lymph node status | | | | | | |
| Negative | 182 (56.9) | 152 (58.7) | 0.661 | 194 (56.6) | 140 (29.3) | 0.509 |
| Positive | 138 (43.1) | 107 (41.3) | | 149 (43.4) | 96 (40.7) | |
| ER status | | | | | | |
| Negative | 86 (26.5) | 79 (29.9) | 0.352 | 131 (37.9) | 34 (14.0) | **<0.001** |
| Positive | 239 (73.5) | 185 (70.1) | | 215 (62.1) | 209 (86.0) | |
| PR status | | | | | | |
| Negative | 149 (45.8) | 140 (53.0) | 0.083 | 191 (55.2) | 98 (40.3) | **<0.001** |
| Positive | 176 (54.2) | 124 (47.0) | | 155 (44.8) | 145 (59.7) | |
| Her-2 status | | | | | | |
| Negative | 280 (87.8) | 209 (79.5) | **0.006** | 286 (83.6) | 203 (84.6) | 0.756 |
| Positive | 39 (12.2) | 54 (20.5) | | 56 (16.4) | 37 (15.4) | |
| Proliferation (Ki67) | | | | | | |
| Low | 222 (73.0) | 150 (58.6) | **<0.001** | 221 (68.4) | 151 (63.7) | 0.244 |
| High | 82 (27.0) | 106 (41.4) | | 102 (31.6) | 86 (36.3) | |
| Molecular subtype | | | | | | |
| Luminal A | 163 (52.6) | 99 (38.5) | **0.002** | 140 (42.2) | 122 (51.9) | **<0.001** |

| | | | | | | |
|---|---|---|---|---|---|---|
| Luminal B | 69 (22.3) | 81 (31.5) | | 68 (20.5) | 82 (34.9) | |
| TNBC | 59 (19.0) | 48 (18.7) | | 89 (26.8) | 18 (7.7) | |
| HER2 | 19 (6.1) | 29 (11.3) | | 35 (10.5) | 13 (5.5) | |

**Table 3.6 Association of IKKα with clinic-pathological characteristics of the 1800-cohort.** *Chi-squared analysis explored the relationship of IKKα with various characteristics of the cohort*

### 3.3.2.3 Expression of IKKα and clinical outcome in different luminal subtypes

Association between expression of IKKα and clinical outcome was also assessed in four breast cancer subtypes as previously described. Cytoplasmic expression of IKKα was not associated with cancer specific survival within each subtype (luminal A, HR 1.17 95% CI 0.59-2.29, P=0.656; luminal B, HR 0.98 95% CI 0.53-1.80, P=0.936: TNBC, HR 1.23 95% CI 0.65-2.35, P=0.523; HER2-positive HR 0.64 95% CI 0.23-1.84, P=0.408) (Figure 3.15).

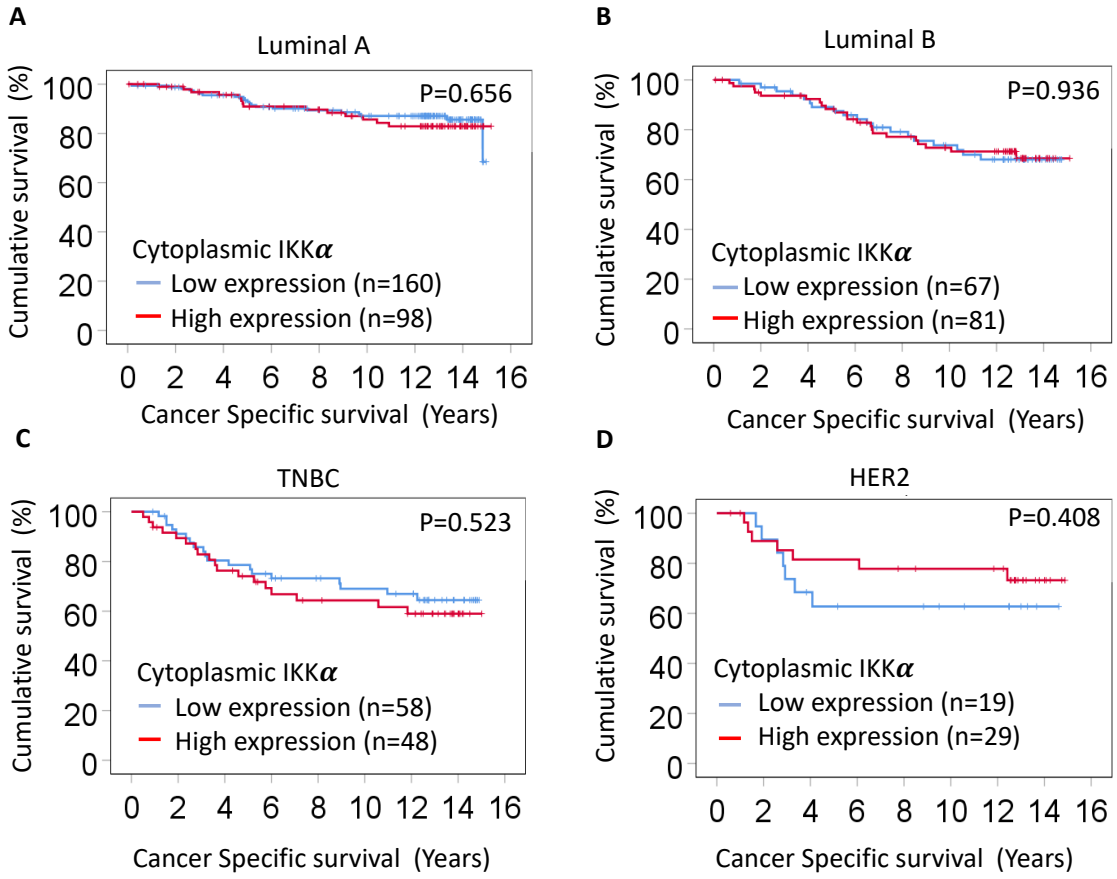


**Figure 3.15 Expression of cytoplasmic IKKα and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic IKKα and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positve tumours (D).*

Similar to cytoplasmic expression of IKKα, nuclear expression of IKKα was not associated with cancer specific survival in each subtype (luminal A, HR 0.88 95% CI 0.45-1.73, P=0.715; luminal B, HR 1.08 95% CI 0.58-2.00, P=0.805; TNBC, HR 0.83 95% CI 0.33-2.14, P=0.706; HER2-positive HR 0.42 95% CI 0.09-1.87, P=0.238), (Figure 3.16).

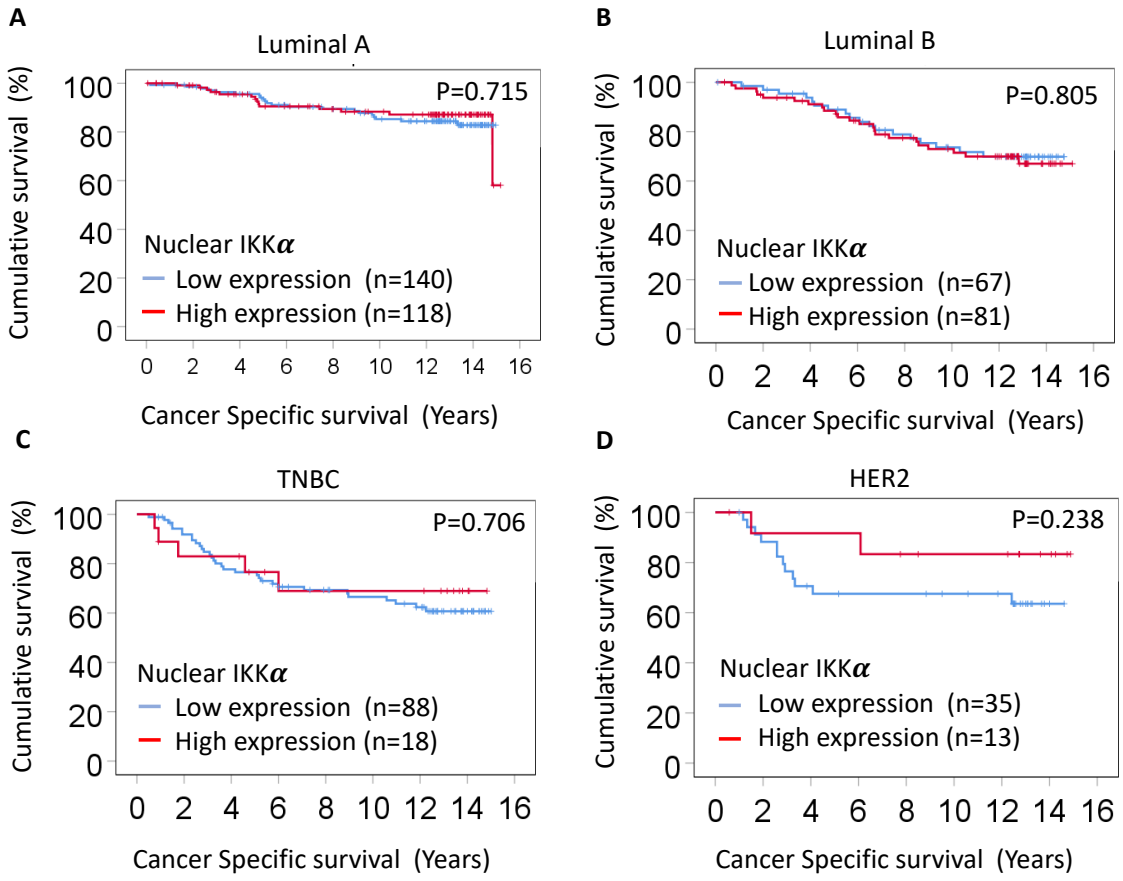


**Figure 3.16 Expression of nuclear IKKα and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between nuclear IKKα and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positve tumours (D).*

In terms of recurrence free survival, no association was observed with cytoplasmic expression of IKKα within each subtype (luminal A, HR 1.13 95% CI 0.55-2.35, P=0.740; luminal B, HR 1.20 95% CI 0.61-2.35, P=0.597: TNBC, HR 1.19 95% CI 0.60-2.36, P=0.618; HER2-positive HR 0.52 95% CI 0.19-1.39, P=0.182) (Figure 3.17).

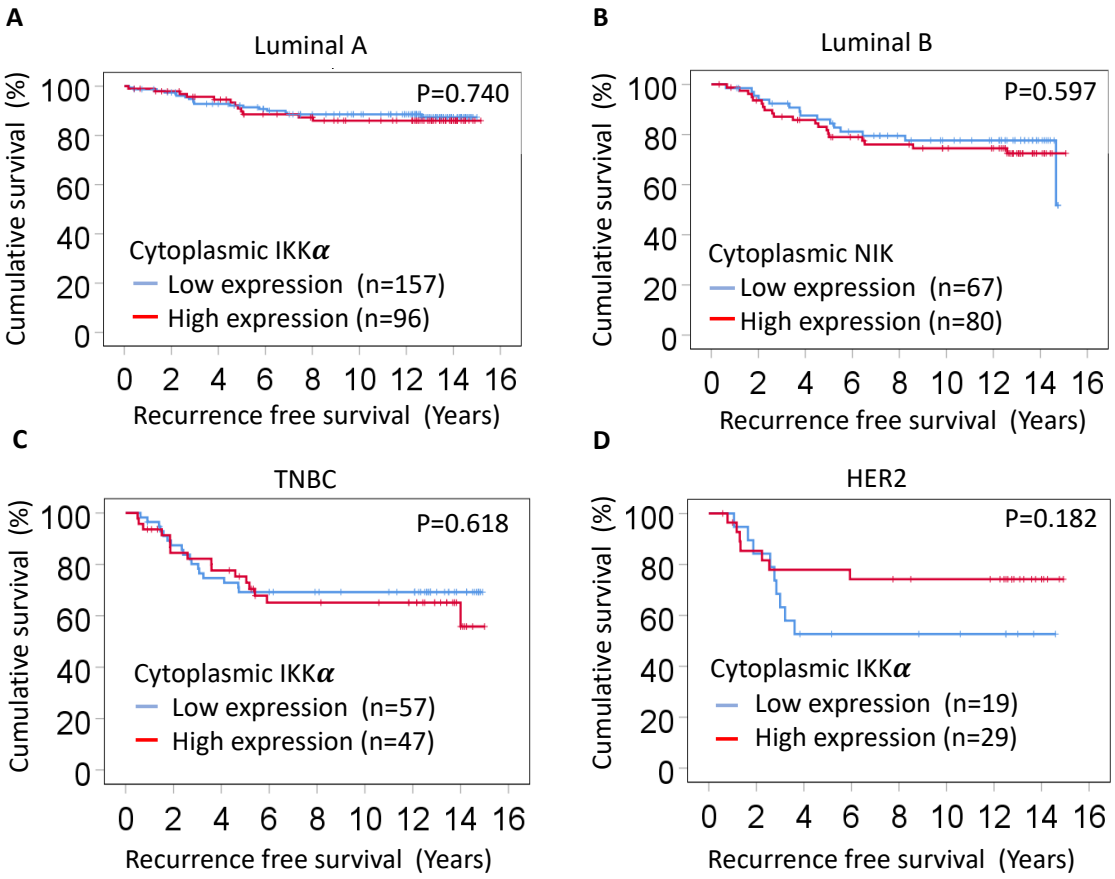


**Figure 3.17 Expression of cytoplasmic IKKα and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic IKKα and recurrence free survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours(C) and HER2-positive tumours (D)*

Expression of nuclear IKKα was not associated with recurrence free survival within each subtype (luminal A, HR 1.27 95% CI 0.62-2.60, P=0.514; luminal B, HR 0.99 95% CI 0.51-1.93, P=0.983: TNBC, HR 1.10 95% CI 0.45-2.66, P=0.836; HER2-positive HR 0.34 95% CI 0.08-1.49, P=0.132) (Figure 3.18).

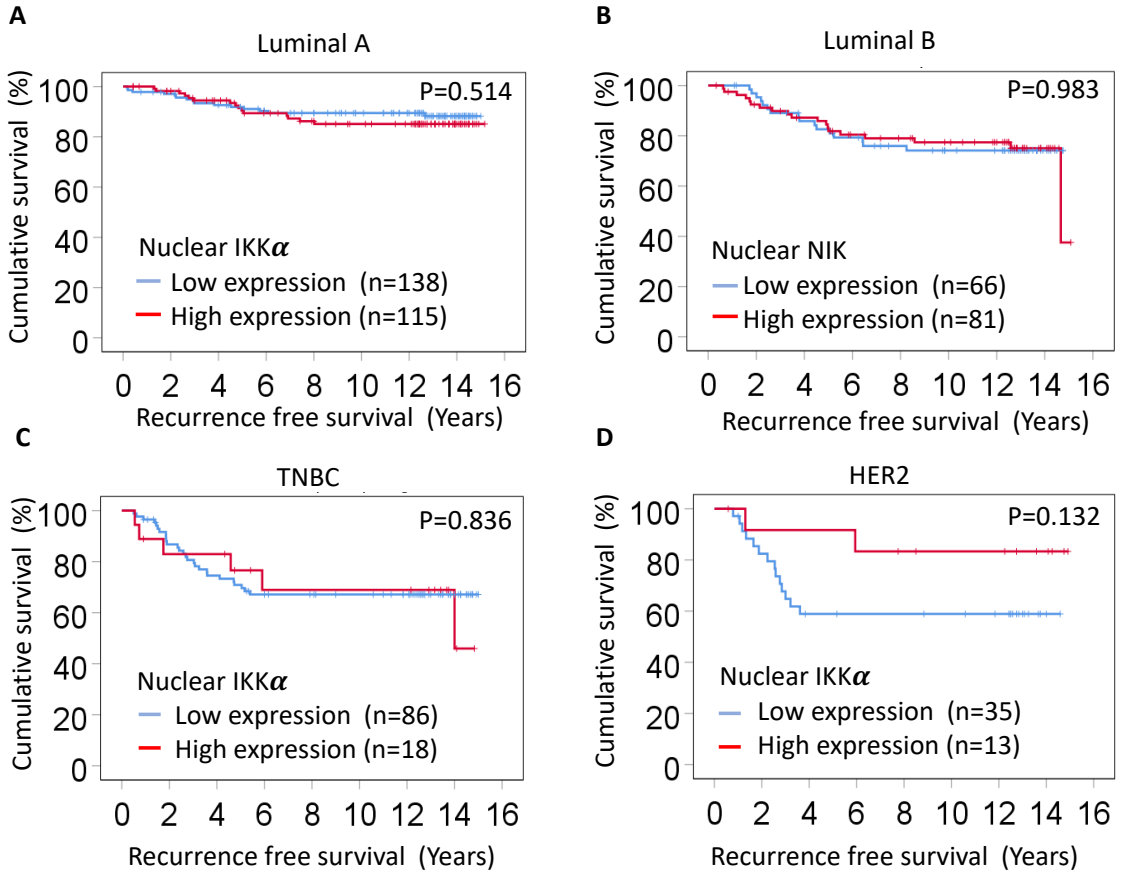


**Figure 3.18 Expression of nuclear IKKα and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic IKKα and recurrence free survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

## 3.3.3 Expression of pIKKα-Ser176 in the 1800-cohort

### 3.3.3.1 Expression of pIKKα-Ser176 and clinical outcomes

Expression of IKKα phosphorylation at serine residue 176 (Ser176) was assessed in 598 patients. The histoscore for cytoplasmic pIKKα-Ser176 ranged from 0 to 300 with a median histoscore of 100 (IQR=66.3-115.5). The histoscore for nuclear pIKKα-Ser176 ranged from 0 to 225 with a median histoscore of 70 (IQR=45.0.3-98.3). An example of immunostaining of low and high expression of pIKKα-Ser176 is shown in Figure 3.19. Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of pIKKα-Ser176 are

shown in Figure 3.19. There was good and moderate correlation between observers with ICCC score of 0.77 for cytoplasmic and a score of 0.74 for nuclear expression.

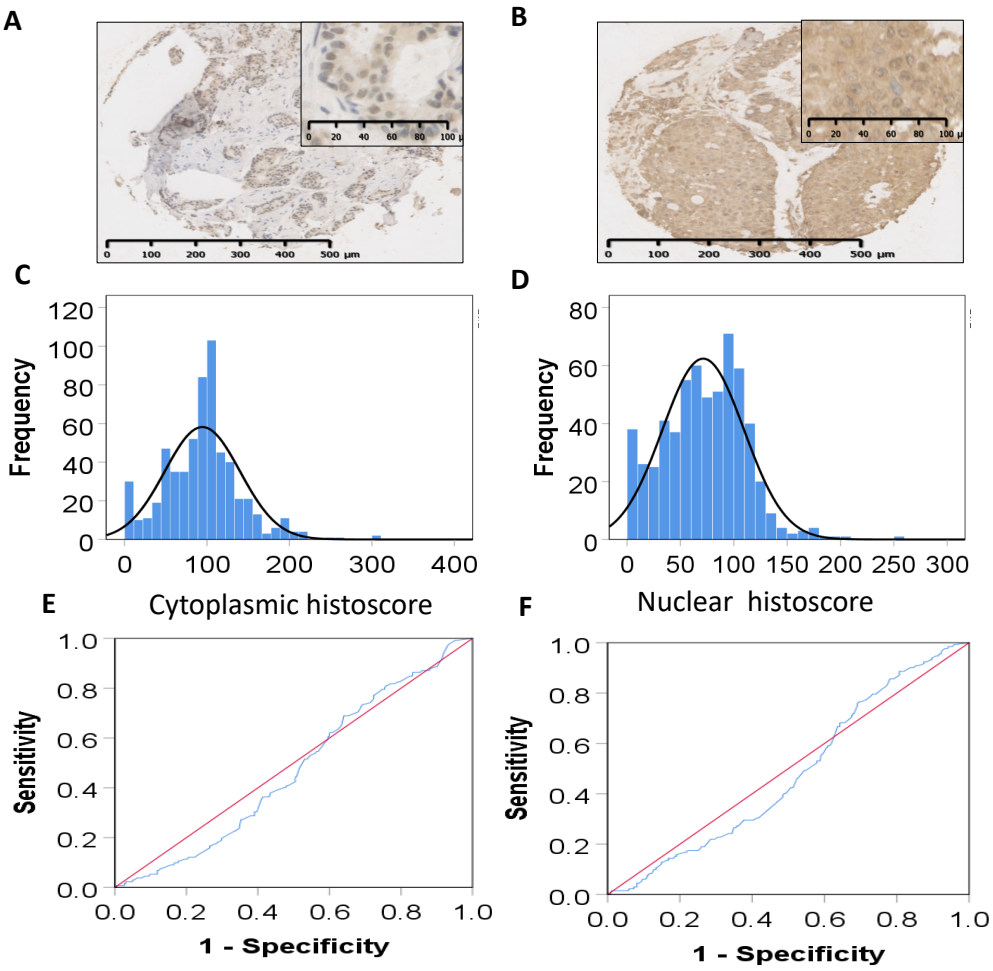


**Figure 3.19 Expression of pIKKα-Ser176.** *(A) Example of low expression and (B) of high expression of pIKKα-Ser176 (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of pIKKα-Ser176. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of pIKKα-Ser176.*

The ROC curve was plotted to determine the cut-off in order to classify low and high expression of cytoplasmic and nuclear pIKKα-Ser176 (Figure 3.19). Since the AUC was 0.427 (P=0.463) and 0.54 (P=0.336) for cytoplasmic and nuclear pIKKα-Ser176, the values from ROC curves were unable to be used to determine optimal cut-off. Therefore, the median histoscore was used as the optimal cut-off. Tumours with cytoplasmic histoscore over 100 and tumour with nuclear histoscore over 70 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-off values, 233 patients had high cytoplasmic expression and 365 patients had low cytoplasmic expression. 304 patients had high nuclear expression and 294 patients had low nuclear expression.

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pIKKα-Ser176 were plotted as shown in Figure 3.20. The log rank test was used to compare low and high expression. A significant association with decreased cancer specific survival was observed in tumours with low expression of cytoplasmic pIKKα-Ser176 (HR 0.63 95% CI 0.44-0.92, P=0.014) but was not observed in tumour with low expression of nuclear pIKKα-Ser176 (HR 0.84 95% CI 0.60-1.18, P=0.308) (Figure 3.20). Furthermore, a trend toward significance was observed between decreased recurrence free survival in tumours with low expression of cytoplasmic pIKKα-Ser176 (HR 0.69 95% CI 0.47-1.01, P=0.052) and low expression of nuclear pIKKα-Ser176 (HR 0.70 95% CI 0.49-1.00, P=0.052, Figure 3.20).

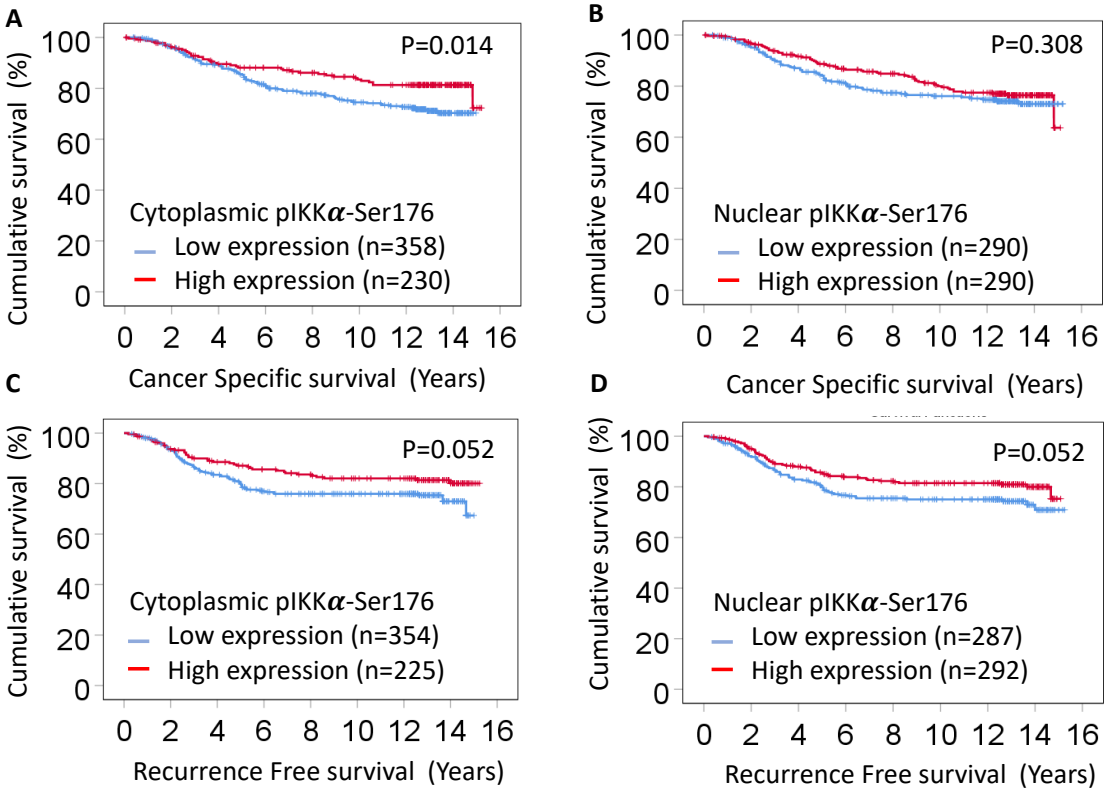


**Figure 3.20 Expression of pIKKα-Ser176 and clinical outcome in the 1800-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pIKKα-Ser176 with cancer specific survival (A and B) and recurrence free survival (C and D)*

Multivariate analysis suggested that cytoplasmic expression of pIKKα-Ser176 was not an independent prognostic marker for cancer specific survival when combined with histological type, tumour size, tumour grade, lymph node status, ER status, PR status, HER2 status, Ki67 and molecular subtypes, (HR 0.72 95% CI 0.49-1.05, P= 0.090) (Table 3.7).

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| Patient characteristics | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 1.05 (0.77-1.45) | 0.748 | | |
| Histological type<br>Other/ Lobular/Ductal | 1.87 (1.21-2.89) | **0.005** | 1.46 (0.82-2.58) | 0.200 |
| Size (mm)<br>20/ 21-50/ 21-50/>50 | 2.14 (1.71-2.68) | **<0.001** | 1.81 (1.35-2.43) | **<0.001** |
| Grade<br>I/II/III | 1.98 (1.57-2.49) | **<0.001** | 1.38 (1.01-1.89) | **0.046** |
| Lymph node status<br>Negative/ Positive | 3.25 (2.37-4.45) | **<0.001** | 2.77 (1.87-4.10) | **<0.001** |
| ER status<br>Negative/ Positive | 0.57 (0.42-0.77) | **<0.001** | 1.57 (0.71-3.46) | 0.262 |
| PR status<br>Negative/ Positive | 0.54 (0.39-0.74) | **<0.001** | 0.85 (0.54-1.34) | 0.485 |
| Her-2 status<br>Negative/ Positive | 1.84 (1.28-2.62) | **0.001** | 1.35 (0.79-2.31) | 0.270 |
| Proliferation (Ki67)<br>Low/ High | 1.64 (1.20-2.25) | **0.002** | 1.11 (0.68-1.83) | 0.674 |
| Molecular subtype<br>Luminal A/Luminal B/TNBC/HER2 | 1.46 (1.27-1.67) | **<0.001** | 1.31 (1.08-1.59) | **0.007** |
| Cytoplasmic pIKKα-Ser176<br>Low/High | 0.63 (0.44-0.92) | **0.015** | 0.72 (0.49-1.05) | 0.090 |
| Nuclear pIKKα-Ser176<br>Low/High | 0.84 (0.60-1.18) | 0.310 | | |

**Table 3.7 Univariate and multivariate analysis for cancer specific survival of pIKKα-Ser176 and clinicopathological characteristic in the full cohort of the 1800-cohort**

### 3.3.3.2 Associated of pIKKα-Ser176 and clinic-pathological characteristics

Chi-squared analysis was performed in order to determine whether expression of pIKKα-Ser176 was associated with any clinicopathological characteristic of the patients in the 1800-cohort as shown in Table 3.8. Expression of cytoplasmic pIKKα-Ser176 was associated only with tumour grade (P=0.006). Expression of nuclear pIKKα-Ser176 was associated with tumour grade (P<0.001), ER status (P<0.001) and molecular subtypes (P<0.001).

| Clinicopathological characteristics | Cytoplasmic Ser176 | | | Nuclear Ser176 | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 105 (28.8) | 78 (33.5) | 0.223 | 94 (32.0) | 89 (29.3) | 0.474 |
| >50 years) | 260 (71.2) | 155 (66.5) | | 200 (68.0) | 215 (70.7) | |
| Histological type | | | | | | |
| Other | 18 (4.9) | 15 (6.4) | 0.482 | 17 (5.8) | 16 (5.3) | 0.287 |
| Lobular | 26 (7.1) | 12 (5.2) | | 14 (4.8) | 24 (7.9) | |
| Ductal | 321 (87.9) | 206 (88.4) | | 263 (89.5) | 264 (86.8) | |
| Size (mm) | | | | | | |
| ≤20 | 204 (56.0) | 129 (55.4) | 0.975 | 164 (56.0) | 169 (55.6) | 0.607 |
| 21-50 | 141 (38.7) | 91 (39.1) | | 116 (39.6) | 116 (38.2) | |
| >50 | 19 (5.2) | 13 (5.6) | | 13 (4.4) | 19 (6.3) | |
| Grade | | | | | | |
| I | 60 (16.5) | 57 (24.5) | **0.006** | 43 (14.7) | 74 (24.3) | **<0.001** |
| II | 163 (44.8) | 112 (48.1) | | 123 (42.0) | 152 (50.0) | |
| III | 141 (38.7) | 64 (27.5) | | 127 (43.3) | 78 (25.7) | |
| Lymph node status | | | | | | |
| Negative | 210 (58.3) | 132 (57.9) | 0.916 | 175 (60.1) | 167 (56.2) | 0.337 |
| Positive | 150 (41.7) | 96 (42.1) | | 116 (39.9) | 130 (43.8) | |
| ER status | | | | | | |
| Negative | 104 (28.5) | 50 (21.5) | 0.055 | 110 (37.4) | 44 (14.5) | **<0.001** |
| Positive | 261 (71.5) | 183 (78.5) | | 184 (62.6) | 260 (85.5) | |
| PR status | | | | | | |
| Negative | 187 (51.2) | 101 (43.3) | 0.060 | 167 (56.8) | 121 (39.8) | **<0.001** |
| Positive | 178 (48.8) | 132 (56.7) | | 127 (43.2) | 183 (60.2) | |
| Her-2 status | | | | | | |
| Negative | 297 (83.0) | 202 (87.1) | 0.177 | 237 (82.6) | 262 (86.5) | 0.191 |
| Positive | 61 (17.0) | 30 (12.9) | | 50 (17.4) | 41 (13.5) | |
| Proliferation (Ki67) | | | | | | |
| Low | 221 (64.4) | 156 (69.3) | 0.226 | 177 (64.6) | 200 (68.0) | 0.387 |
| High | 122 (35.6) | 69 (30.7) | | 97 (35.4) | 94 (32.0) | |
| Molecular subtype | | | | | | |
| Luminal A | 155 (44.2) | 121 (53.8) | 0.157 | 108 (38.7) | 168 (56.6) | **<0.001** |
| Luminal B | 100 (28.5) | 55 (24.4) | | 69 (24.7) | 86 (29.0) | |
| TNBC | 65 (18.5) | 34 (15.1) | | 71 (25.4) | 28 (9.4) | |
| HER2 | 31 (8.8) | 15 (6.7) | | 31 (11.1) | 15 (5.1) | |

**Table 3.8 Association of pIKKα-Ser76 with clinic-pathological characteristics of the 1800-cohort.** *Chi-squared analysis explored the relationship of pIKKα-Ser176 with various characteristics of the cohort.*

### 3.3.3.3 Expression of pIKKα-Ser176 and clinical outcome in different breast cancer subtypes

Expression of pIKKα-Ser176 was also assessed in different breast cancer subtype (Figure 3.21). Low expression of cytoplasmic pIKKα-Ser176 was associated with decreased cancer specific survival in luminal B tumours (HR 0.47 95% CI 0.23-0.96, P=0.035). However, the association was not observed in luminal A tumours (HR 0.47 95% CI 0.23-0.96, P=0.593), TNBC patients (HR 0.84 95% CI 0.44-1.61, P=0.095) and HER2-positive tumours (HR 0.86 95% CI 0.27-2.75, P=0.802).

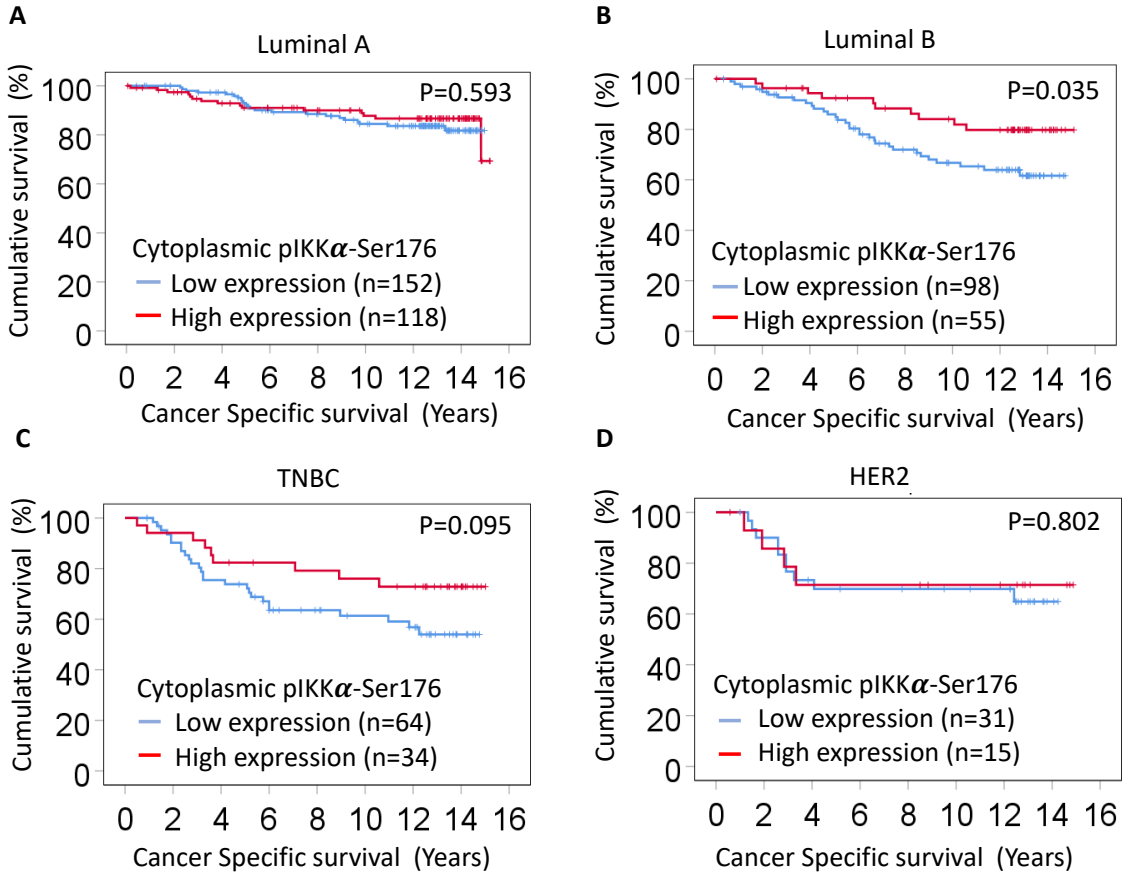


**Figure 3.21 Expression of cytoplasmic pIKKα-Ser176 and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα-Ser176 and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

The univariate analysis was performed first to investigate the survival with respect to a single variable, but it was ignoring the impact of any others. In breast cancer, several factors potential affect patient prognosis. Thus, when investigating survival in relation to any one factor, it was often desirable to adjust for the impact of others. Therefore, multivariable was used to analyse survival with respect to several factors simultaneously.

In univariate analysis, tumour size, lymph node involvement, and cytoplasmic pIKKα-Ser176 were associated with cancer specific survival in luminal B tumours. The association between pIKKα-Ser176 and cancer specific survival was also found to be significant on multivariate analysis, indicating an independent prognostic role of pIKKα-Ser176 (HR 0.47 95% CI 0.23-0.95, P= 0.036). (Table 3.9).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 1.02 (0.55-1.91) | 0.945 | | |
| Histological type<br>Other/ Lobular/Ductal | 1.59 (0.68-3.69) | 0.285 | | |
| Size (mm)<br>20/ 21-50/ 21-50/>50 | 1.77 (0.14-2.76) | **0.012** | 1.47 (0.92-2.33) | 0.104 |
| Grade<br>I/II/III | 1.55 (0.99-2.42) | 0.051 | | |
| Lymph node status<br>Negative/ Positive | 2.62 (1.46-4.71) | **0.001** | 2.73 (1.46-5.09) | **0.002** |
| ER status<br>Negative/ Positive | 0.73 (0.100-5.29) | 0.754 | | |
| PR status<br>Negative/ Positive | 1.07 (0.59-1.93) | 0.831 | | |
| Her-2 status<br>Negative/ Positive | 1.30 (0.70-2.39) | 0.408 | | |
| Proliferation (Ki67)<br>Low/ High | 0.84 (0.38-1.87) | 0.669 | | |
| Cytoplasmic pIKKα-Ser176<br>Low/High | 0.47 (0.23-0.96) | **0.039** | 0.47 (0.23-0.95) | **0.036** |
| Nuclear pIKKα-Ser176<br>Low/High | 0.98 (0.53-1.81) | 0.958 | | |

**Table 3.9 Univariate and multivariate analysis for cancer specific survival of pIKKα-Ser176 and clinicopathological characteristic in luminal B tumours**

**Expression of nuclear pIKKα-Ser176 was not associated with cancer specific survival in any of four breast cancer subtypes (luminal A, HR 0.84 95% CI 0.44-1.60, P=0.598; luminal B; HR 0.98 95% CI 0.53-1.81, P=0.958; TNBC tumour (HR 0.83 95% CI 0.41-1.67, P=0.596) and HER2-positive tumour (HR 0.72 95% CI 0.23-2.31, P=0.581) (**
Figure 3.22).

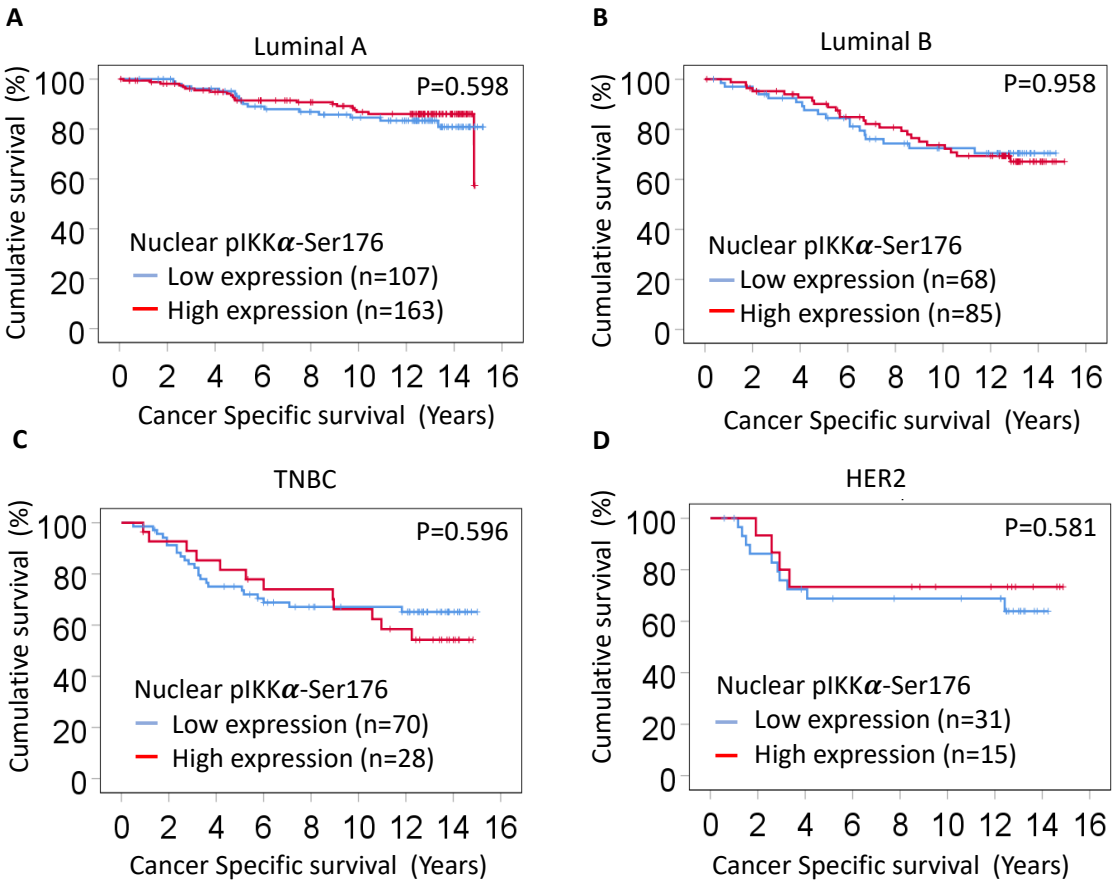


**Figure 3.22 Expression of nuclear pIKKα-Ser176 and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα-Ser176 and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumour (C) and HER2-positive tumour (D).*

**In terms of recurrence free survival, a trend towards improved recurrence-free survival was noted in patients with luminal B disease (HR 0.52 95% CI 0.25-1.06, P=0.068). But no association was found between expression of cytoplasmic pIKKα-Ser176 and this outcome in any of the other three breast cancer subtypes. (luminal A, HR 0.84 95% CI 0.43-1.66, P=0.618; TNBC tumour (HR 0.66 95% CI 0.30-1.43, P=0.283 and HER2-positive tumour HR 1.11 95% CI 0.39-3.22, P=0.837) (**
Figure 3.23).

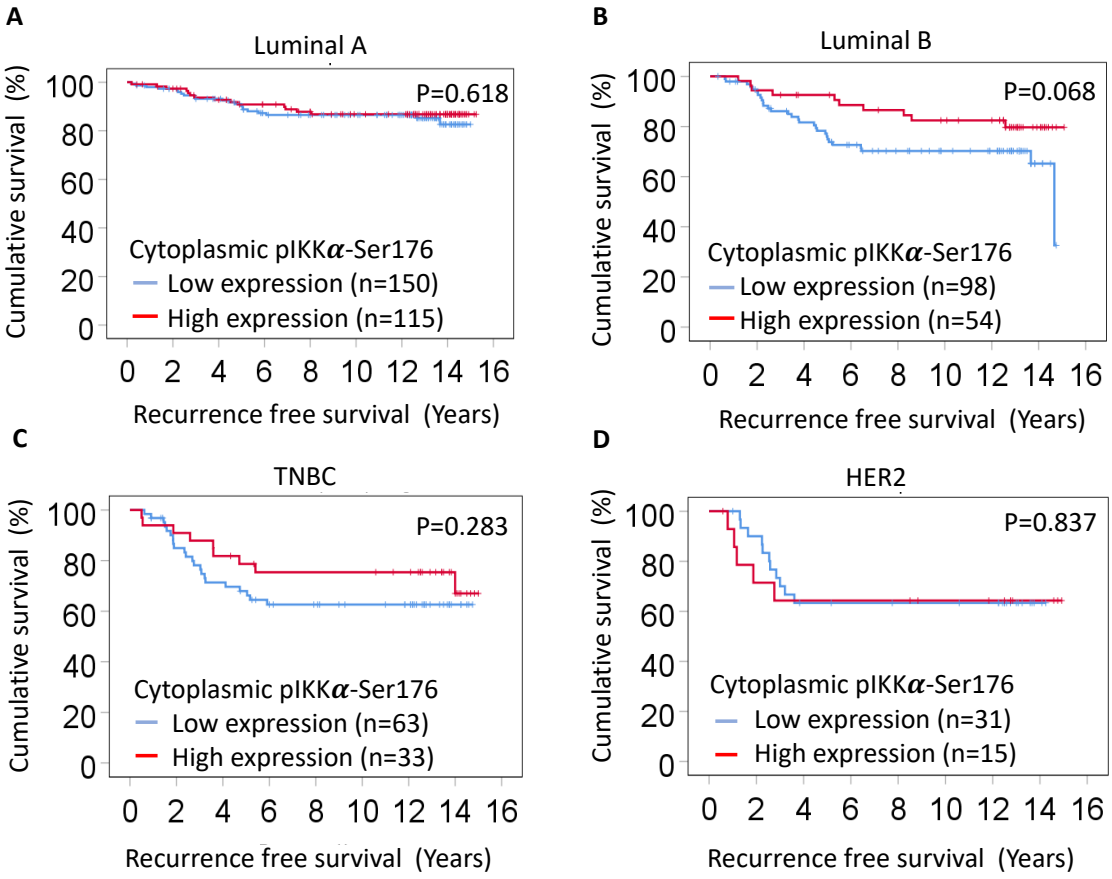


**Figure 3.23 Expression of cytoplasmic pIKKα-Ser176 and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα-Ser176 and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

Similarly, expression of nuclear pIKKα-Ser176 was not associated with recurrence free survival in any of four breast cancer subtypes (luminal A, HR 1.29 95% CI 0.66-2.51, P=0.451; luminal B; HR 1.12 95% CI 0.60-2.11, P=0.724; TNBC tumour HR 1.55 95% CI 0.67-3.60, P=0.306 and HER2-positive tumour HR 1.21 95% CI 0.42-3.48, P=0.725) (Figure 3.24).

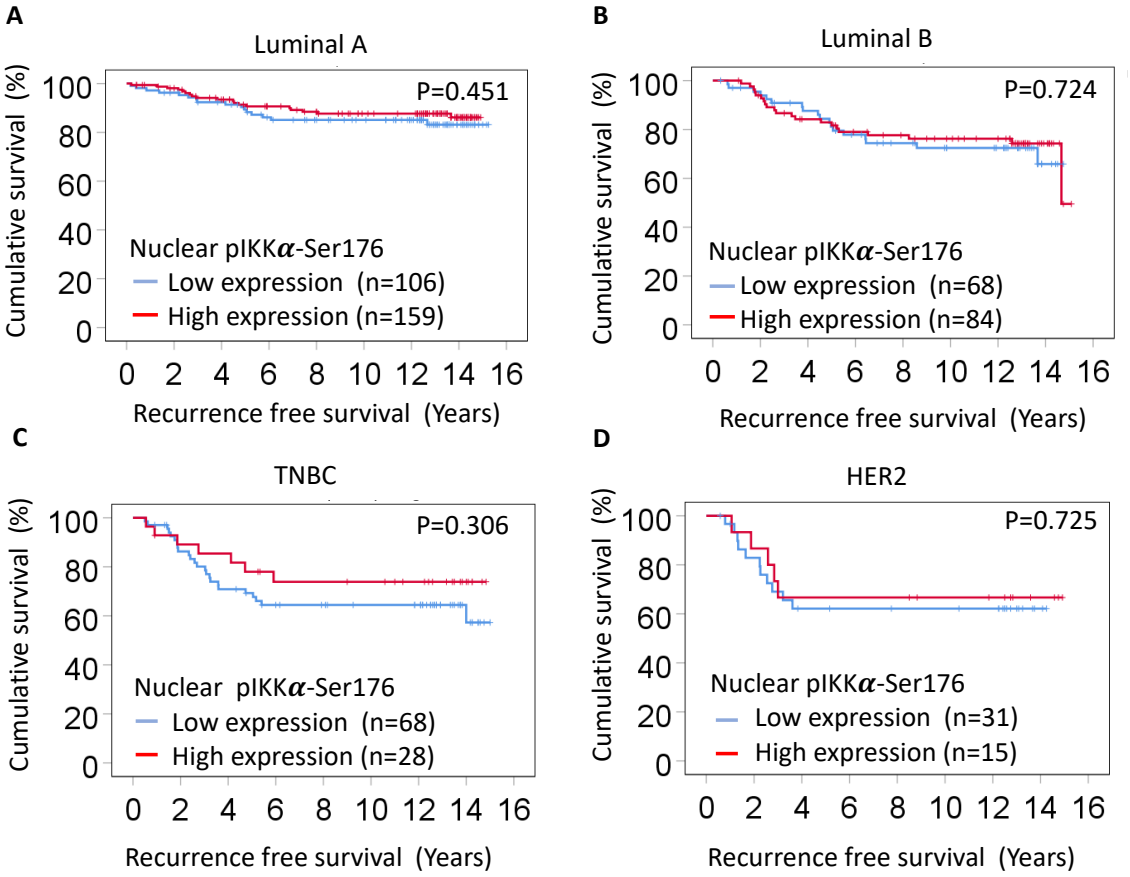


**Figure 3.24 Expression of nuclear pIKKα-Ser176 and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα-Ser176 and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

## 3.3.4 Expression of pIKKα-Thr23

### 3.3.4.1 Expression of pIKKα- Thr23 and clinical outcome in the 1800-cohort

Expression of IKKα phosphorylation at threonine residue 23 (pIKKα-Thr23) was assessed in 599 patients within the 1800-cohort. The histoscore for cytoplasmic expression of pIKKα-Thr23 ranged from 0 to 208 with a median histoscore of 80 (IQR=48.3-98.0). The histoscore for nuclear phosphorylation of pIKKα-Thr23 ranged from 0 to 300 and a median histoscore of 90 (IQR=48.3-148.3). An example of immunostaining of low and high expression of pIKKα-Thr23 is shown in Figure 3.25. Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of pIKKα-Thr23 are shown (Figure 3.25). There was moderated correlation between observers with ICCC score of 0.74 and 0.73 for cytoplasmic and nuclear expression.

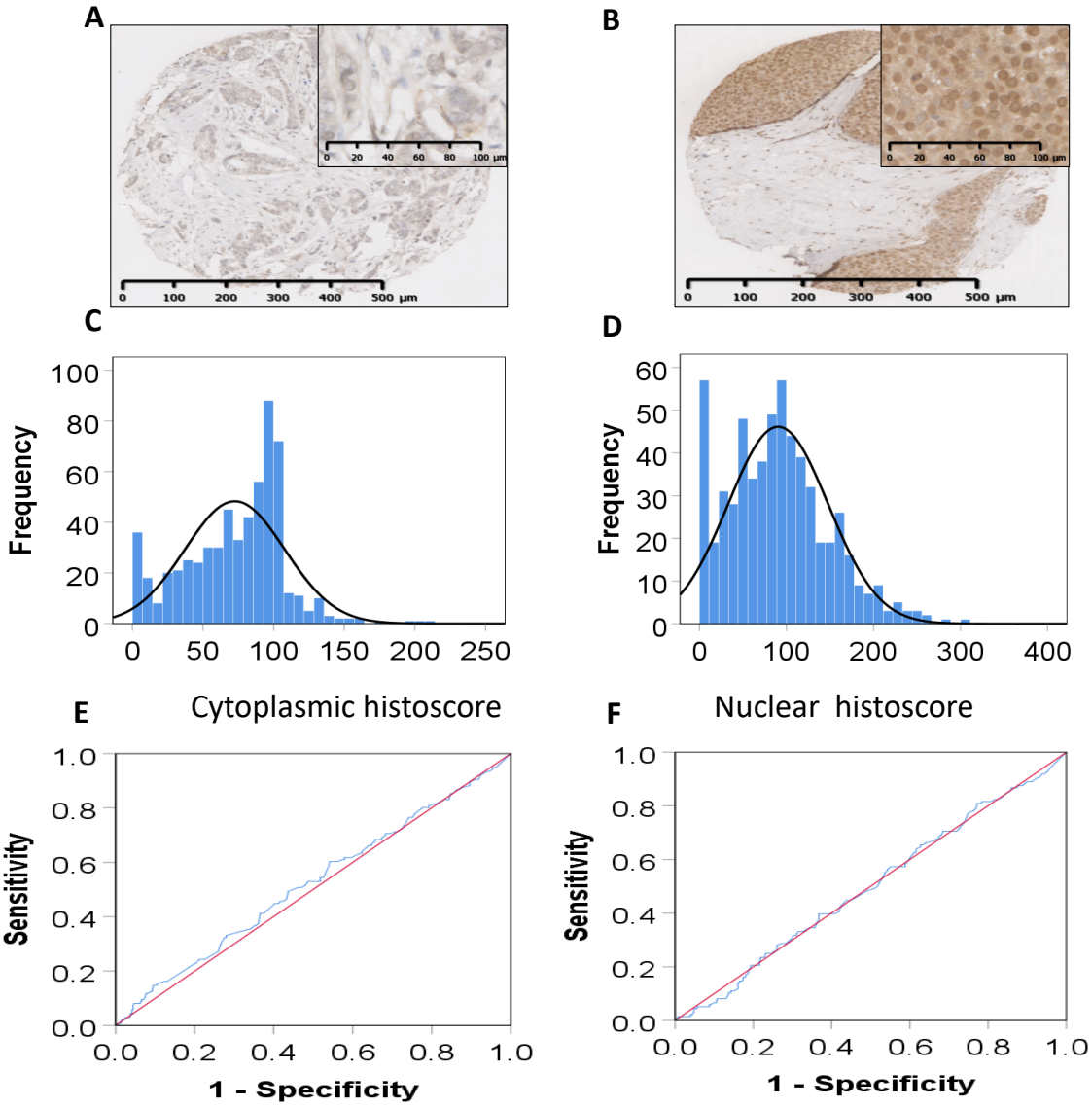


**Figure 3.25 Expression of pIKKα-Thr23.** *(A) Example of low expression and (B) of high expression of pIKKα-Thr23 (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic (D) and nuclear expression of pIKKα-ThrT23. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of pIKKα-Thr23.*

The ROC curve was plotted to determine the cut-off in order to classify low and high expression of cytoplasmic and nuclear pIKKα-Thr23 (Figure 3.25). Since the AUC was 0.52 (P=0.467) and 0.50 (P=0.940) for cytoplasmic and nuclear pIKKαhr-Thr23, the values from ROC curves were unable to be used to determine optimal cut-off. Therefore, the median histoscore was used as the optimal cut-off. Tumours with cytoplasmic histoscore over 80 and tumour with nuclear histoscore over 90 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-off, 296 patients had high cytoplasmic

expression and 303 patients had low cytoplasmic expression. 279 patients had high nuclear expression and 320 patients had low nuclear expression.

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pIKKα-Thr23 were plotted as shown in Figure 3.26. The log rank test was used to compare low and high expression. High expression of cytoplasmic pIKKα-Thr23 was not associate with cancer specific survival (HR 1.15 95% CI 0.82-1.62, P= 0.407) or recurrence free survival (HR 1.23 95% CI 0.87-1.74, P= 0.245). Similarly, high expression of nuclear pIKKα-Thr23 was not associated with cancer specific survival (HR 0.94 95% CI 0.67-1.32, P= 0.734) or recurrence free survival (HR 0.97 95% CI 0.69-1.38, P= 0.877).

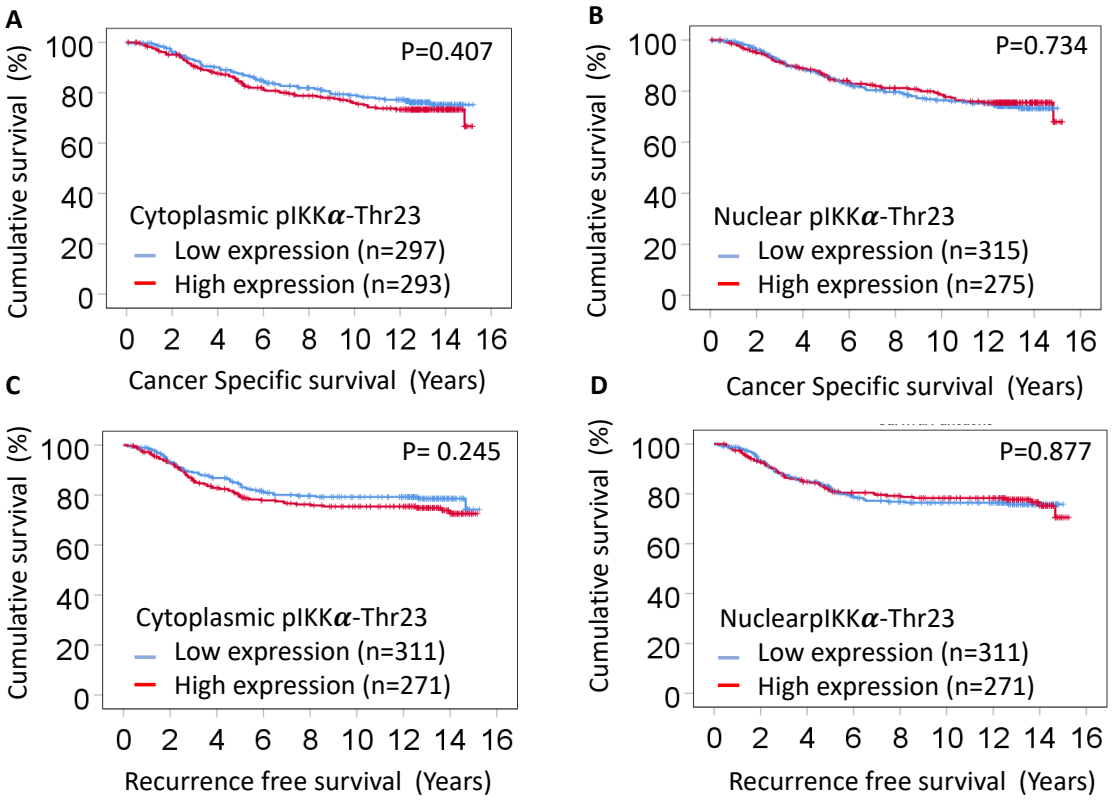


**Figure 3.26 Expression of pIKKα-Thr23 and clinical outcome in the 1800-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pIKKα-Ser176 with cancer specific survival (A and B) and recurrence free survival (C and D)*

### 3.3.4.2 Associations between pIKKα-Thr23 and clinicopathological characteristics of patients in the 1800-cohort

Chi-squared analysis was performed in order to determine whether expression of pIKKα-Thr23 was associated with any clinicopathological characteristic of the patients in the 1800-cohort. Expression of cytoplasmic pIKKα-Thr23 was associated with PR status (P=0.037) and Ki67 (P=0.007) (Table 3.10). Whereas expression of nuclear pIKKα-Thr23 was associated with histological type (P=0.032), tumour grade (P=0.014), ER status (P=0.017), Ki67 (P<0.001) and molecular subtype (P=0.001) (Table 3.10).

| Clinicopathological characteristics | Cytoplasmic Thr23 | | | Nuclear Thr23 | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 89 (29.4) | 97 (32.8) | 0.369 | 102 (31.9) | 84 (30.1) | 0.641 |
| >50 years) | 214 (70.6) | 199 (67.2) | | 218 (68.1) | 195 (69.9) | |
| Histological type | | | | | | |
| Other | 15 (5.0) | 16 (5.4) | 0.815 | 18 (5.6) | 13 (4.7) | **0.032** |
| Lubular | 18 (5.9) | 21 (7.1) | | 13 (4.1) | 26 (9.3) | |
| Ductal | 270 (89.1) | 259 (87.5) | | 289 (90.3) | 240 (86.0) | |
| Size (mm) | | | | | | |
| ≤20 | 174 (57.4) | 149 (50.5) | 0.202 | 168 (52.5) | 155 (55.8) | 0.210 |
| 21-50 | 112 (37.0) | 130 (44.1) | | 138 (43.1) | 104 (37.4) | |
| >50 | 17 (5.6) | 16 (5.4) | | 14 (4.4) | 19 (6.8) | |
| Grade | | | | | | |
| I | 52 (17.2) | 64 (21.6) | 0.376 | 50 (15.6) | 66 (23.7) | **0.014** |
| II | 141 (46.5) | 128 (43.2) | | 142 (44.4) | 127 (45.5) | |
| III | 110 (36.3) | 104 (36.1) | | 128 (40.0) | 86 (30.8) | |
| Lymph node status | | | | | | |
| Negative | 176 (58.5) | 164 (56.7) | 0.672 | 184 (58.0) | 156 (57.1) | 0.825 |
| Positive | 125 (41.5) | 125 (43.3) | | 133 (42.0) | 117 (42.9) | |
| ER status | | | | | | |
| Negative | 86 (28.4) | 79 (26.7) | 0.643 | 101 (31.6) | 64 (22.9) | **0.018** |
| Positive | 217 (71.6) | 217 (73.3) | | 219 (68.4) | 215 (77.1) | |
| PR status | | | | | | |
| Negative | 138 (45.5) | 160 (54.1) | **0.037** | 158 (49.4) | 140 (50.2) | 0.844 |
| Positive | 165 (54.5) | 136 (45.9) | | 162 (50.6) | 139 (4938) | |
| Her-2 status | | | | | | |
| Negative | 247 (82.9) | 252 (85.7) | 0.344 | 259 (82.2) | 240 (86.6) | 0.140 |
| Positive | 51 (17.1) | 42 (14.3) | | 56(17.8) | 37 (13.4) | |
| Proliferation (Ki67) | | | | | | |
| Low | 179 (62.4 | 207 (72.9) | **0.007** | 181 (60.1) | 205 (75.9) | **<0.001** |
| High | 108 (37.6) | 77 (27.1) | | 120 (39.9) | 65 (24.1) | |
| Molecular subtype | | | | | | |
| Luminal A | 126 (42.9 | 151 (52.8) | 0.053 | 123 (39.7) | 154 (57.0) | **0.001** |
| Luminal B | 88 (29.9) | 60 (21.0) | | 92 (29.7) | 56 (20.7) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| TNBC<br>HER2 | 54 (18.4)<br>26 (8.8) | 52 (18.2)<br>23 (8.0) | | 66 (21.3)<br>29 (9.4) | 40 (14.8)<br>20 (7.4) | |

**Table 3.10 Association of pIKKα-Thr23 with clinic-pathological characteristics of the 1800-cohort.** *Chi-squared analysis explored the relationship of pIKKα-Thr23 with various characteristics of the cohort*

### 3.3.4.3 Expression of pIKKα- Thr23 and clinical outcome in different subtypes

Expression of pIKKα-Thr23 was also assessed in different breast cancer subtype (Figure 3.27). Patients with high expression of cytoplasmic pIKKα-Thr23 was not associated with cancer specific survival in luminal A tumours (HR 1.50 95% CI 0.78-2.89, P=0.222) and in luminal B tumours (HR 1.04 95% CI 0.56-1.93, P=0.910). Decreased cancer specific survival was observed in patients with high expression of cytoplasmic pIKKα-Thr23 in TNBC tumours (HR 2.25 95% CI 1.12-4.51, P=0.018). However, high expression of cytoplasmic pIKK-Thr23 was associated with improved cancer specific survival in HER2-positive tumour (HR 0.31 95% CI 0.10-0.94, P=0.027).

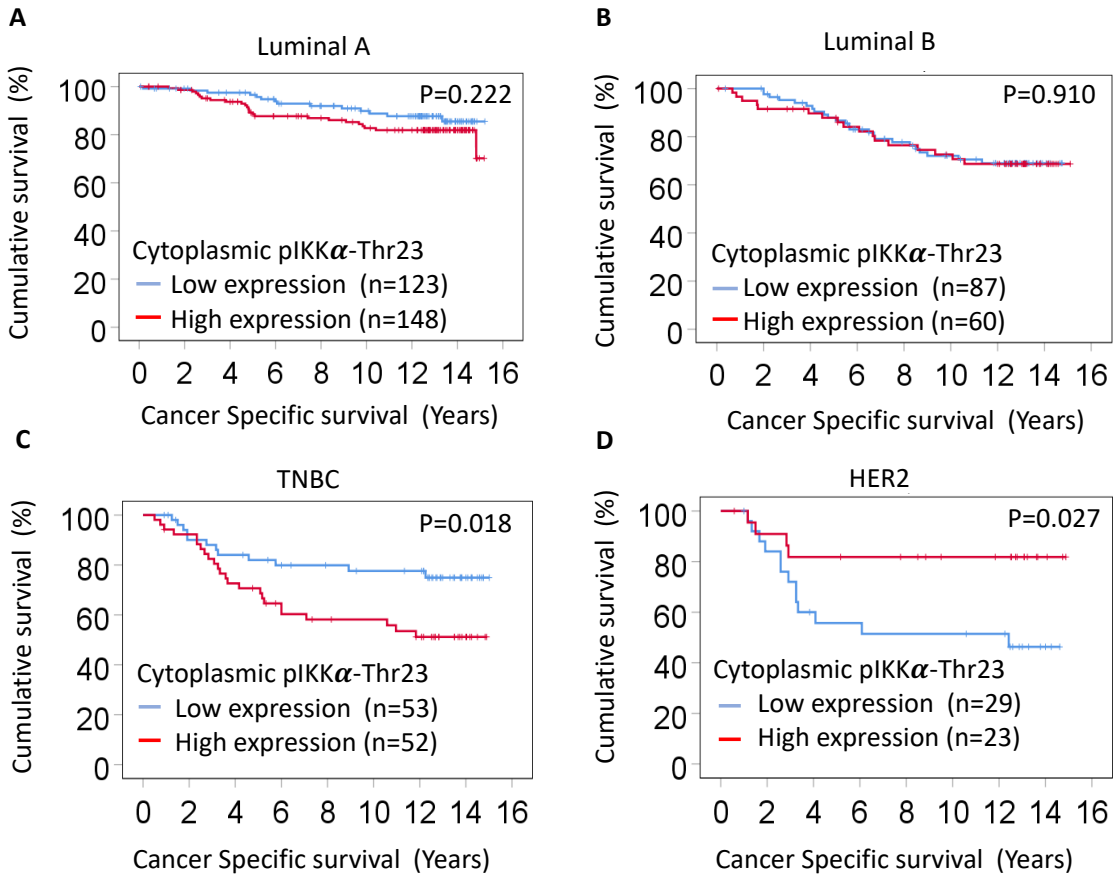


**Figure 3.27 Expression of cytoplasmic pIKKα-Thr23 and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα - Thr23 and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumour (C) and HER2-positive tumour (D).*

Similarly, nuclear pIKKα-Thr23 was not associated with cancer specific survival in luminal A tumour (HR 1.37 95% CI 0.71-2.63, P=0.348) and in luminal B tumour (HR 0.76 95% CI 0.40-1.44, P=0.395). High expression of nuclear pIKKα -Thr23 was associated with shorter cancer specific survival in TNBC tumour (HR 2.05 95% CI 1.07-3.95, P=0.028). However, high expression of nuclear pIKKα -Thr23 was associated with improved cancer specific survival in HER2-positive tumour (HR 0.26 95% CI 0.07-0.90, P=0.021) (Figure 3.28).

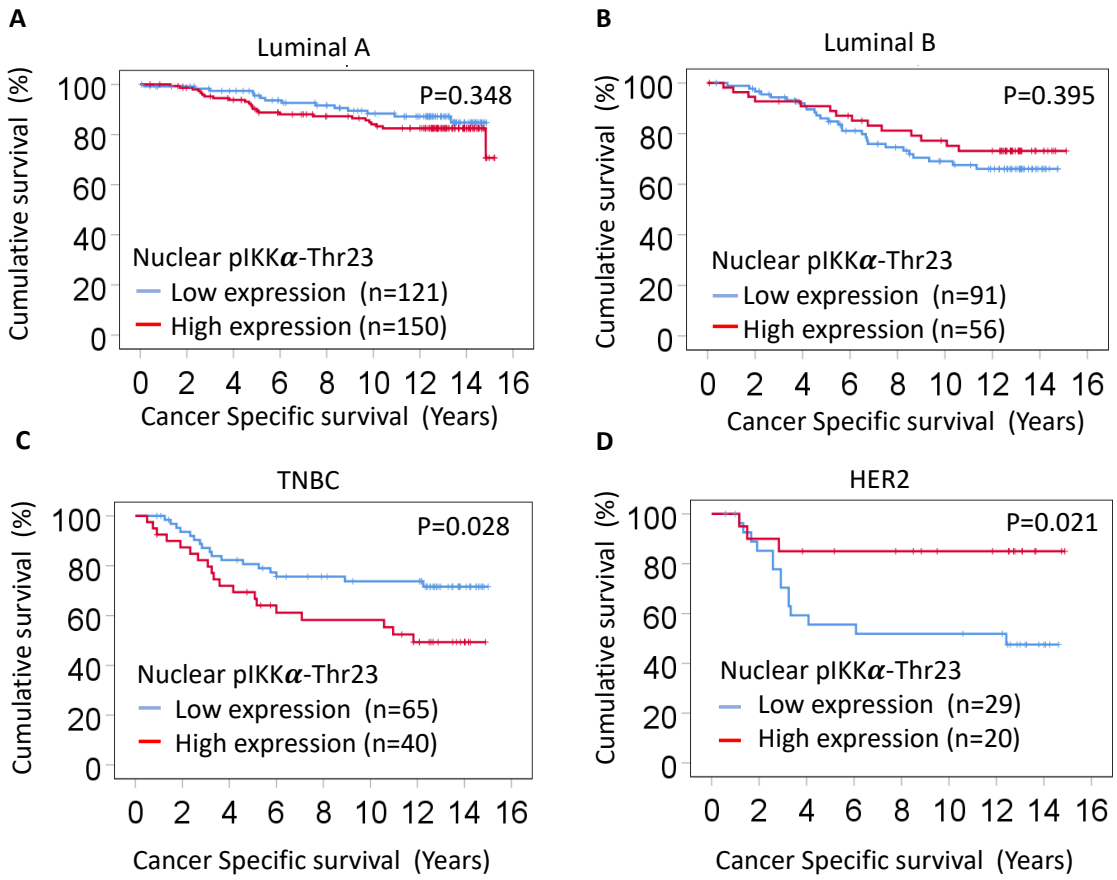


**Figure 3.28 Expression of nuclear pIKKα-Thr23 and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between nuclear pIKKα -Thr23 and cancer specific survival in luminal A tumours (A), luminal B tumours (B), TNBC tumour (C) and HER2-positive tumour (D).*

Multivariate analysis suggested that cytoplasmic and nuclear expression of pIKKα-Thr23 was not an independent prognostic marker for cancer specific survival when combined with tumour size and lymph node status in TNBC (Cytoplasmic, HR 1.35 95% CI 0.65-2.80, P=0.420; Nuclear, HR 1.00 95% CI 0.38-2.64, P=0.996) (Table 3.11).

| Patients characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 1.28 (0.72-2.29) | 0.397 | | |
| Histological type<br>Other/ Lobular/Ductal | 3.66 (0.82-16.41) | 0.090 | | |
| Size (mm)<br>20/ 21-50/ 21-50/>50 | 1.07 (1.04-1.07) | **<0.001** | 4.09 (2.03-8.23) | **<0.001** |
| Grade<br>I/II/III | 1.32 (0.75-2.31) | 0.340 | | |
| Lymph node status<br>Negative/ Positive | 4.14 (2.26-7.59) | **<0.001** | 3.78 (1.75-8.17) | **0.001** |
| Proliferation (Ki67)<br>Low/ High | 0.99 (0.97-1.01) | 0.261 | | |
| Cytoplasmic pIKKα-Thr23<br>Low/High | 2.25 (1.12-4.51) | **0.022** | 1.35 (0.65-2.80) | 0.420 |
| Nuclear pIKKα-Thr23<br>Low/High | 2.05 (1.07-3.95) | **0.032** | 1.00 (0.38-2.64) | 0.996 |

**Table 3.11 Univariate and multivariate analysis for cancer specific survival of pIKKα-Thr23 and clinicopathological characteristic in the TNBC tumours**

High expression of cytoplasmic pIKKα-Thr23 was associated with decreased recurrence free survival in luminal A tumours (HR 2.47 95% CI 0.16-5.28, P=0.016) and in TNBC tumours (HR 2.25 95% CI 1.12-4.51, P=0.033) ( Figure 3.29). However, the association was not found in luminal B tumours (HR 2.47 95% CI 0.16-5.28, P=0.666) and in HER2-positive tumours (HR 0.46 95% CI 0.17-1.21, P=0.105) (Figure 3.29).

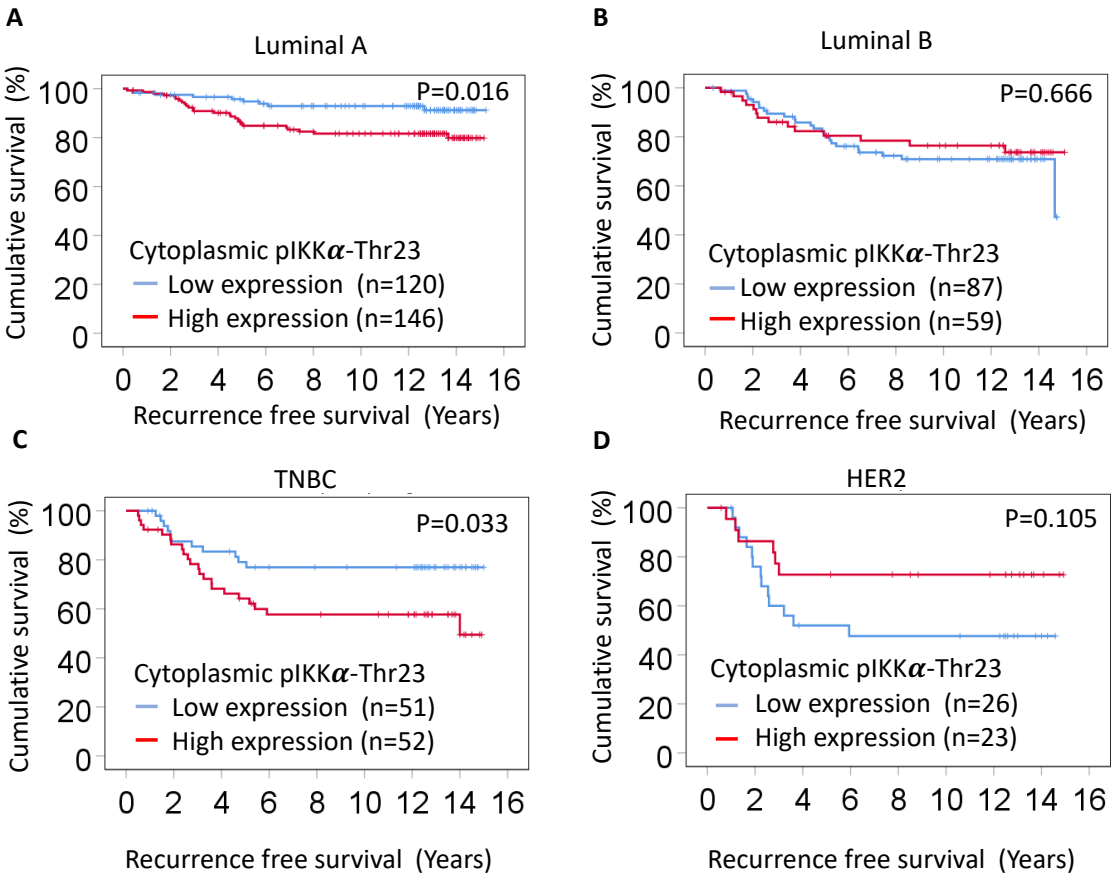


**Figure 3.29 Expression of cytoplasmic pIKKα-Thr23 and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα -Thr23 and recurrence free survival in luminal A tumours (A), luminal B tumours (B), TNBC tumour (C) and HER2-positive tumour (D).*

High expression of nuclear pIKKα-T23 had a trend towards decreased recurrence free survival in luminal A tumours (HR1.37 95% CI 0.71-2.63, P=0.062) and toward significance with decreased recurrence free survival in TNBC tumours (HR 1.94 95% CI 0.98-3.85, P=0.052) (Figure 3.30). However, this association was not found in luminal B tumours (HR 0.59 95% CI 0.29-1.19, P=0.136) but a trend was observed in HER2-positive tumours towards increase recurrence free survival (HR 0.43 95% CI 0.16-1.20, P=0.097) (Figure 3.30).

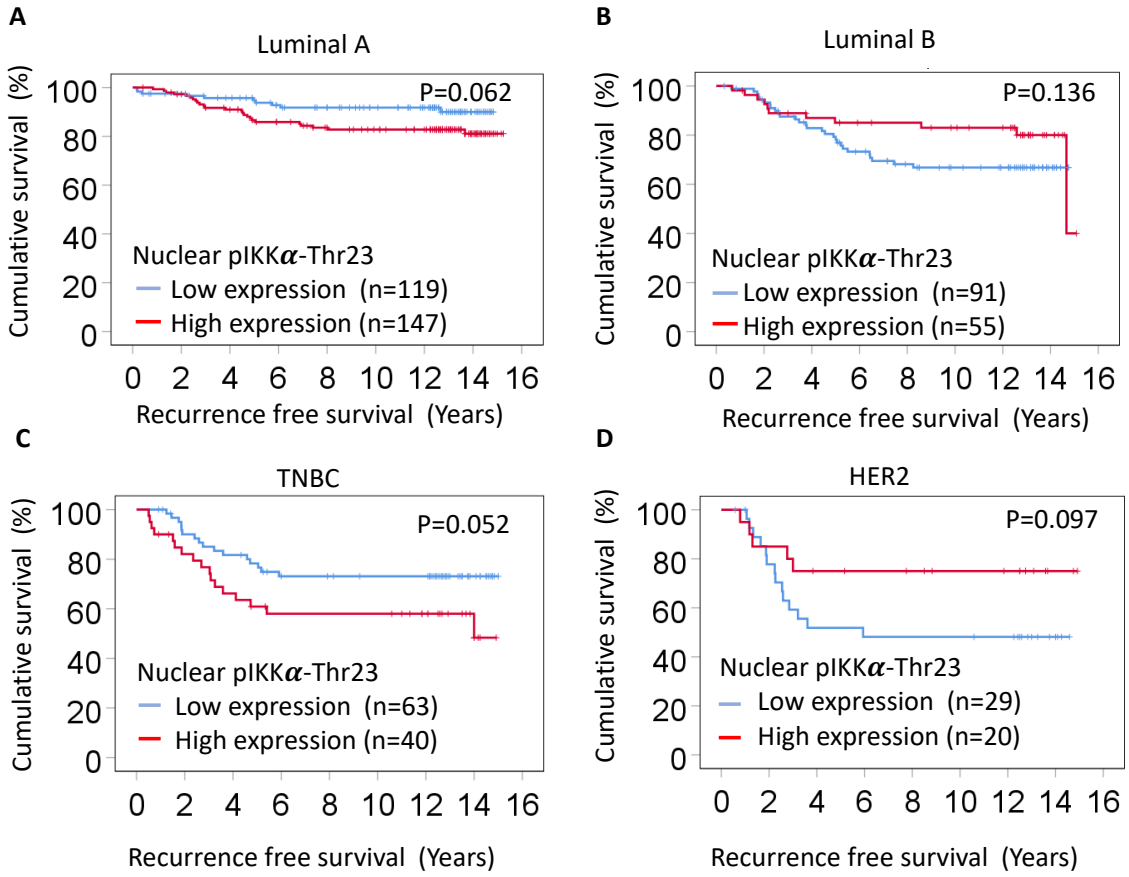


**Figure 3.30 Expression of nuclear pIKKα-Thr23 and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between nuclear pIKKα - Thr23 and recurrence free survival in luminal A tumours (A), luminal B tumours (B), TNBC tumour (C) and HER2-positive tumour (D).*

In luminal A tumours, multivariate analysis suggested that cytoplasmic pIKKα-Thr23 was an independent prognostic marker for recurrence free survival when combined with tumour size and tumour grade (HR 2.20 95% CI 1.02-4.73, P=0.044) (Table 3.12).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 1.13 (0.59-2.14) | 0.715 | | |
| Histological type<br>Other/ Lobular/Ductal | 1.19 (0.69-2.06) | 0.539 | | |
| Size (mm)<br>20/ 21-50/ 21-50/>50 | 1.90 (1.24-2.91) | 0.003 | 2.10 (1.23-3.61) | 0.007 |
| Grade<br>I/II/III | 2.07 (1.31-2.27) | 0.002 | 1.71 (1.00-2.92) | **0.052** |
| Lymph node status<br>Negative/ Positive | 2.56 (1.41-4.65) | 0.002 | 1.78 (0.89-3.57) | 0.106 |
| Cytoplasmic pIKKα-Thr23<br>Low/High | 2.47 (1.16-5.28) | **0.019** | 2.20 (1.02-4.73) | **0.044** |

| | | | | |
|---|---|---|---|---|
| Nuclear pIKKα-Thr23<br>Low/High | 1.98 (0.95-4.13) | 0.068 | | |

**Table 3.12 Univariate and multivariate analysis for recurrence free survival of pIKKα-Thr23 and clinicopathological characteristic in luminal A tumour**

In TNBC tumours, multivariate analysis suggested that cytoplasmic pIKKα-Thr23 was not an independent prognostic marker for recurrence free survival when combined with tumour size and lymph node status (HR 1.36 95% CI 0.64-2.90), P=0.425) (Table 3.13).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 1.01 (0.58-1.78) | 0.957 | | |
| Histological type<br>Other/ Lobular/Ductal | 3.78 (0.84-16.93) | 0.082 | | |
| Size (mm)<br>20/ 21-50/ 21-50/>50 | 3.14 (1.98-4.96) | **<0.001** | 4.66 (2.16-10.05) | **<0.001** |
| Grade<br>I/II/III | 1.28 (0.74-2.20) | 0.380 | | |
| Lymph node status<br>Negative/ Positive | 3.03 (1.72-3.56) | **<0.001** | 2.89 (1.36-6.13) | **0.006** |
| Cytoplasmic pIKKα-Thr23<br>Low/High | 2.16 (1.05-4.46) | **0.037** | 1.36 (0.64-2.90) | 0.425 |
| Nuclear pIKKα-Thr23<br>Low/High | 1.94 (0.98-3.85) | 0.057 | | |

**Table 3.13 Univariate and multivariate analysis for recurrence free survival of pIKKα-T23 and clinicopathological characteristic in TNBC tumour**

## 3.3.5 Expression of pAKT in the 1800-cohort

As pAKT has been demonstrated as one of the upstream regulators that influence IKKα activation, expression of pAKT was also assessed in the 1800-cohort

### 3.3.5.1 Association of pAKT with clinical outcome

The expression of pAKT was analyse in 654 (94.6%) patients. The histoscore for cytoplasmic expression of pAKT ranged from 0 to 300 with a median histoscore of 90 (IQR=36.7-115.0) and the histoscore for nuclear expression ranged from 0-188 with a median histoscore of 50 (IQR=11.7-90.0). An example of immunostaining for low and high expression of pAKT is shown in Figure 3.31. Distribution of histoscores for cytoplasmic and nuclear expression of pAKT are also shown in Figure 3.31. There was good correlation

between observers with an ICCC score of 0.81 for cytoplasmic and 0.85 for nuclear expression. The receiver operating characteristic (ROC) curve was then plotted to determine the cut-off to classify low and high expression of cytoplasmic pAKT and nuclear p-AKT (Figure 3.31). The AUC was 0.54 (P=0.112) and was 0.53 (P=0.279), the values from the ROC curves were unable to be used to determine the optimal cut-off.

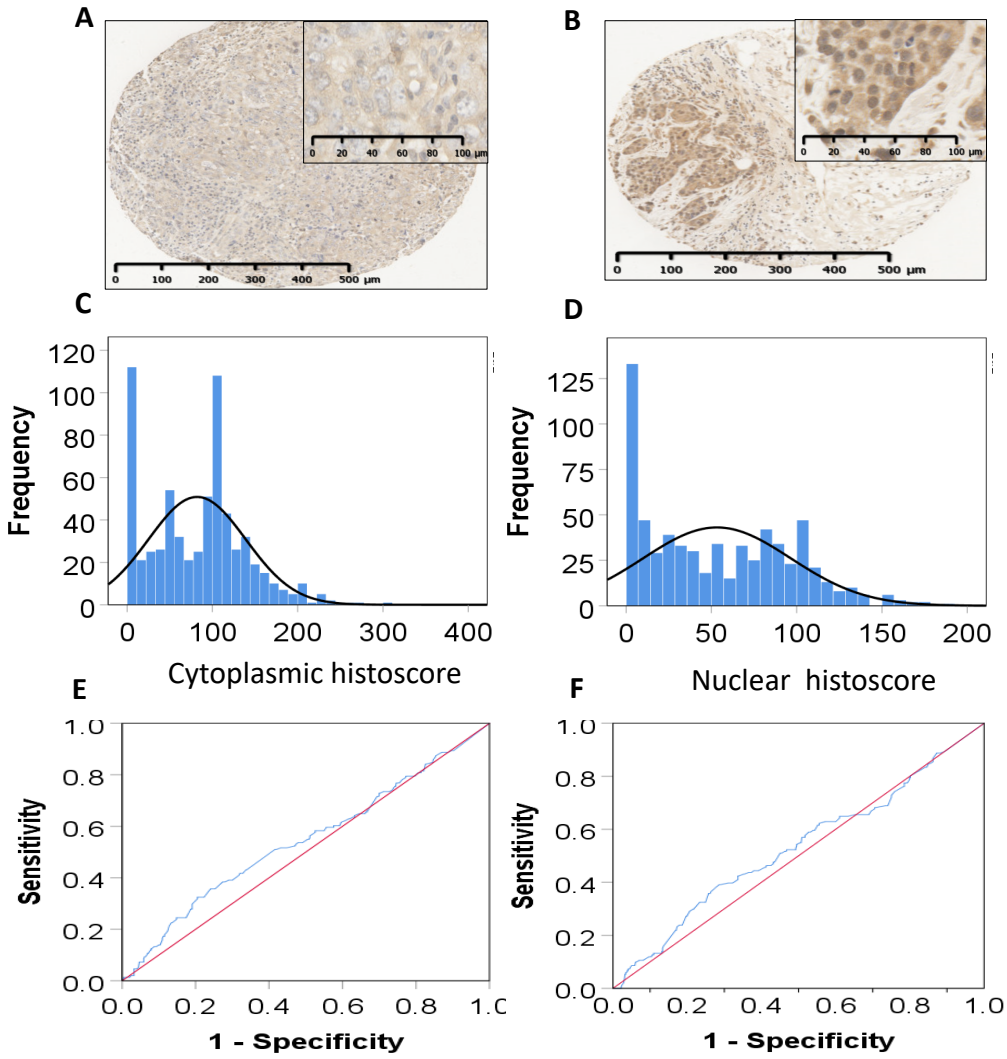


**Figure 3.31 Expression of pAKT.** *(A) Example of low expression and (B) of high expression of pAKT (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of pAKT. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of pAKT.*

The cohort was therefore subdivided into low and high expression by using the median histoscore as a cut-off. Tumours with cytoplasmic histoscore over 90 and tumour with nuclear histoscore over 50 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-off, 329 patients had high cytoplasmic expression and 325

patients had low cytoplasmic expression. 316 patients had high nuclear expression and 338 patients had low nuclear expression.

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pAKT were plotted as shown in Figure 3.32. The log rank test was used to compare low and high expression. Expression of cytoplasmic pAKT (HR 1.14 95% CI 0.83-1.57, P=0.431) and nuclear pAKT (HR 1.18 95% CI 0.86-1.62, P=0.314) was not associated with cancer specific survival. High expression of cytoplasmic pAKT (HR 1.38 95% CI 1.00-1.91, P=0.050) but not nuclear pAKT (HR 1.27 95% CI 0.92-1.75, P=0.152) was associated with decreased recurrence free survival.

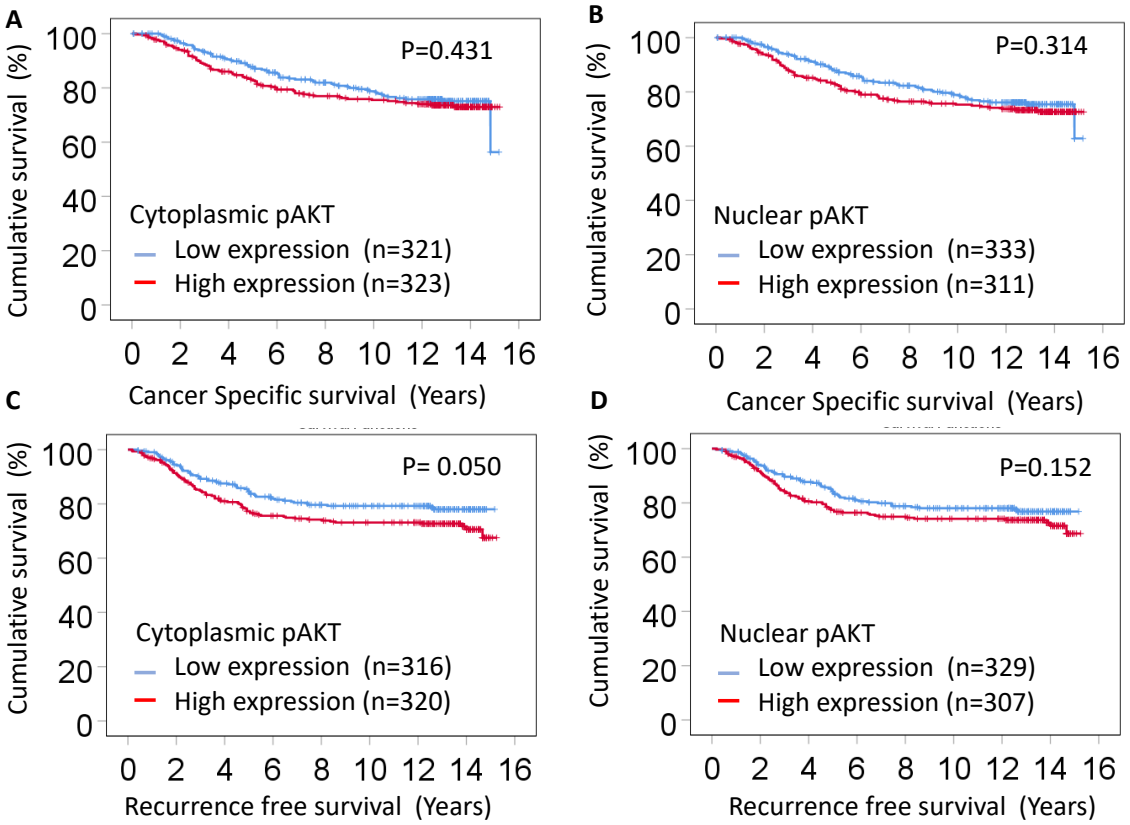


**Figure 3.32 Expression of pAKT and clinical outcome in 1800-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pAKT with cancer specific survival (A and B) and recurrence free survival (C and D).*

Multivariate analysis suggested that cytoplasmic pAKT was not an independent prognostic marker for recurrence free survival when combined with tumour size, grade, lymph node status, ER status, PR status, HER2 status, Ki67, and molecular subtype (HR 1.23 95% CI 0.85-1.76), P=0.273) (Table 3.14).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 1.32 (0.98-1.79) | 0.073 | | |
| Histological type<br>Other/ Lobular/Ductal | 1.55 (1.06-2.25) | **0.022** | 1.11 (0.69-1.78) | 0.658 |
| Size (mm)<br>20/ 21-50/ 21-50/>50 | 1.88 (1.51-2.35) | **<0.001** | 1.65 (1.24-2.18) | **0.001** |
| Grade<br>I/II/III | 2.01 (1.60-2.53) | **<0.001** | 1.68 (1.22-2.32) | **0.001** |
| Lymph node status<br>Negative/ Positive | 2.70 (1.99-3.66) | **<0.001** | 2.35 (1.64-3.36) | **<0.001** |
| ER status<br>Negative/ Positive | 0.48 (0.36-0.65) | **<0.001** | 0.68 (0.34-1.37) | 0.277 |
| PR status<br>Negative/ Positive | 0.43 (0.31-0.69) | **<0.001** | 0.77 (0.49-1.22) | 0.259 |
| Her-2 status<br>Negative/ Positive | 2.19 (1.56-3.08) | **<0.001** | 1.29 (0.75-2.22) | 0.361 |
| Proliferation (Ki67)<br>Low/ High | 1.52 (1.11-2.07) | **0.008** | 1.01 (0.66-1.56) | 0.963 |
| Molecular subtype<br>Luminal A/Luminal B/TNBC/HER2 | 1.54 (1.35-1.77) | **<0.001** | 1.27 (1.06-1.51) | **0.009** |
| Cytoplasmic pAKT<br>Low/High | 1.38 (1.01-1.91) | **0.050** | 1.23 (0.85-1.76) | 0.273 |
| Nuclear pAKT<br>Low/High | 1.27 (0.92-1.75) | 0.153 | | |

**Table 3.14 Univariate and multivariate analysis for recurrence free survival of pAKT and clinicopathological characteristic in the 1800-cohort**

### 3.3.5.2 Associations between pAKT and clinicopathological characteristics of patients in the 1800-cohort

Chi-squared analysis was performed to determine whether expression of pAKT was associated with any clinicopathological characteristic in the 1800-cohort as shown in Table 3.15. Expression of cytoplasmic pAKT was associated with patient age (P=0.004), histological type (P=0.001), tumour grade (P<0.001), ER status (P<0.001), PR status (P<0.001), HER2 status (P=0.041) and molecular subtypes (P<0.001). Similar to cytoplasmic pAKT, expression of nuclear pAKT was associated with patient age (P<0.001), histological type (P=0.018), tumour grade (P<0.001), ER status (P<0.001), PR status (P<0.001), HER2 status (P=0.002) and molecular subtypes (P<0.001).

| Clinicopathological characteristics | Cytoplasmic AKT | | | Nuclear AKT | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 80 (24.6) | 115 (35.0) | **0.004** | 78 (23.1) | 117 (37.0) | **<0.001** |
| >50 years) | 245 (75.4) | 214 (65.0) | | 260 (76.9) | 199 (63.0) | |
| Histological type | | | | | | |
| Other | 11 (3.4) | 18 (5.5) | **0.001** | 14 (4.1) | 15 (4.7) | **0.018** |
| Lobular | 32 (9.8) | 14 (4.3) | | 33 (9.8) | 13 (4.1) | |
| Ductal | 282 (86.8) | 297 (90.3) | | 291 (86.1) | 288 (91.1) | |
| Size (mm) | | | | | | |
| ≤20 | 177 (54.5) | 181 (55.2) | 0.368 | 184 (54.4) | 174 (55.2) | 0.719 |
| 21-50 | 126 (38.8) | 133(40.5) | | 133 (39.3) | 126 (40.0) | |
| >50 | 22 (6.8) | 14 (4.3) | | 21 (6.2) | 15 (4.8) | |
| Grade | | | | | | |
| I | 67 (20.6) | 46 (14.0) | **<0.001** | 67 (19.8) | 46 (14.6) | **<0.001** |
| II | 164 (50.5) | 117 (35.7) | | 169 (50.0) | 112 (35.6) | |
| III | 94 (28.9) | 165 (50.3) | | 102 (30.2) | 157 (49.8) | |
| Lymph node status | | | | | | |
| Negative | 179 (56.1) | 187 (57.2) | 0.783 | 186 (56.0) | 180 (57.3) | 0.739 |
| Positive | 140 (43.9) | 140 (42.8) | | 146 (44.0) | 134 (42.7) | |
| ER status | | | | | | |
| Negative | 61 (18.8) | 164 (49.8) | **<0.001** | 66 (19.6) | 159 (50.3) | **<0.001** |
| Positive | 263 (81.2) | 165 (50.2) | | 271 (80.4) | 157 (49.7) | |
| PR status | | | | | | |
| Negative | 150 (46.3) | 202 (61.4) | **<0.001** | 155 (46.0) | 197 (62.3) | **<0.001** |
| Positive | 174 (53.7) | 127 (39.6) | | 182 (54.0) | 119 (37.7) | |
| Her-2 status | | | | | | |
| Negative | 274 (86.2) | 262 (80.1) | **0.041** | 289 (87.6) | 247 (78.4) | **0.002** |
| Positive | 44 (13.8) | 65 (19.9) | | 41 (12.4) | 68 (21.6) | |
| Proliferation (Ki67) | | | | | | |
| Low | 219 (68.9) | 219 (71.3) | 0.501 | 222 (67.5) | 216 (73.0) | 0.134 |
| High | 99 (31.1) | 88 (28.7) | | 107 (32.5) | 80 (27.0) | |
| Molecular subtype | | | | | | |
| Luminal A | 175 (55.7) | 105 (33.0) | **<0.001** | 172 (52.8) | 108 (35.3) | **<0.001** |
| Luminal B | 85 (27.1) | 55 (17.3) | | 95 (29.1) | 45 (14.7) | |
| TNBC | 31 (9.9) | 115 (36.2) | | 39 (12.0) | 107 (35.0) | |
| HER2 | 23 (7.3) | 43 (13.5) | | 20 (6.1) | 46 (15.0) | |

**Table 3.15 Association of pAKT with clinic-pathological characteristics of the 1800-cohort.** *Chi-squared analysis exploring associations between pAKT with clinicopathological characteristics of the cohort*

### 3.3.5.3 Expression of pAKT and clinical outcome in different subtypes

Association between expression of pAKT and clinical outcome was also assessed in different breast cancer subtypes (Figure 3.33). In terms of cancer specific survival, cytoplasmic pAKT

was not associated with survival within each subtype (luminal A, HR 0.67 95% CI 0.34-1.33, P=0.252; luminal B, HR 1.19 95% CI 0.65-2.17, P=0.583; TNBC, HR 1.50 95% CI 0.67-3.37, P= 0.318; HER2-positive, HR 0.62 95% CI 0.27-1.42, P=0.251).

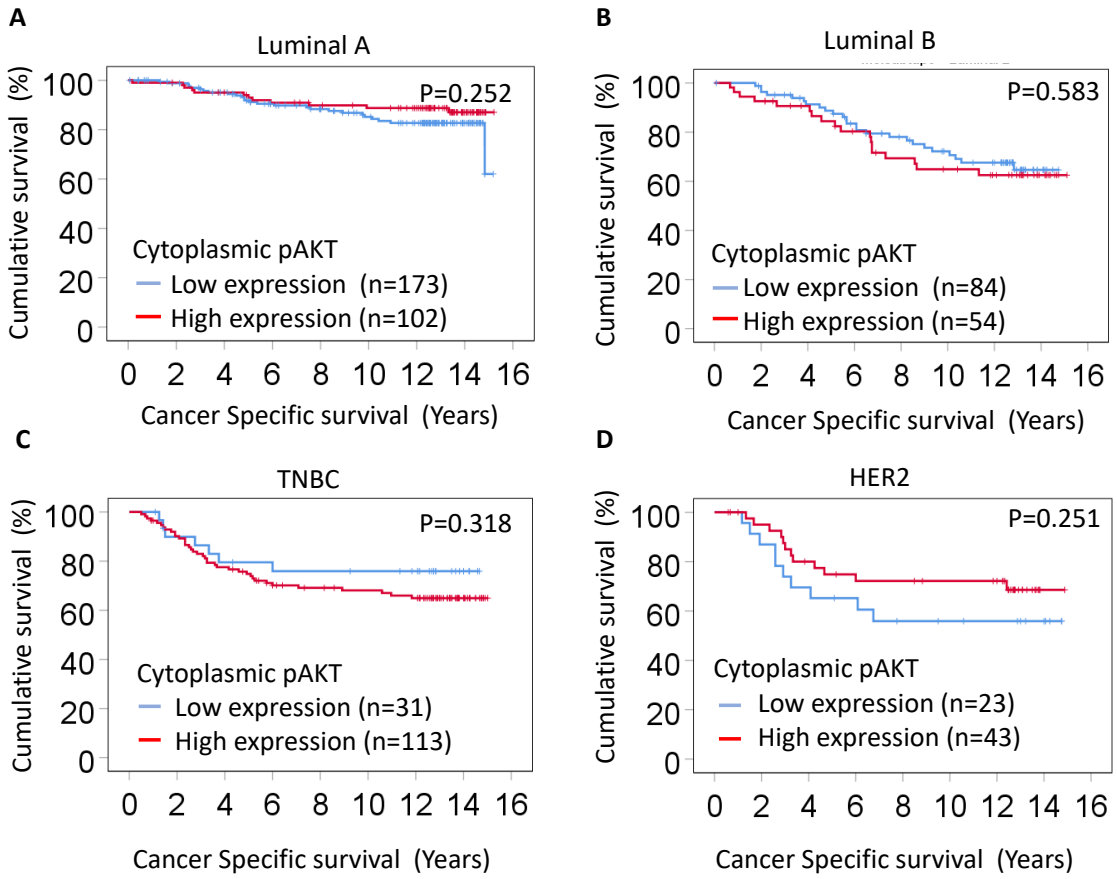


**Figure 3.33 Expression of cytoplasmic pAKT and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pAKT and recurrence survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

Similarly, nuclear pAKT was not associated with cancer specific survival within each subtype (luminal A, HR 0.57 95% CI 0.28-1.15, P=0.114; luminal B, HR 1.18 95% CI 0.63-2.22, P=0.601; TNBC, HR 1.79 95% CI 0.83-3.84, P= 0.130; HER2-positive, HR 0.99 95% CI 0.40-2.42, P=0.979) (Figure 3.34).

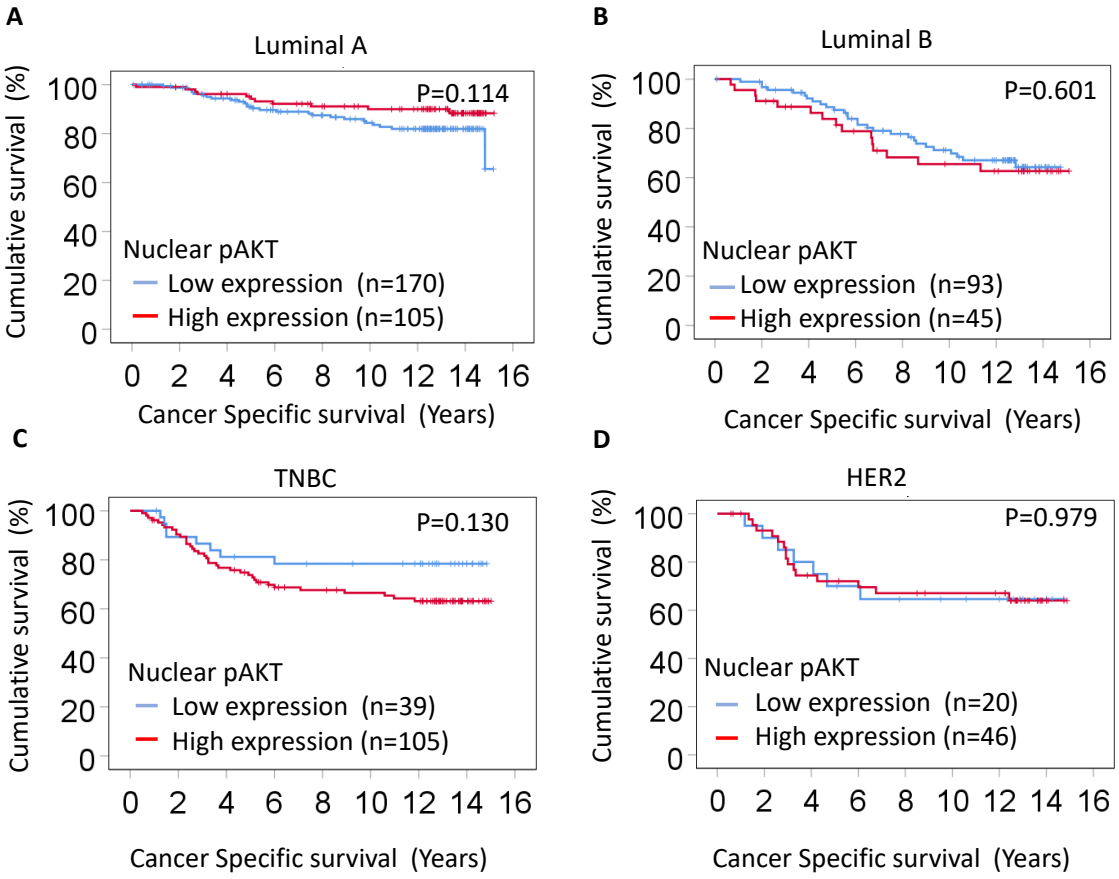


**Figure 3.34 Expression of nuclear pAKT and cancer specific survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pAKT and recurrence survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

Comparable with cancer specific survival, cytoplasmic pAKT was not associated with recurrence free survival within each subtype (luminal A, HR 0.77 95% CI 0.38-1.54, P=0.457; luminal B, HR 1.67 95% CI 0.90-2.12, P=0.102; TNBC, HR 1.53 95% CI 0.68-3.42, P= 0.302; HER2-positive, HR 0.80 95% CI 0.37-1.76, P=0.584) ( Figure 3.35).

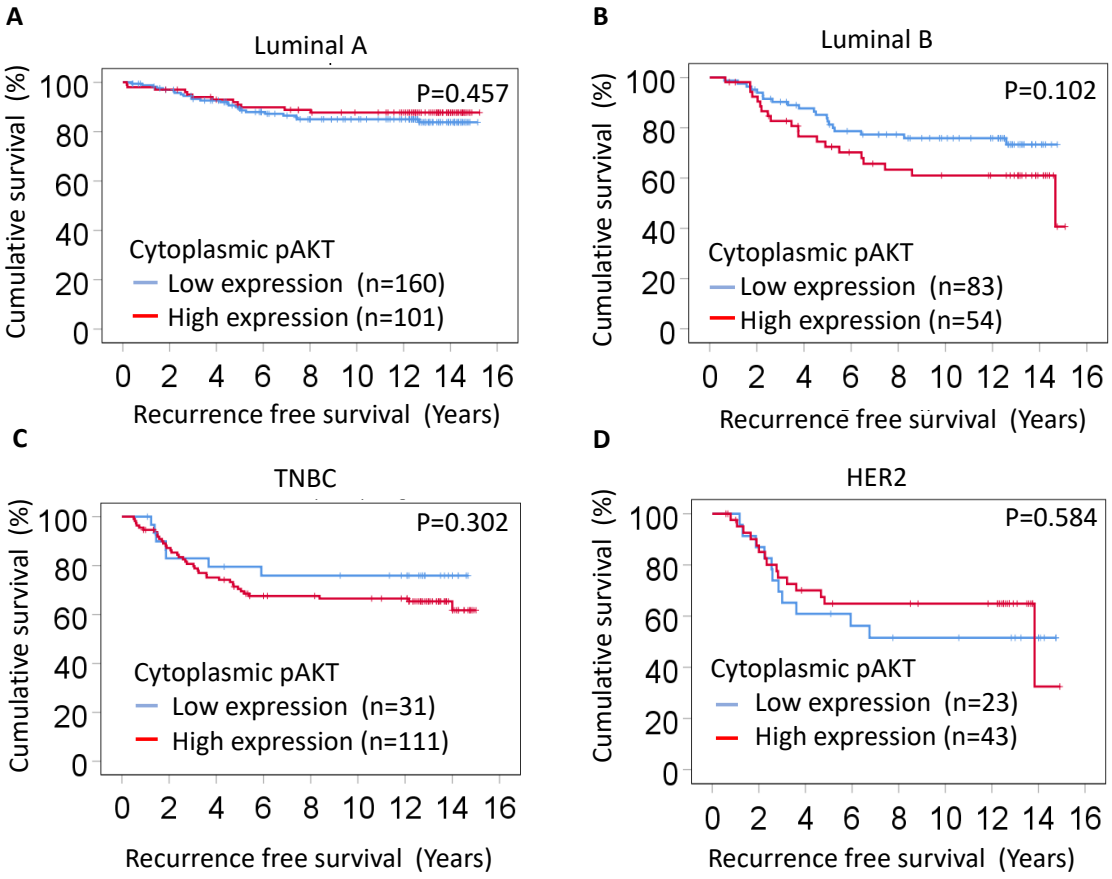


**Figure 3.35 Expression of cytoplasmic pAKT and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pAKT and recurrence survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

Similar to cytoplasmic pAKT, nuclear pAKT was not associated with recurrence free survival within each subtype (luminal A, HR 0.56 95% CI 0.27-1.16, P=0.111; luminal B, HR 1.48 95% CI 0.78-2.80, P=0.227; TNBC, HR 1.59 95% CI 0.77-3.30, P= 0.210; HER2-positive, HR 0.91 95% CI 0.41-2.05, P=0.821) (Figure 3.36).

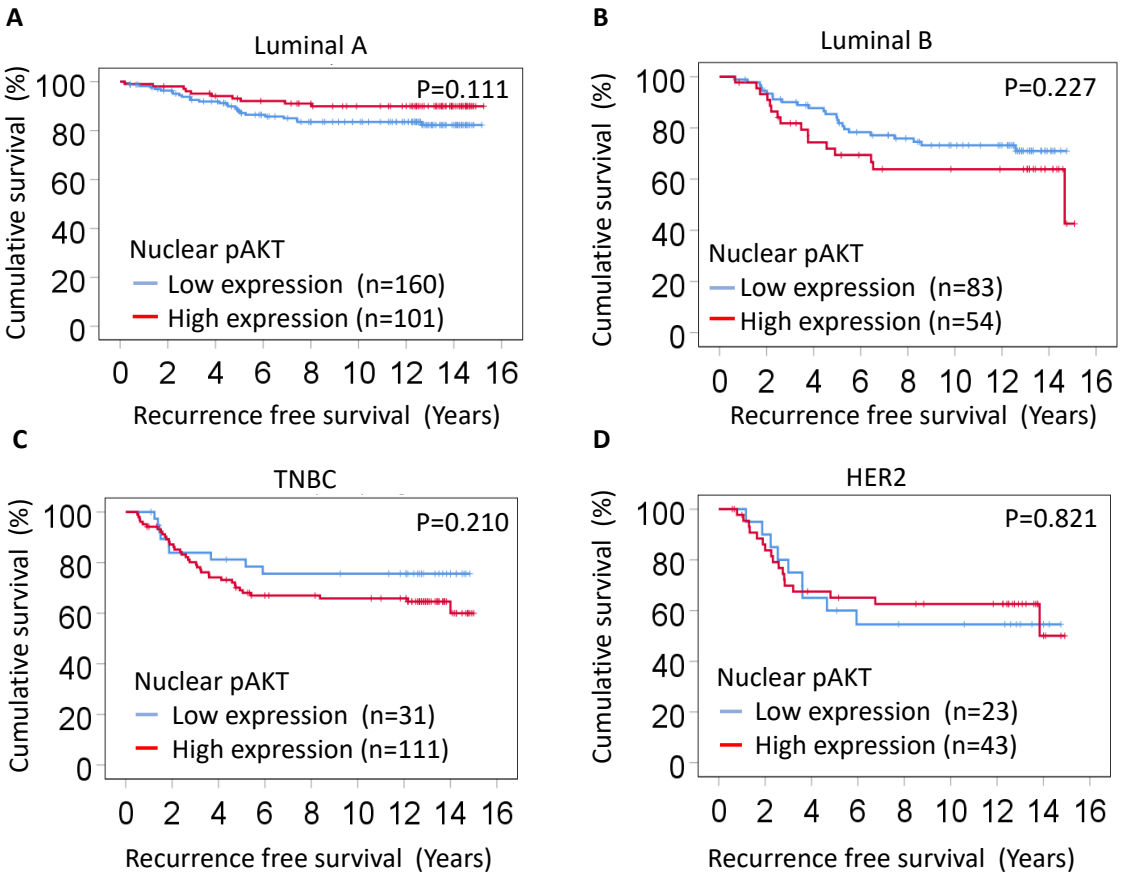


**Figure 3.36 Expression of nuclear pAKT and recurrence free survival in different breast cancer subtypes.** *Kaplan Meier curves showing associations between nuclear pAKT and recurrence survival in luminal A tumours (A), luminal B tumours (B), TNBC tumours (C) and HER2-positive tumours (D).*

## 3.3.6 Correlation between members of the non-canonical NF-κB pathway and upstream regulators

### 3.3.6.1 Correlation between expression of NIK, IKKα, pIKKα-Ser176 and pIKKα-Thr23

Correlation between the upstream regulators and IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (199, 200). There was no correlation between expression of cytoplasmic NIK and cytoplasmic IKKα (Spearman's rho=0.082, P = 0.057). There was a statistically significant, weak correlation between cytoplasmic NIK and nuclear IKKα; Spearman's rho=0.037, P = 0.039). There was a statistically significant, weak positive correlation between cytoplasmic NIK and cytoplasmic pIKKα-Ser176 (Spearman's rho=0.256, P<0.001) and nuclear pIKKα-Ser176 (Spearman's rho=0.158, P<0.001). There was a statistically significant, weak positive correlation between expression of cytoplasmic NIK and expression of pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.163, P<0.001; Nuclear; Spearman's rho=0.160, P< 0.001).

There was no correlation between the expression of nuclear NIK and cytoplasmic IKKα (Spearman's rho=-0.081, P = 0.062). There was a weak correlation between nuclear NIK and nuclear IKKα (Spearman's rho=0.100, P = 0.020). There was a weak correlation between nuclear NIK and cytoplasmic pIKKα-Ser176 (Spearman's rho=0.101, P=0.018) and nuclear pIKKα-Ser176 (Spearman's rho=0.213, P<0.001). There was a statistically significant, weak positive correlation between the expression of nuclear NIK and expression of pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.090, P=0.034; Nuclear; Spearman's rho=0.207, P< 0.001).

### 3.3.6.2 Correlation between expression of pAKT, IKKα, pIKKα-Ser176 and pIKKα-Thr23

Correlation between pAKT, IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (199). Expression of cytoplasmic pAKT did not correlate with expression of cytoplasmic IKKα (Spearman's rho=0.004, P < 0.923). There was a statistically significant, weak negative correlation between expression of cytoplasmic pAKT and expression of nuclear IKKα (Spearman's rho=-0.140, P=0.001). Expression of cytoplasmic pAKT did weakly correlate with cytoplasmic pIKKα-Ser176 (Spearman's rho=0.092, P=0.034) but not nuclear pIKKα-Ser176 (Spearman's rho=-0.077,

P=0.076). There was a statistically significant, weak positive correlation between expression of cytoplasmic pAKT and expression of pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.176, P<0.001; Nuclear; Spearman's rho=0.121, P=0.005).

#### 3.3.6.3 Correlation between expression of IKKα, pIKKα-Ser176 and pIKKα-Thr23

Correlation between IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot. There was a statistically significant, moderate positive correlation between expression of cytoplasmic IKKα and cytoplasmic pIKKα-Ser176 (Spearman's rho=0.407, P<0.001) and a statistically significant, weak positive correlation between cytoplasmic IKKα and nuclear pIKKα-Ser176 (Spearman's rho=0.258, P<0.001). There was a statistically significant, weak positive correlation between expression of cytoplasmic IKKα and expression of pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.373, P<0.001; Nuclear; Spearman's rho=0.226, P<0.001).

There was a statistically significant, weak positive correlation between nuclear IKKα and cytoplasmic pIKKα-Ser176 (Spearman's rho=0.339, P<0.001) and a statistically significant, moderate correlation between nuclear IKKα and nuclear pIKKα-Ser176 (Spearman's rho=-0.427, P<0.001). There was a statistically significant, weak positive correlation between expression of cytoplasmic IKKα and expression of pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.296, P<0.001; Nuclear; Spearman's rho=0.311, P=0.005).

#### 3.3.6.4 Cytoplasmic NIK and phospho-IKKα activity (pIKKα-Ser176, pIKKα-Thr23) as a combined biological marker of clinical outcomes

Expression of nuclear NIK showed a significant correlation with expression of pIKKα-Ser176 and pIKKα-Thr23. Low expression of nuclear NIK was associated with shorter cancer specific survival and recurrence free survival in the entire cohort and luminal B tumour. We hypothesised that the combined expression of nuclear NIK with pIKKα-Ser176 might enhance the association.

### 3.3.6.5 Combined expression nuclear NIK and cytoplasmic pIKKα-Ser176

As a low expression of cytoplasmic pIKKα-Ser176 was associated with decreased cancer specific survival in the entire cohort and luminal B subtype, combined expression of cytoplasmic pIKKα-Ser176 and nuclear NIK was assessed. Patients with both low expressions of cytoplasmic pIKKα-Ser176 and low expression of nuclear NIK were classified as 'high risk'. A combination of at least one low was classified as an 'intermediate risk'. A combination of both high was classified as a 'low risk'. Patients with high risk were associated with decreased cancer specific survival in the entire cohort (HR 1.61 95% CI 1.24-2.10, P=0.001) and in luminal B tumour (HR 2.43 95% CI 1.36-4.33, P=0.007) (Figure 3.37). Similarly, patients with high risk were associated with decreased recurrence free survival in the entire cohort (HR 1.44 95% CI 1.11-1.89, P=0.014) and luminal B tumour (HR 1.93 95% CI 1.11-3.37, P=0.048).

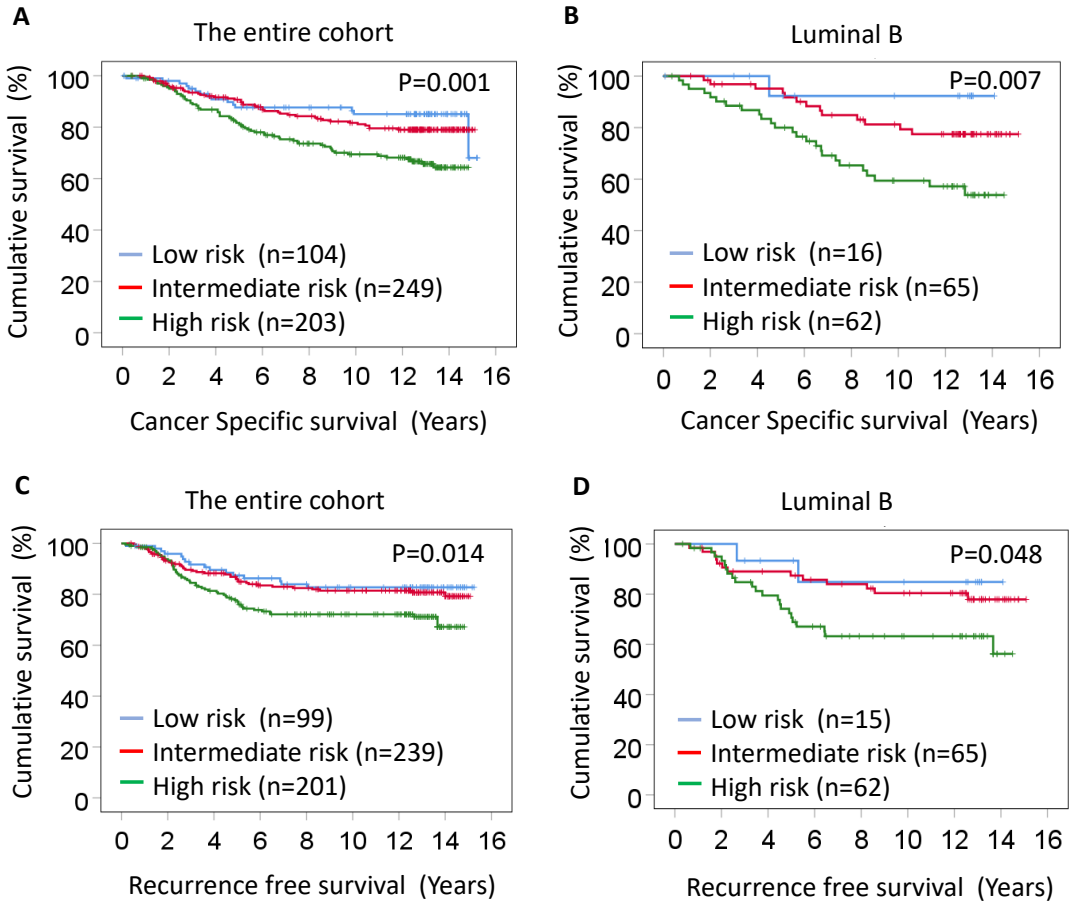


**Figure 3.37 Combined expression of nuclear NIK and cytoplasmic pIKKα-Ser176.** *Kaplan Meier curves showing associations between combined nuclear NIK and cytoplasmic pIKKα-Ser176 and cancer specific survival in the full cohort (A) and TNBC tumour (B). Kaplan Meier curves showing associations between combined nuclear NIK and cytoplasmic pIKKα-Ser176 and recurrence free survival in the full cohort (C) and luminal B tumour (D).*

### 3.3.6.6 Cytoplasmic pAKT and phospho-IKKα activity (pIKKα-Ser176, pIKKα-Thr23) as a combined biological marker of clinical outcomes

High cytoplasmic pAKT was associated with shorter recurrence free survival in the entire cohort and was significantly correlated with IKKα, pIKKα-Ser176, pIKKα-Thr23. As low expression of pIKKα-Ser176 was associated with recurrence free survival in the whole cohort, it was hypothesised that the combined expression of cytoplasmic pAKT with pIKKα-Ser176 might provide additive prognostic power.

### 3.3.6.7 Combined expression of cytoplasmic pAKT and cytoplasmic pIKKα-Ser176

As low cytoplasmic pIKKα-Ser176 and high cytoplasmic pAKT was associated with decreased recurrence free survival in the entire cohort, combined expression of cytoplasmic pIKKα-Ser176 and cytoplasmic pAKT was assessed. Patients with low cytoplasmic pIKKα-Ser176 and high expression of cytoplasmic pAKT were classified as a 'high risk', patients with at least one low expression of cytoplasmic pIKKα-Ser176 or high expression of cytoplasmic pAKT were classified as an 'intermediate risk' and patients with high expression of cytoplasmic pIKKα-Ser176 and low expression of cytoplasmic pAKT was classified as 'low risk'. Patients with high risk had shorter cancer specific survival (HR 1.41 95% CI 1.08-1.85, P=0.011) and shorter reurrence free survival (HR 1.44 95% CI 1.10-1.90, P=0.015), (Figure 3.38).

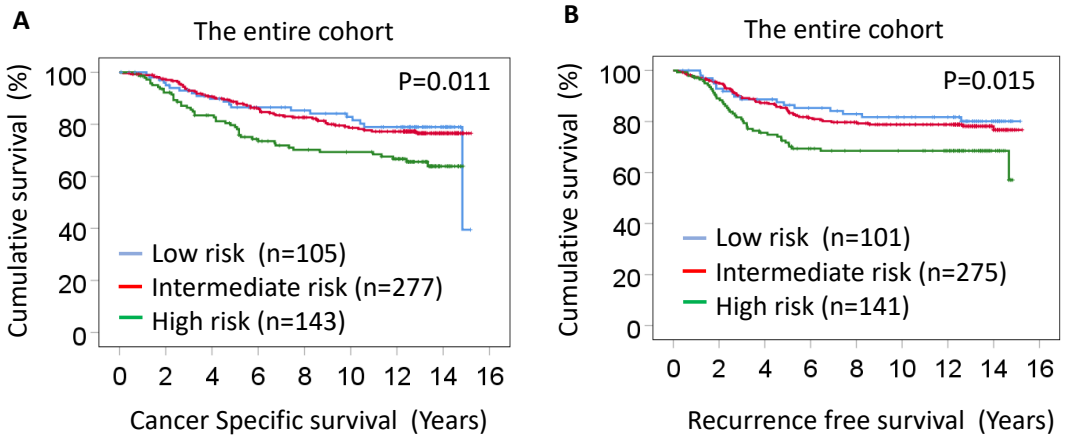


**Figure 3.38 Combined expression of cytoplasmic pAKT and cytoplasmic pIKKα-Ser176.**
*Kaplan Meier curves showing association between combined cytoplasmic pAKT and cytoplasmic pIKKα-Ser176 and recurrence free survival in the full cohort.*

## 3.4 Discussion

Breast cancer treatment has significant changed in the past decades due to the discovery of more specific prognostic and predictive biomarkers. In addition to the classical clinicopathological prognostic factors, several of the established molecular biomarkers such as ER and HER2-play a key role in the selection of patients benefiting from endocrine and HER2 targeting therapy, respectively. Despite advances in treatment of breast cancer, some of the patients experience relapse and resistance to treatment. Identification of new biomarkers are need in order to provide a definite insight into breast cancer biology to provide maximal benefit and avoid unnecessary treatment to the patients.

The NF-κB signalling pathway is a key factor of innate and adaptive immune responses and has been acknowledged as a critical mechanistic link between inflammation and cancer. One role is as a transcription factor, NF-κB regulates transcriptional processing of various genes that are involved in cell proliferation, angiogenesis, and anti-apoptotic genes, to promote cancer development and progression (201-203). Constitutive activation of NF- κB has been reported in primary human breast cancer tissue, breast cancer cell lines and in carcinogen-induced primary rat mammary tumours (142). IKKα, a key kinase in non-canonical NF-κB pathway has previously been investigated in breast cancer (204). However, the role of phosphorylation has not been considered. Therefore, the current chapter performed IHC to assess an association of IKKα, its upstream kinase (NIK and pAKT) and clinical outcomes in patient with breast cancer and further focused on the phosphorylation sites serine 176 and threonine 23 to demonstrate if they can provide additional prognostic power.

In this chapter, the expression of IKKα was not associated with clinical outcomes for the entire cohort or when stratified by tumour subtype. This finding was contrary to previous study from our group, suggesting that although cytoplasmic IKKα was not associated with cancer specific survival but was associated with decreased recurrence-free survival and recurrence free survival on tamoxifen for the whole cohort (173). A significant association with recurrence free survival and recurrence on tamoxifen was also observed in luminal A tumours (173). A possible explanation for this might be that this chapter included 850 breast cancer patients with invasive ductal, invasive lobular and other histological types, unlike the previous study that consists of 362 patients with invasive ductal breast cancer. A difference in patients' number and those might have different clinicopathological characteristics, leading to different clinical outcomes.

Another possible explanation for this is that the median histoscore, which is used as a cut-off value to categorise tumours with low and high expression of IKKα, is different. In the previous study, the cut-off value for cytoplasmic IKKα and nuclear IKKα was 100 and 126, respectively. Unlikely, the cut-off value for IKKα in this thesis was 120 and 100 for cytoplasmic and nuclear, respectively.

Although there was no data of pIKKα-Thr23 in TNBC, a number of recent studies demonstrated the role of IKKα in TNBC. IKKα has been indicated as a crucial factor for Notch signalling in TNBC cells and TNBC cancer stem cell (CSC), mediating AKT activation, mitochondrial metabolism as well as NF-κB-dependent transcription of anti-apoptotic genes cIAP-2, in TNBC (205). A study has shown that rapid AKT phosphorylation was observed after exposure of MDA-MB-231 breast cancer cell lines to recombinant Notch ligand, Jagged1. Phosphorylation of AKT was decreased following IKKα knockdown, and this result indicates that IKKα is required for Notch function (206). Constitutively expressed Notch1 was observed in patient-derived xenografts (PDX) from a TNBC patient. Remarkably inhibition mammosphere growth was observed after treated with a combination of AKT inhibitor and IKK inhibitor. Co-culture of MDA-MB-231 cells with mouse leukocyte receptor tyrosine kinase (LTK) fibroblasts engineered to express Notch ligand, Jagged-1 (LTK–JAG1), markedly induced recruitment of Notch1, p65, and IKKα to the cIAP-2 promoter compared to control vector-transduced parental fibroblasts, indicating a cooperative role of Notch and IKKα to promote NF-κB dependent gene transcription in TNBC cells. It was observed that the expression of NOTCH1 and conserved helix-loop-helix ubiquitous kinase (CHUK is gene symbol of IKKα) was significantly correlated with reduced relapse-free survival in TNBC.

In contrast to TNBC, pIKKα-Thr23 was associated with favourable outcome in the HER2-positive subgroup. This was unexpected finding as activation of NF-κB has been reported to associated with invasive phenotype of HER2-positive breast cancer. Activation of NF-κB has been reported in HER2-positive tumours, especially in HER2-positive/ER-negative tumours as compared to HER2-positive/ER-positive tumours (207, 208). Inhibition of NF-κB in an ER-negative/HER-2 positive breast cancer cell line (SKBr3) led to inhibited heregulin-induced proliferation and resulted in apoptosis (207, 208). In addition, IKKα has been indicated as a crucial link between NF-κB and EGF pathway that is involved in an invasive phenotype of HER2-positive breast cancer cell lines (209). Upon treatment with the dual EGFR/Her2 inhibitor Lapatinib, a significant decrease in p65 phosphorylation at serine 536 was observed in HER2-overexpressing tumour cell lines, including SKBr3 and MDA-

MB-453 (209). At the same time, lapatinib can inhibited phosphorylation of AKT at serine 473 in SKBr3 cell lines (209). Knockdown of IKKα led to a marked–decrease in p65 phosphorylation than knockdown of IKKβ (209). A significant decreased in expression of pro-inflammatory cytokines, including TNF, IL-6, IL-8, CCL-2, and the serine-protease uPA, were observed after siRNA knockdown of IKKα (209).

High expression of cytoplasmic pIKKα-Ser176 was associated with favourable clinical outcomes for the entire cohort and in luminal B tumours. This finding might suggest that pIKKα-Ser176 play contradictory role with pIKKα-Thr23 in breast cancer. However, no previous study on the association of pIKKα-Ser176 and outcomes in breast cancer, it need to confirm this finding in the other cohort of breast cancer.

High nuclear NIK was associated with longer cancer specific survival in the entire cohort and luminal B tumours. This finding is in line with a study that demonstrated an association of NIK with improved recurrence free survival in breast cancer patients (172). However, it is also an unexpected result as NIK is a key upstream kinase in non-canonical NF-κB pathway and a previous study demonstrated an unfavourable role of NIK in breast cancer (210).

When patients were classified as high risk, intermediate risk and low risk group regarding their combined expression as previously described earlier, a combined expression of the biomarkers demonstrated additive prognostic power. Combined expression of cytoplasmic pIKKα-Ser176 with nuclear NIK provide additive prognostic power for cancer specific survival and recurrence free survival in the entire cohort and in luminal B tumours. Combined expression of cytoplasmic pIKKα-Ser176 and cytoplasmic pAKT provide additive prognostic power for recurrence free survival in the entire cohort.

In this chapter, cytoplasmic pAKT was associated with shorter recurrence free survival but not cancer specific survival for the whole cohort or any outcomes when stratified by tumour subtypes. This finding suggests pAKT is an unfavourable prognostic marker, which supports the previous study from our group that demonstrated cytoplasmic pAKT was associated with decreased overall survival but was not recurrence free survival in 402 ER-positive breast cancer patients treated with tamoxifen (211). To confirm this result, external validation of a prognostic role of pAKT from a cohort of breast cancer patients with different breast cancer subtypes should be considered.

In conclusion, this chapter has shown that pIKKα-S176, pIKKα-Thr23 and their upstream kinases NIK and pAKT were associated with clinical outcomes in a cohort of mixed breast cancer subtypes. However, the association of these biomarkers with survival varies between subtypes. Therefore, each subtype should be analysed individually and validated in a larger cohort that consists of an adequate number of patients per subtype.

# Chapter 4 : Expression of members of the non-canonical NF-κB pathways in the cohort of ER-positive breast cancer

## 4.1 Introduction

It was previously demonstrated that pIKKα-Ser176 was associated with longer cancer specific survival in luminal B. pIKKα-Thr23 was associated with decreased cancer specific survival and recurrence free survival in TNBC, decreased recurrence free survival in luminal A but was associated with increased cancer specific survival and recurrence free survival in HER2-positive. NIK was associated with longer cancer specific survival and recurrence free survival. pAKT was associated with decreased recurrence free survival. Although IKKα was not associated with the outcome measures in this cohort, expression of IKKα was also assessed in the cohort of ER-positive patients. This finding may be inconclusive since the 95% CI for IKKα was broad. Therefore, we decided to confirm this finding in the ER-positive cohort.

## 4.2 Expression and clinical outcome of members of non-canonical NF-κB pathway in the patient with ER-positive breast cancer

The STB-cohort included 456 patients presenting with invasive breast cancer between 1980 and 1999 within the Glasgow Royal Infirmary. Patients with unknown ER status (n=44) and ER-negative status (n=20) were excluded, leaving 392 patients available for investigation as shown in Figure 4.1.

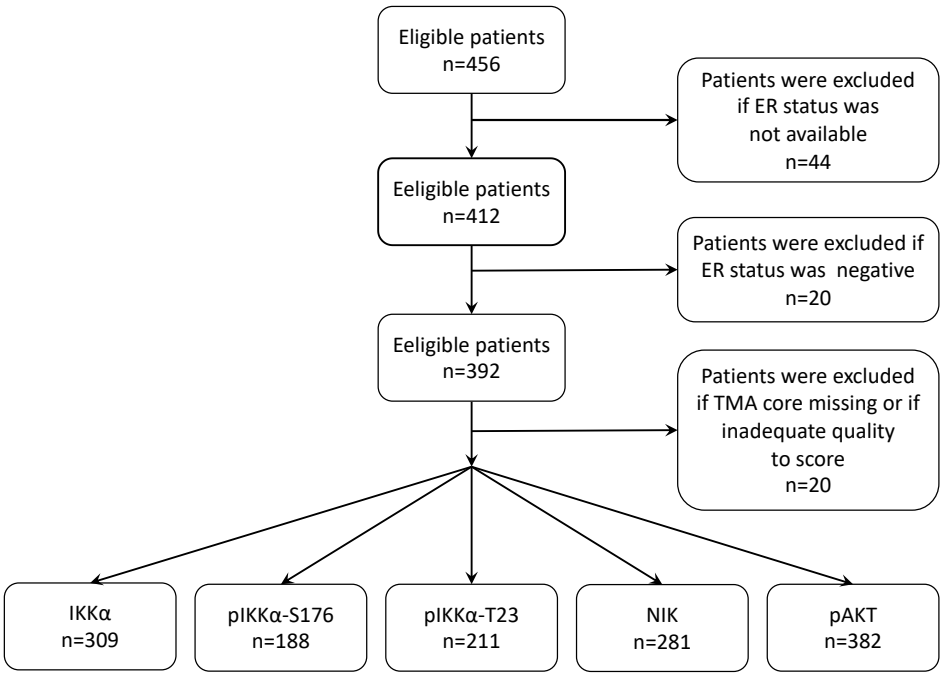

**Figure 4.1 Flow diagram demonstrating patient exclusions.** *Patients with missing cores or insufficient tumour for analysis were excluded*

The strength of this cohort is that the patients were treated with adjuvant tamoxifen and time on tamoxifen was available. A median time of adjuvant tamoxifen was 5 years (ranged 0.03-18 years). A median followed up time was 7.33 years (ranged 0.11-25.93 years). Clinicopathological data, including patient age, tumour size, grade, lymph node status, progesterone receptor (PR) status, HER2 status, Ki67 proliferation index, was available. The patient characteristics are shown in

| Clinicopathological characteristic | The STB cohort (N=456) |
|---|---|
| Age | |
| ≤50 | 72 (18.4) |
| >50 | 319 (81.4) |
| Missing | 1 (0.3) |
| Histological type | |
| Other | 26 (6.6) |
| Lobular | 44 (11.2) |
| Ductal | 314 (80.1) |
| Missing | 8 (2) |
| Size (mm) | |
| ≤20 | 193 (49.2) |
| 21-50 | 155 (39.5) |
| >50 | 22 (5.6) |
| Missing | 22 (5.6) |
| Grade | |
| I | 96 (24.5) |
| II | 188 (48) |
| III | 97 (24.7) |
| Missing | 97 (24.7) |
| Lymph node status | |
| Negative | 188 (48) |
| Positive | 171 (43.6) |
| Missing | 33 (8.4) |
| ER status | |
| Negative | 20 (0.04) |
| Positive | 392 (0.85) |
| Missing | 44 (0.09) |
| PR status | |
| Negative | 145(37) |
| Positive | 234(59.7) |
| Missing | 13(3.3) |
| Her-2 status | |
| Negative | 362(92.3) |
| Positive | 27(6.9) |
| Missing | 3(0.8) |
| Proliferation (Ki67) | |

| | |
|---|---|
| Low | 235(59.9) |
| High | 72(18.4) |
| Missing | 85(21.7) |
| Molecular subtype | |
| Luminal A | 221(56.4)/ |
| Luminal B | 90(23)/ |
| Missing | 81(20.7) |
| Adjuvant endocrine treatment | |
| No | 0 (0%) |
| Yes | 100 (100%) |
| Adjuvant Chemotherapy | |
| No | 296(75.5) |
| Yes | 95(24.2) |
| Missing | 1(0.3) |
| Adjuvant radiotherapy | |
| No | 283(72.2)/ |
| Yes | 109(27.8) |

Table 4.1.

| **Clinicopathological characteristic** | **The STB cohort (N=456)** |
|---|---|
| Age | |
| ≤50 | 72 (18.4) |
| >50 | 319 (81.4) |
| Missing | 1 (0.3) |
| Histological type | |
| Other | 26 (6.6) |
| Lobular | 44 (11.2) |
| Ductal | 314 (80.1) |
| Missing | 8 (2) |
| Size (mm) | |
| ≤20 | 193 (49.2) |
| 21-50 | 155 (39.5) |
| >50 | 22 (5.6) |
| Missing | 22 (5.6) |
| Grade | |
| I | 96 (24.5) |
| II | 188 (48) |
| III | 97 (24.7) |
| Missing | 97 (24.7) |
| Lymph node status | |
| Negative | 188 (48) |
| Positive | 171 (43.6) |
| Missing | 33 (8.4) |
| ER status | |
| Negative | 20 (0.04) |
| Positive | 392 (0.85) |
| Missing | 44 (0.09) |
| PR status | |
| Negative | 145(37) |
| Positive | 234(59.7) |
| Missing | 13(3.3) |
| Her-2 status | |
| Negative | 362(92.3) |

| | |
|---|---|
| Positive | 27(6.9) |
| Missing | 3(0.8) |
| Proliferation (Ki67) | |
| Low | 235(59.9) |
| High | 72(18.4) |
| Missing | 85(21.7) |
| Molecular subtype | |
| Luminal A | 221(56.4)/ |
| Luminal B | 90(23)/ |
| Missing | 81(20.7) |
| Adjuvant endocrine treatment | |
| No | 0 (0%) |
| Yes | 100 (100%) |
| Adjuvant Chemotherapy | |
| No | 296(75.5) |
| Yes | 95(24.2) |
| Missing | 1(0.3) |
| Adjuvant radiotherapy | |
| No | 283(72.2)/ |
| Yes | 109(27.8) |

**Table 4.1 Patient characteristic of the STB-cohort**

## 4.2.1 Expression of NIK in a cohort of patients with ER-positive breast cancer

### 4.2.1.1 Association of NIK with clinical outcome in a cohort of patients with ER-positive breast cancer

To assess expression of members of the pathway and its upstream regulators in patients with ER-positive breast cancer the STB-cohort was utilised. The expression of NIK, the kinase upstream of IKKα, was analysed in 281 (71.68%) of the patients from the STB-cohort (N=392). The histoscore for cytoplasmic expression of NIK ranged from 0 to 300 with a median histoscore of 180 (IQR=135.0-224.5). The histoscore for nuclear expression of NIK ranged from 0-300 with a median histoscore of 130 (IQR=101.8-167.2). An example of immunostaining of a tumour with low and high NIK expression is shown in Figure 4.2. Histograms showing the distribution of histoscore for cytoplasmic and nuclear expression of NIK are shown in Figure 4.2. There was moderate and good correlation between observers with ICCC score of 0.73 for cytoplasmic and 0.96 or nuclear expression, respectively.

The cut-offs from the previous cohort were not applied for analysis as the STB cohort consists of only ER-positive patients and it was deemed inappropriate to use a median from the cohort of mixed breast cancer subtype. Therefore, the ROC curve was plotted to determine the cut-off point in order to classify low and high expression of cytoplasmic and

nuclear expression of NIK. Regarding the traditional academic point system guidance for classifying the accuracy of a diagnostic test, the AUC under the curve above 0.8 is considered to be "good" at separate low and high expression (212). Since the AUC was 0.52 (P=0.685) for cytoplasmic and 0.47 (P=0.507) for nuclear expression of NIK, the values from ROC curves were unable to be used to define the optimal cut-off (Figure 4.2).

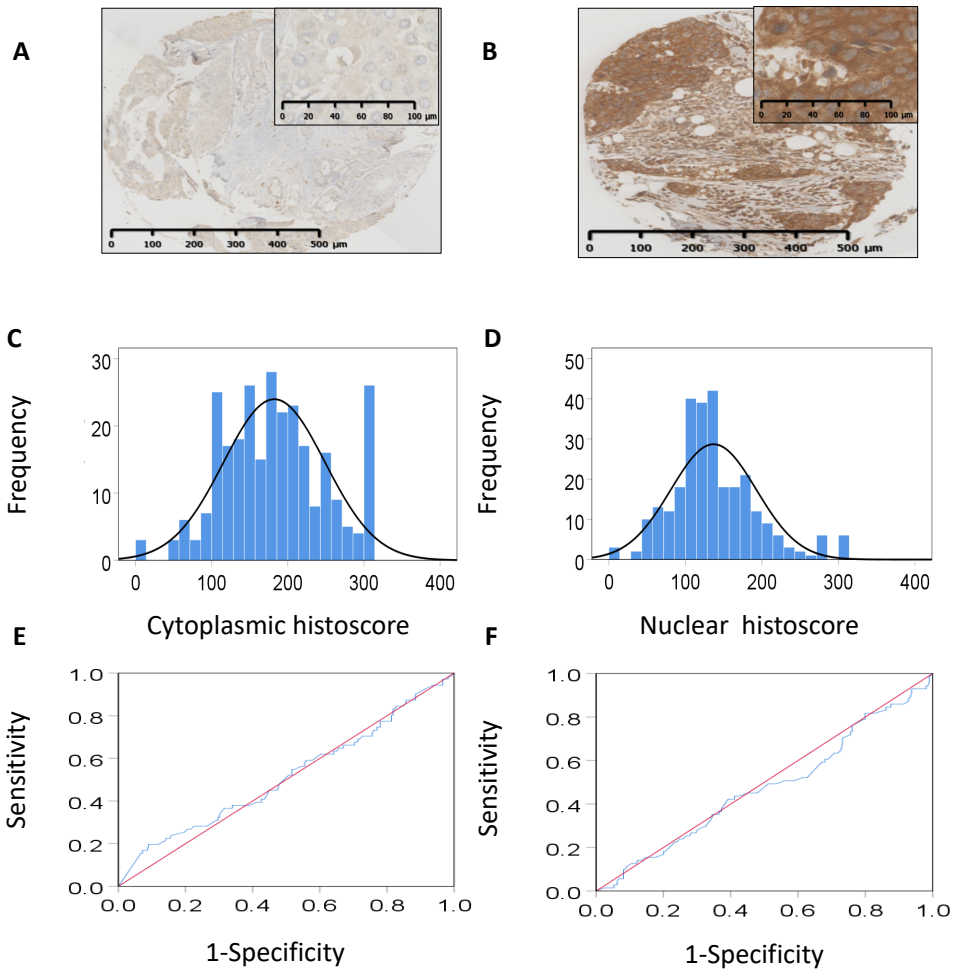


**Figure 4.2 Expression of NIK.** *(A) Example of low expression and (B) of high expression of NIK (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of NIK. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of NIK.*

The median histoscore was therefore used as an optimal cut-off in order to subdivide the cohort into low and high expression. Tumours with cytoplasmic histoscore over 180 and tumour with nuclear histoscore over 130 were classified as. high cytoplasmic and high nuclear expression, respectively. Using these cut-off values, 135 patients had high

cytoplasmic expression and 146 patients had low cytoplasmic expression. 133 patients had high nuclear expression and 148 patients had low nuclear expression.

In order to determine whether NIK expression was associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of NIK were plotted as shown in Figure 4.3. Low and high expression were compared by using the log rank test. Expression of NIK was not associated with cancer specific survival (cytoplasmic expression , HR 1.14 95% CI 0.72-1.83, P=0.575; nuclear expression , HR 1.07 95% CI 0.67-1.701, P=0.793, Figure 4.3; recurrence free survival (cytoplasmic expression, HR 1.00 95% CI 0.64-1.58 P=0.989; nuclear expression, HR 0.99 95% CI 0.63-1.56, P=0.979) nor recurrence on tamoxifen (cytoplasmic expression, HR 1.01 95% CI 0.61-1.69, P=0.960; nuclear expression, HR 0.90 95% CI 0.54-1.51, P=0.691, Figure 4.3).

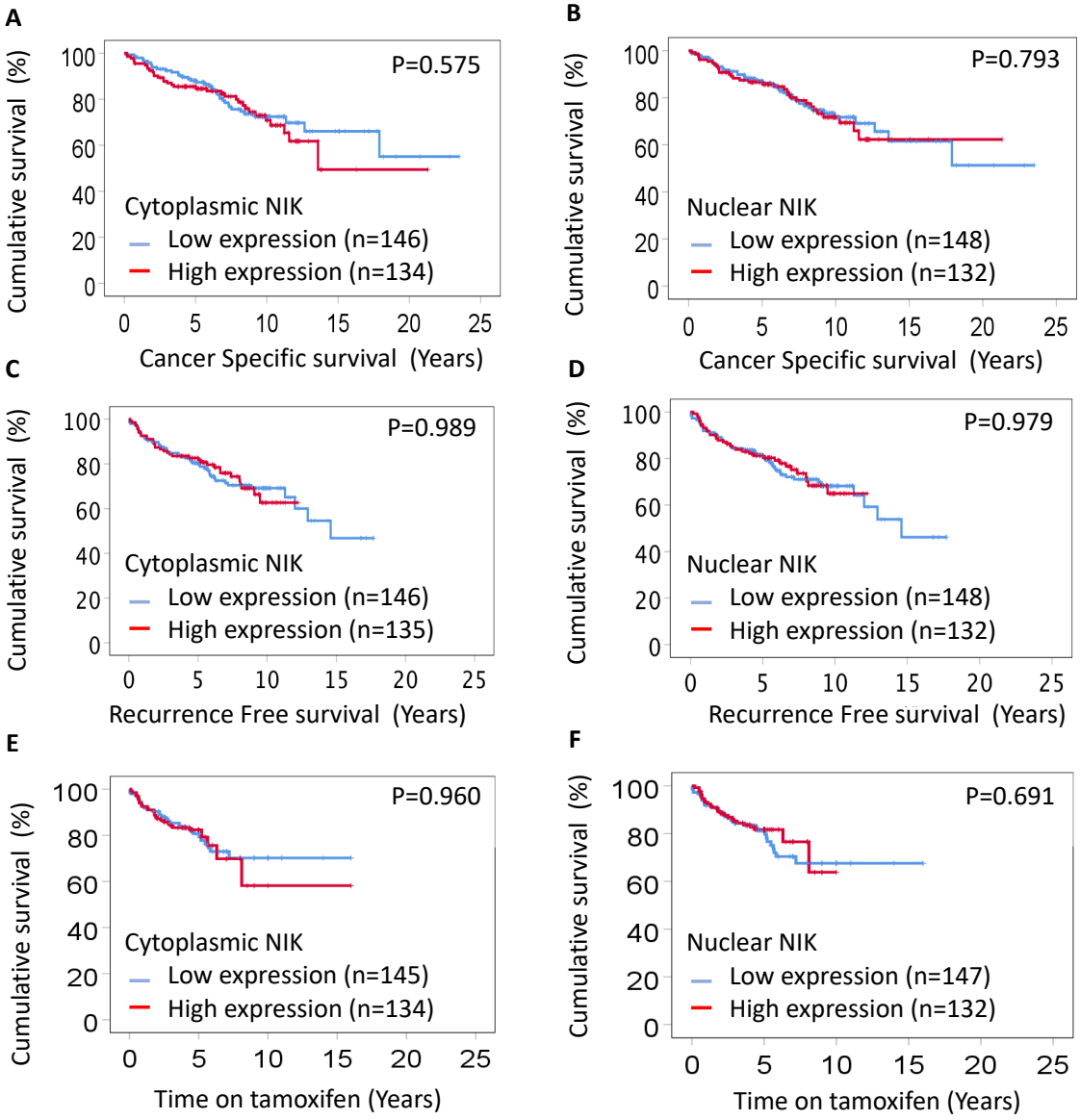


**Figure 4.3 Expression of NIK and clinical outcome in the STB-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear NIK with cancer specific survival (A and B), recurrence free survival (C and D), and recurrence free survival on tamoxifen (E and F).*

### 4.2.1.2 Associations between NIK and clinicopathological characteristics of patients with ER-positive breast cancer

Chi-squared analysis was performed in order to determine whether expression of NIK was associated with any clinicopathological characteristics of ER-positive patients with the STB-cohort as shown in Table 4.2. Expression of cytoplasmic NIK was associated with Ki67 (P=0.002), molecular subtype (P=0.006) and chemotherapy (P=0.023). Expression of nuclear NIK was associated with PR status (P=0.024).

| Clinicopathological characteristics | Cytoplasmic NIK | | | Nuclear NIK | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 22(15.) | 29(21.6) | 0.155 | 26(17.6) | 25(18.9) | 0.767 |
| >50 years) | 124(84.9) | 105(78.4) | | 25(18.9) | 107(81.1) | |
| Histological type | | | | | | |
| Ductal | 119(83.8) | 117(86.7) | 0.797 | 122(84.7) | 114(85.7) | 0.951 |
| Lobular | 13(9.2) | 10(7.4) | | 12(8.3) | 11(8.3) | |
| Other | 10(7.0) | 8(5.9) | | 10(6.9) | 8(6.0) | |
| Size (mm) | | | | | | |
| ≤20 | 58(43.9) | 69(52.7) | 0.317 | 57(41.9) | 70(55.1) | 0.096 |
| 21-50 | 66(50.0) | 57(43.5) | | 72(52.9) | 51(40.2) | |
| >50 | 8(6.1) | 5(3.8) | | 7(5.1) | 6(4.7) | |
| Grade | | | | | | |
| I | 37(25.9) | 25(19.4) | 0.430 | 34(23.3) | 28(22.2) | 0.359 |
| II | 68(47.6) | 65(50.4) | | 66(45.2) | 67(53.2) | |
| III | 38(26.6) | 39(30.2) | | 46(31.5) | 31(24.6) | |
| Lymph node status | | | | | | |
| Negative | 73(57.5) | 58(45.7) | 0.060 | 67(51.9) | 64(54.2) | 0.906 |
| Positive | 54(42.5) | 69(54.3) | | 62(48.1) | 61(48.8) | |
| NPI | | | | | | |
| I | 41(35.3) | 38(31.9) | 0.527 | 36(29.8) | 43(37.7) | 0.431 |
| II | 58(50.0) | 57(47.9) | | 63(52.1) | 52(45.6) | |
| III | 17(14.7) | 24(20.0) | | 22(18.2) | 19(16.7) | |
| PR status | | | | | | |
| Negative | 57(40.1) | 52(39.1) | 0.86 | 67(45.9) | 42(32.6) | **0.024** |
| Positive | 85(59.9) | 81(60.9) | | 79(54.1) | 87(67.4) | |
| Her-2 status | | | | | | |
| Negative | 137(93.8) | 125(93.3) | 0.851 | 136(91.9) | 126(95.5) | 0.225 |
| Positive | 9(6.2) | 9(6.7) | | 12(8.1) | 6(4.5) | |
| Proliferation (Ki67) | | | | | | |
| Low | 111(84.7) | 82(68.30 | **0.002** | 110(81.5) | 83(71.6) | 0.063 |
| High | 20(15.30 | 38(31.7) | | 25(18.5) | 33(28.4) | |
| Molecular subtype | | | | | | |
| Luminal A | 105(80.8) | 79(65.3) | **0.006** | 104(77.6) | 80(68.4) | 0.099 |
| Luminal B | 25(19.2) | 42(34.7) | | 30(22.4) | 37(31.6) | |

**Table 4.2 Association of NIK with clinicopathological characteristics of the STB-cohort.** *Chi-squared analysis exploring association between NIK with clinicopathological characteristics of the cohort.*

#### 4.2.1.3 Expression of NIK and clinical outcome in different luminal subtypes

In order to determine whether expression of NIK was associated with clinical outcome in specific ER-positive subtype, the cohort was subdivided into luminal A and luminal B tumours. High cytoplasmic expression of NIK was not associated with any of clinical outcomes in terms of cancer specific survival (luminal A, HR 1.01 95% CI 0.54-1.88), P=0.977; luminal B, HR 0.77 95% CI 0.35-1.70, P=0.518, Figure 4.4), recurrence free survival (luminal A, HR 0.96 95% CI 0.52-1.78, P=0.941; luminal B, HR 0.60 95% CI 0.28-1.28, P=0.178, Figure 4.5) and recurrence on tamoxifen (luminal A, HR 0.86 95% CI 0.41-1.81, P=0.715; luminal B, HR 0.61 95% CI 0.29-1.29, P=0.190, Figure 4.6). Similarly, high nuclear expression of NIK was not associated with cancer specific survival (luminal A, HR 1.32 95% CI 0.71-2.45, P=0.382; luminal B, HR 0.65 95% CI 0.30-1.44, P=0.285, Figure 4.4 recurrence free survival (luminal A, HR 1.22 95% CI 0.66-2.34, P=0.551; luminal B, HR 0.54 95% CI 0.25-1.17, P=0.113, Figure 4.5; and recurrence on tamoxifen (luminal A, HR 0.98 95% CI 0.48-2.03, P=0.947; luminal B. HR 0.55 95% CI 0.25-1.18, P=0.118, Figure 4.6)

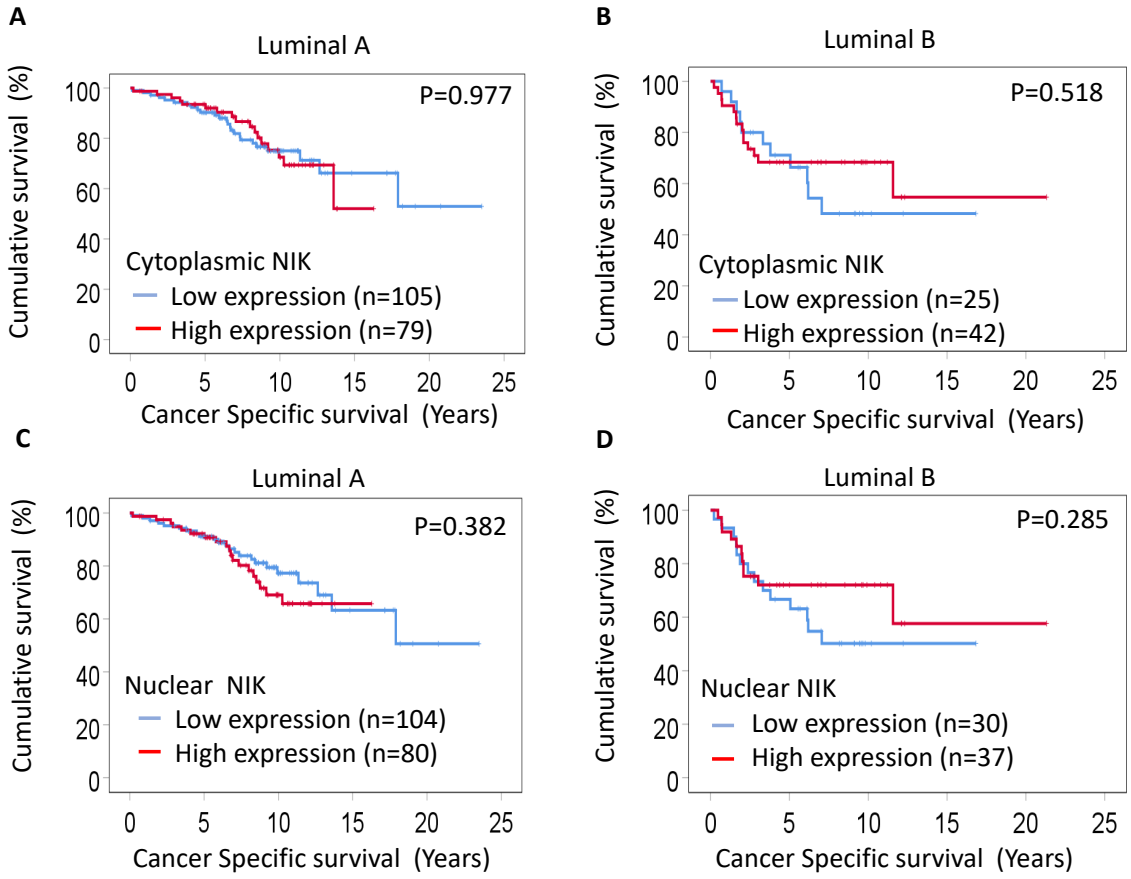


**Figure 4.4 Expression of NIK and cancer specific survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic NIK and cancer specific survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of NIK and cancer specific survival in luminal A tumours (C) or luminal B tumours (D)*

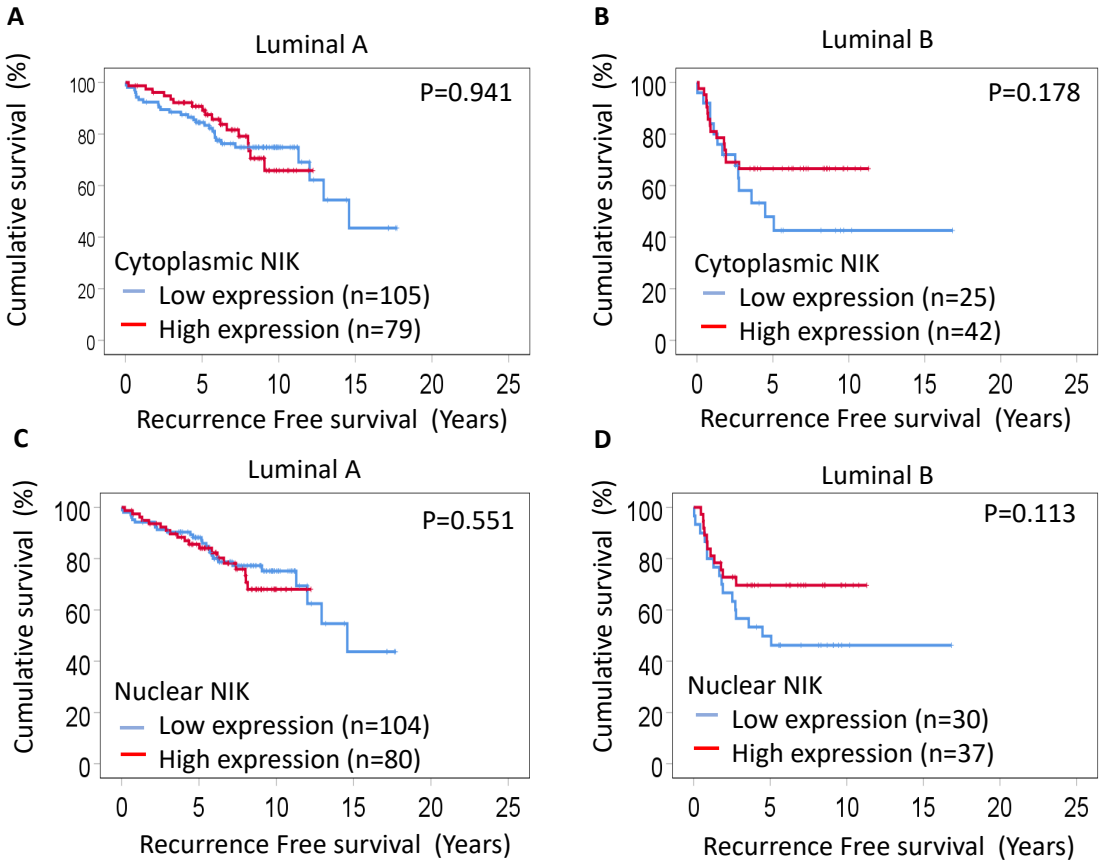


**Figure 4.5 Expression of NIK and recurrence free survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic NIK and recurrence free survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of NIK and recurrence free survival in luminal A tumours (C) or luminal B tumours (D)*

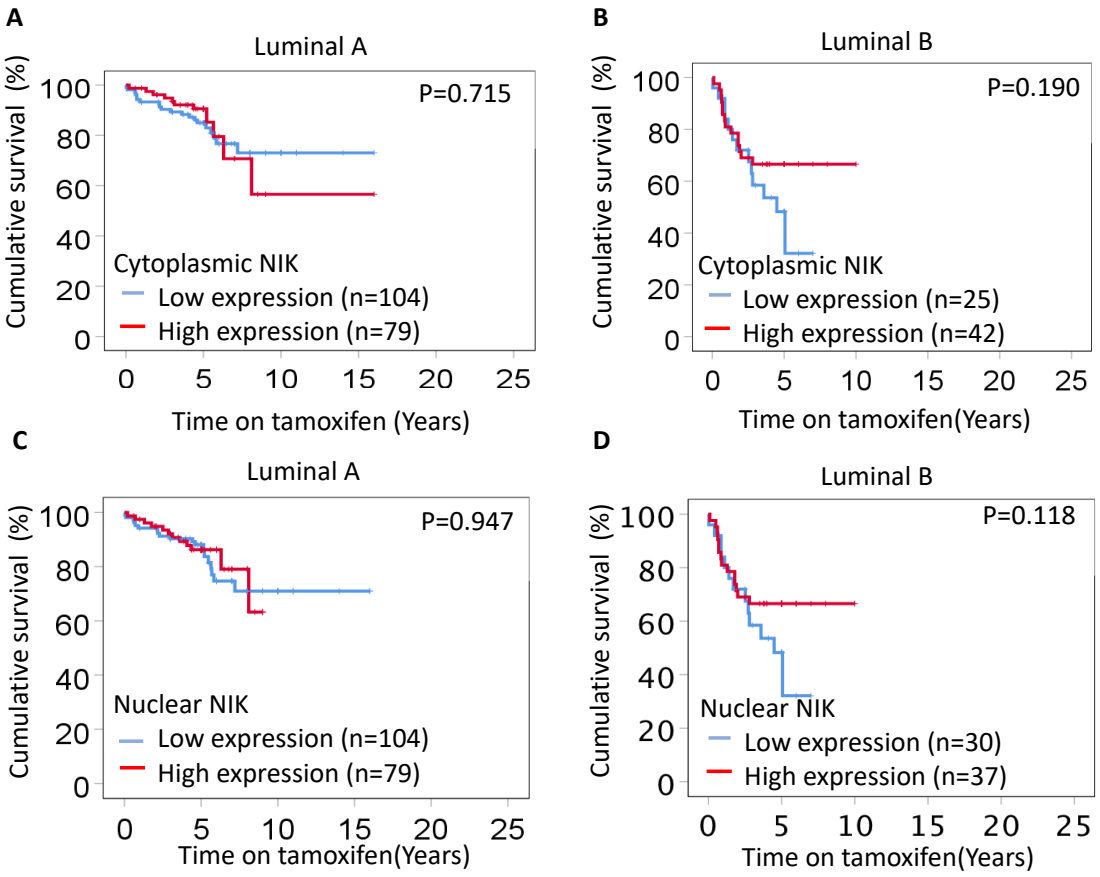


**Figure 4.6 Expression of NIK and recurrence on tamoxifen in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic NIK and recurrence on tamoxifen in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of NIK recurrence on tamoxifen in luminal A tumours (C) or luminal B tumours (D).*

## 4.2.2 Expression of IKKα in a cohort of the patients with ER-positive breast cancer

### 4.2.2.1 Association of IKKα with clinical outcome in a cohort of the patients with ER-positive breast cancer

Expression of IKKα was already available in the STB cohort from a previous study by Dr Lindsay Bennet and was assessed in 309 (79%) patients from the STB-cohort (N=392) (204). The histoscore for cytoplasmic expression of IKKα ranged from 0 to 180 with a median histoscore of 95 (IQR=73.3-116.7). The histoscore for nuclear expression of IKKα ranged from 0 to 146 with a median histoscore of 33.33 (IQR=15.3-33.3). An example of immunostaining of low and high expression of IKKα is shown in Figure 4.7. Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of IKKα are shown in Figure 4.7. There was good correlation between observers with ICCC score of 0.86 for cytoplasmic and 0.81for nuclear expression.

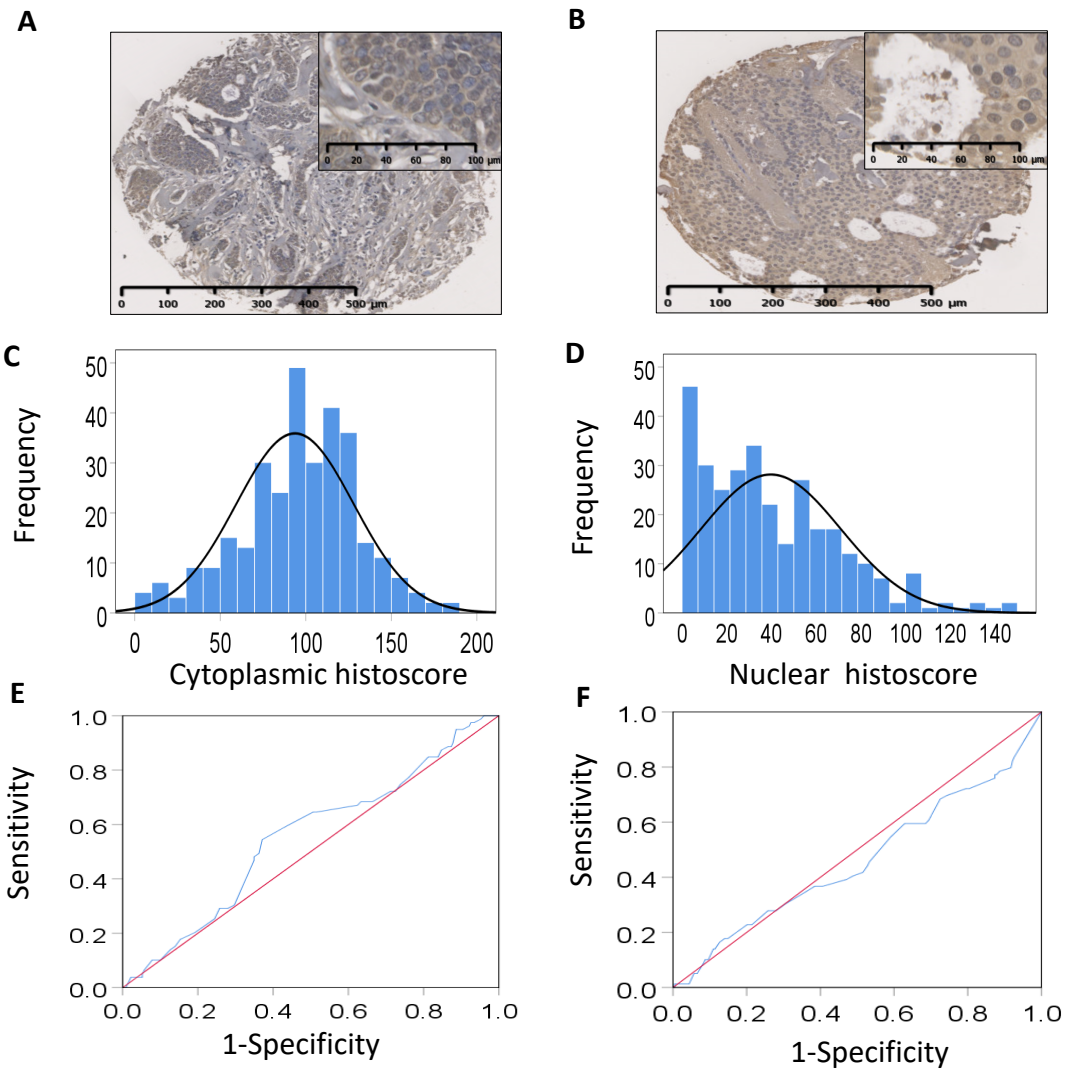


**Figure 4.7 Expression of IKKα.** *(A) Example of low expression and (B) of high expression of IKKα (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of IKKα. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of IKKα*

The ROC curve was plotted to determine the cut-off point in order to classify low and high expression of cytoplasmic IKKα and nuclear IKKα (Figure 4.7). Since the AUC was 0.55 (P=0.174) and 0.46 (P=0.303) for cytoplasmic and nuclear IKKα, the values from ROC curves were unable to be used to determine optimal cut-off points. Therefore, the median histoscore was used as an optimal cut-off point in order to subdivide the cohort into low and high expression. Tumours with cytoplasmic histoscore over 100 and tumour with nuclear histoscore over 35 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-off values, 128 patients had high cytoplasmic expression and 181 patients had low cytoplasmic expression. 145 patients had high nuclear expression and 164 patients had low nuclear expression.

In order to determine whether IKKα expression was associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of IKKα were plotted as shown in Figure 4.8. Low and high expression were compared by using the log rank test. Cytoplasmic expression of IKKα was associated with decreased cancer specific survival (HR =1.14 95% CI 0.25-5.16, P=0.012, recurrence free survival (HR 1.86 95% CI 1.20-2.88, P=0.005) and recurrence on tamoxifen (HR 1.87 95% CI 1.15-3.04, P=0.010). In contrast to cytoplasmic expression, nuclear expression of IKKα was not associated with any clinical outcomes including cancer specific survival (HR 0.68, 95% CI 0.43-1.07, P=0.960), recurrence free survival (HR 0.68 95% CI 0.44-1.07, P=0.092) and recurrence on tamoxifen (HR 0.77 95% CI 0.47-1.25, P =0.289) (Figure 4.8).

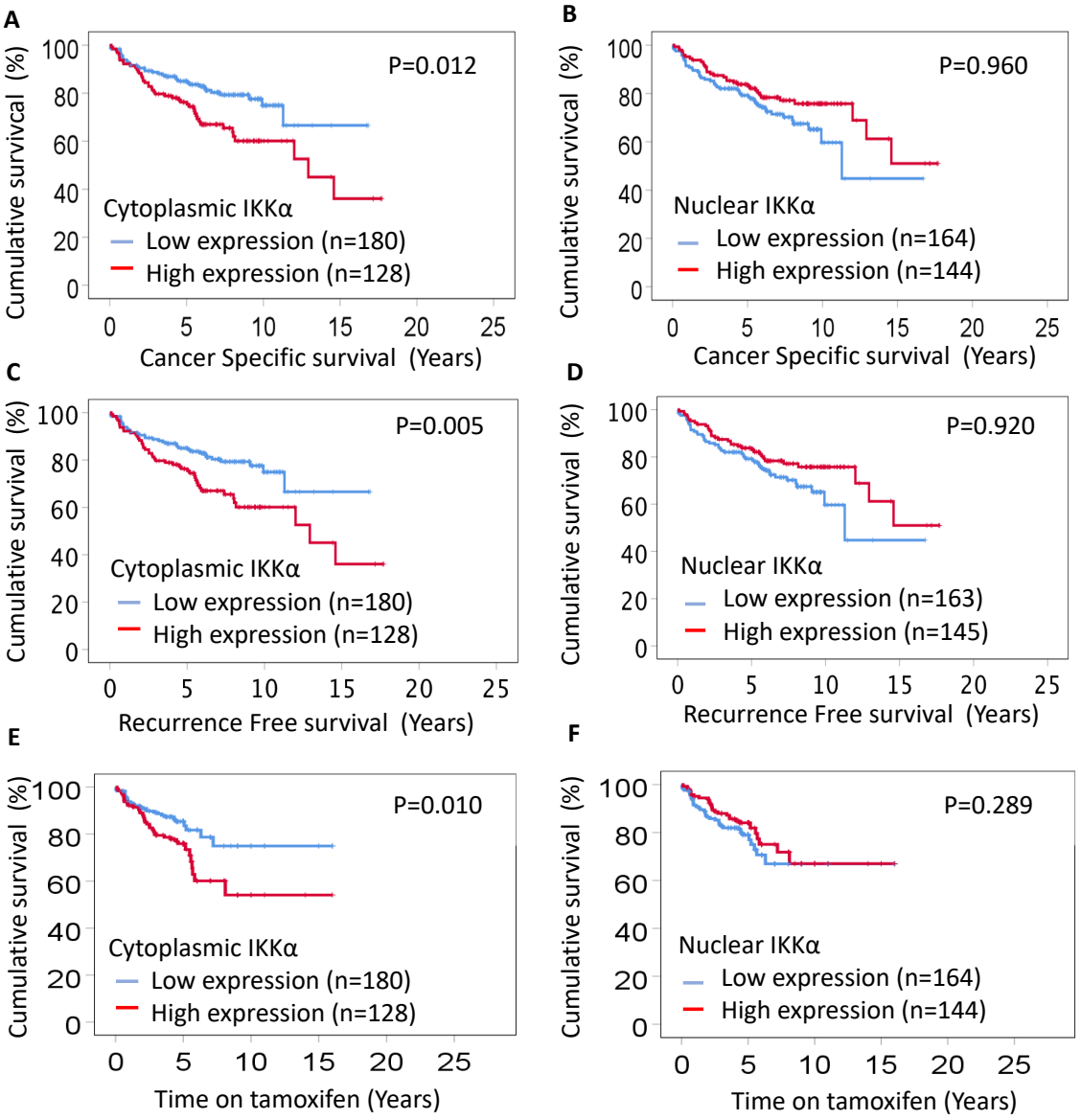


**Figure 4.8 Expression of IKKα and clinical outcome in the STB-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear IKKα with cancer specific survival (A and B), recurrence free survival (C and D), and recurrence free survival on tamoxifen (E and F)*

Multivariate analysis suggested that cytoplasmic IKKα expression was an independent prognostic factor when combined with tumour size, tumour grade, lymph node status, NPI, PR status, Ki 67 and molecular subtypes (HR 1.78 95% CI 1.06-2.98, P=0.502, Table 4.3).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>≤50>50 | 1.90 (0.99-3.70) | 0.055 | | |
| Histological type<br>Ductal/ Lobular/Other | 1.35 (0.89-2.05) | 0.159 | | |
| Size (mm)<br>≤20/ 21-50/ 21-50/>50 | 2.60 (1.88-3.61) | **<0.001** | 1.93 (1.231.15 (0.57- | **0.004** |
| Grade<br>I/II/III | 1.87 (1.39-2.53) | **<0.001** | 1.32 (0.89-1.95) | 0.171 |
| Lymph node status<br>Negative/ Positive | 3.47 (2.15-5.60) | **<0.001** | 2.93 (1.63-5.23) | **<0.001** |
| NPI<br>I (3.5)/ II (3.5-5.5)/III (>5.5) | 2.98 (2.16-4.12) | **<0.001** | 1.15 (0.57-2.33) | 0.698 |
| PR status<br>Negative/ Positive | 0.64 (0.44-0.97) | **0.033** | 0.94 (0.55-1.58) | 0.805 |
| Her-2 status<br>Negative/ Positive | 1.70 (0.82-3.51) | 0.147 | | |
| Proliferation (Ki67)<br>Low/ High | 2.05 (1.27-3.31) | **0.003** | 1.25 (0.27-5.84) | 0.781 |
| Molecular subtype<br>Luminal A/ Luminal B | 2.00 (1.26-3.17) | **0.003** | 1.89 (1.10-3.26) | 0.022 |
| Cytoplasmic IKKα<br>Low/High | 1.76 (1.13-2.74) | **0.013** | 1.78 (1.06-2.98) | **0.029** |
| Nuclear IKKα<br>Low/High | 0.68 (0.43-1.07) | 0.098 | | |

**Table 4.3 Univariate and multivariate analysis of IKKα and clinicopathological characteristic in the STB cohort**

#### 4.2.2.2 Associations between IKKα and clinicopathological characteristics of patients with ER-positive breast cancer

Chi-squared analysis was performed in order to determine whether expression of IKKα was associated with any clinicopathological characteristic of the patients in the STB-cohort as shown in Table 4.8. Expression of cytoplasmic IKKα was associated with NPI (P=0.037). Expression of nuclear IKKα was associated with lymph node status (P=0.005), Ki-67 (P=0.009) and molecular subtype (P=0.008).

| Clinicopathological characteristics | Cytoplasmic IKKα | | | Nuclear IKKα | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 31(17.2) | 22(17.2) | 0.994 | 22(13.5) | 31(21.4) | 0.067 |
| >50 years) | 149(82.8) | 106(82.8) | | 141(86.5) | 114(78.6) | |
| Histological type | | | | | | |
| Ductal | 154(87) | 112(89.6) | 0.189 | 148(90.8) | 118(84.9) | 0.174 |
| Lobular | 9(5.1) | 9(7.2) | | 6(3.7) | 12(8.6) | |
| Other | 14(7.9) | 14(7.9) | | 9(5.5) | 9(6.5) | |
| Size (mm) | | | | | | |
| ≤20 | 91(52.0) | 54(45.4) | 0.513 | 74(46.0) | 71(53.7) | 0.384 |
| 21-50 | 75(42.9) | 57(47.9) | | 76(47.2) | 56(42.1) | |
| >50 | 9(5.1) | 8(6.7) | | 11(6.8) | 6(4.5) | |
| Grade | | | | | | |
| I | 48(27.6) | 20(16) | 0.058 | 33(20.5) | 35(25.4) | 0.095 |
| II | 82(47.1) | 66(52.8) | | 75(46.6) | 73(52.9) | |
| III | 44(25.3) | 39(31.2) | | 53(32.9) | 30(21.7) | |
| Lymph node status | | | | 67(45.0) | 80(62.0) | **0.005** |
| Negative | 91(56.2) | 56(48.3) | 0.193 | 82(55.0) | 49(38.0) | |
| Positive | 71(43.8) | 60(51.7) | | | | |
| NPI | | | | | | |
| I | 63(41.2) | 28(25.9) | **0.037** | 43(29.7) | 48(41.4) | 0.096 |
| II | 65(42.5) | 56(51.9) | | 70(48.3) | 51(44.0) | |
| III | 25(16.3) | 24(22.2) | | 32(22.1) | 17(14.7) | |
| PR status | | | | | | |
| Negative | 64(35.8) | 46(37.4) | 0.770 | 64(40.0) | 46(32.4) | 0.170 |
| Positive | 115(64.2) | 77(62.6) | | 96(60.0) | 96(67.6) | |
| Her-2 status | | | | | | |
| Negative | 167(93.3) | 119(93) | 0.911 | 150(92.0) | 136(94.4) | 0.402 |
| Positive | 12(6.7) | 9(7) | | 13(8.0) | 8(5.6) | |
| Proliferation (Ki67) | | | | | | |
| Low | 117(72.7) | 95(79.2) | 0.211 | 103(69.1) | 109(82.6) | **0.009** |
| High | 44(27.3) | 25(20.8) | | 46(30.9) | 23(17.4) | |
| Molecular subtype | | | | | | |
| Luminal A | 111(69.4) | 89(73.6) | 0.444 | 96(64.4) | 104(78.8) | **0.008** |
| Luminal B | 49(30.6) | 32(26.4) | | 53(35.6) | 28(21.2) | |

**Table 4.4 Association of IKKα with clinic-pathological characteristics of the STB-cohort.**
*Chi-squared analysis explored the relationship of IKKα with various characteristics of the cohort*

#### 4.2.2.3 Expression of IKKα and clinical outcome in different luminal subtypes

In order to determine whether expression of IKKα was associated with clinical outcome in a particular ER-positive subtype, the cohort was subdivided into luminal A and luminal B disease. High cytoplasmic expression of IKKα was associated with poorer clinical outcome

in luminal B tumours, in terms of shorter cancer specific survival (HR 2.43 95% CI 1.14-5.18, P=0.018, Figure 4.9), shorter recurrence free survival (HR 2.41 95% CI 1.18-4.93, P=0.013, Figure 4.10), shorter recurrence on tamoxifen (HR 2.43 95% CI 1.14-5.18, P=0.008, Figure 4.11). Nuclear expression of IKKα was not associated with cancer specific survival (luminal A, HR, 0.68 95% CI 0.38-1.21, P=0.185; luminal B tumours, HR 0.92 95% CI 0.41-2.02, P=0.825, Figure 4.9, recurrence free survival (luminal A, HR 0.68 95% CI 0.38-1.24, P=0.220, Figure 4.10; luminal B tumours, HR 0.81 95% CI 0.38-1.72, P=0.580,) and recurrence on tamoxifen (luminal A, HR 0.82 95% CI 0.41-1.65, P=0.590; luminal B tumours, HR 0.90 95% CI 0.43-1.92, P=0.792, Figure 4.11) in either subtype.

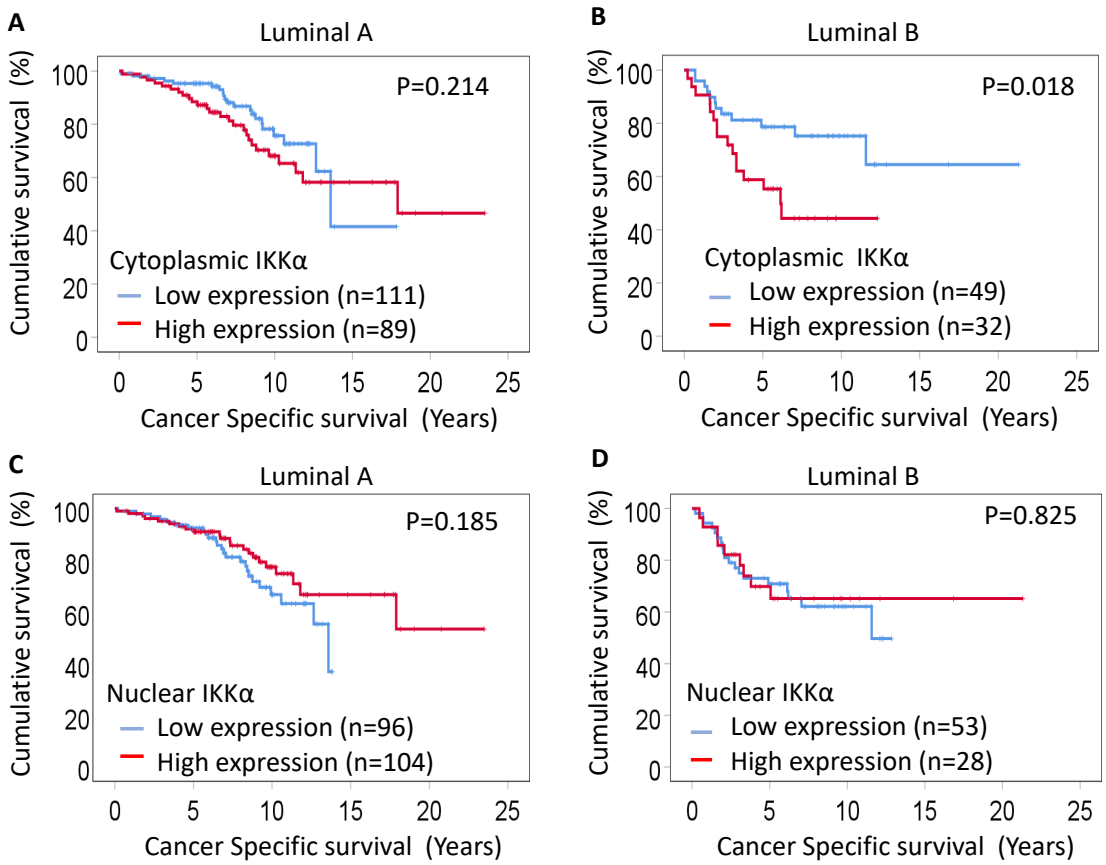


**Figure 4.9 Expression of IKKα and cancer specific survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic IKKα and cancer specific survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of IKKα and cancer specific survival in luminal A tumours (C) or luminal B tumours (D).*

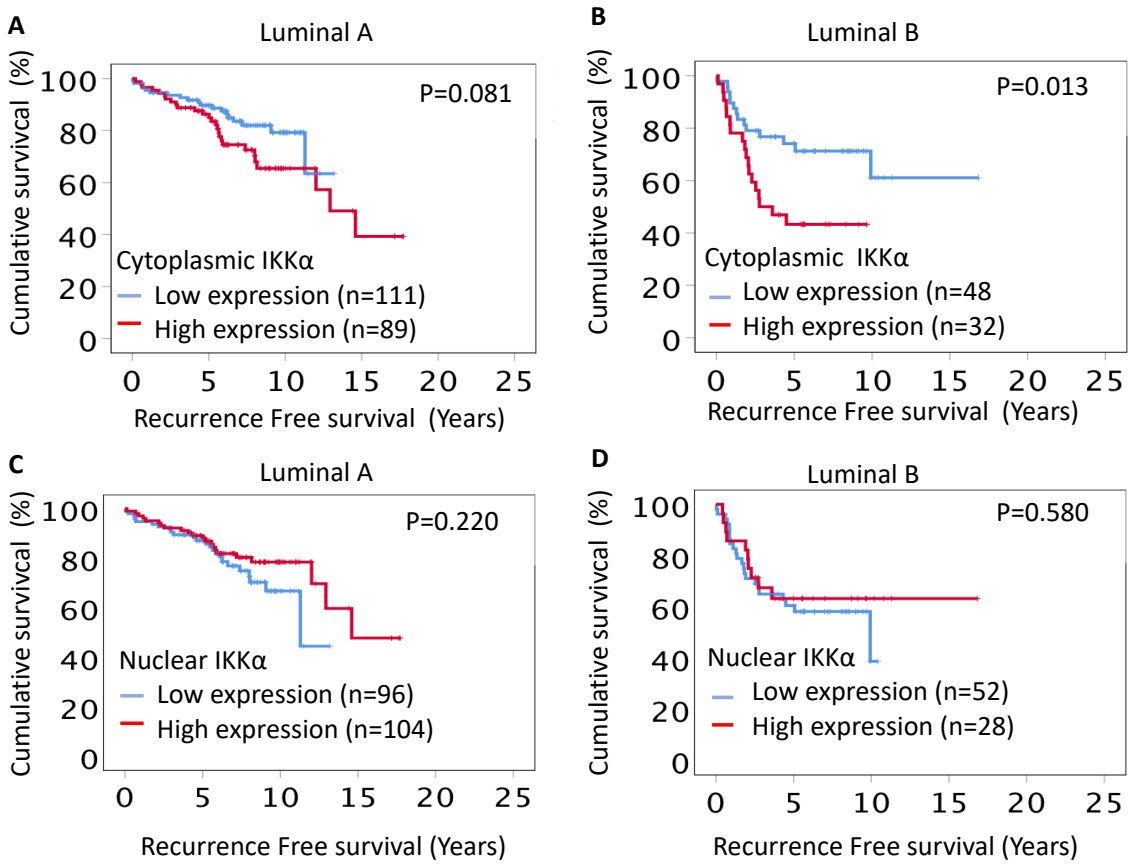


**Figure 4.10 Expression of IKKα and recurrence free survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic IKKα and recurrence free survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of IKKα and recurrence free survival in luminal A tumours (C) or luminal B tumours (D).*

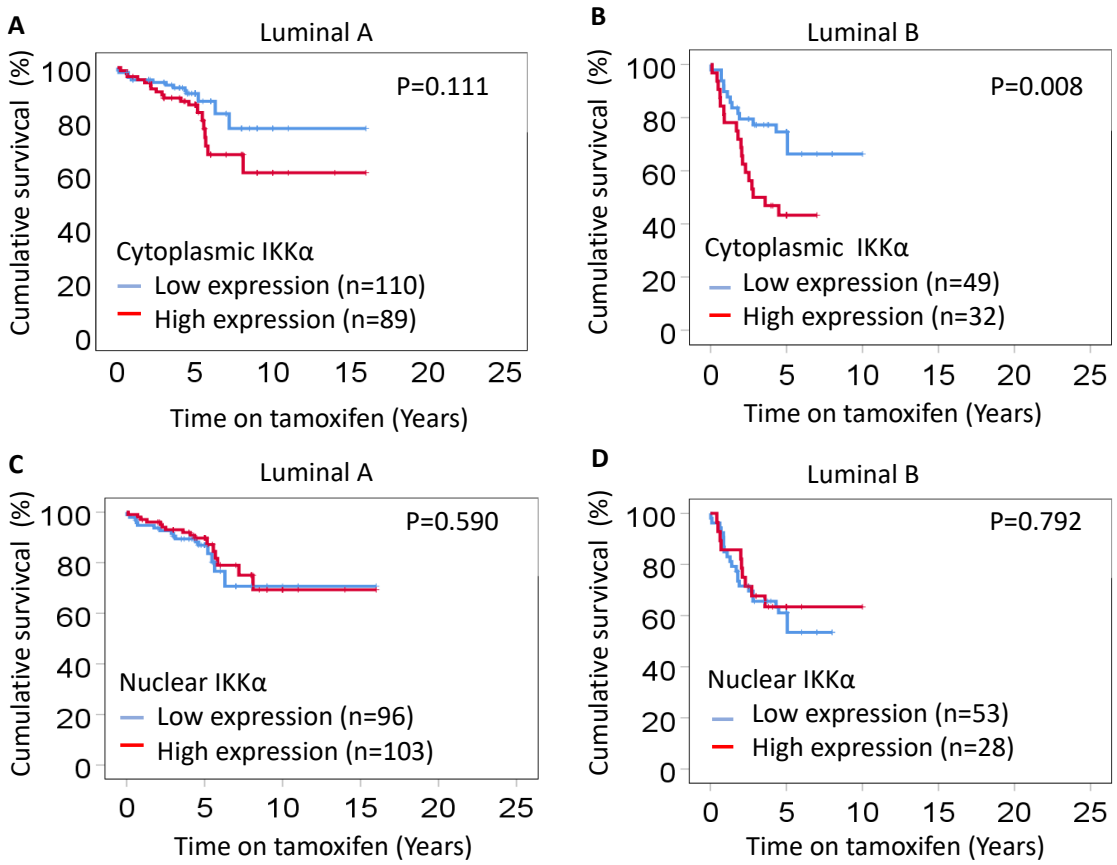


**Figure 4.11 Expression of IKKα and recurrence on tamoxifen in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic IKKα and recurrence on tamoxifen in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of IKKα and recurrence on tamoxifen in luminal A tumours (C) or luminal B tumours (D)*

Multivariate analysis suggested that cytoplasmic IKKα was not an independent prognostic marker when combined with tumour size, lymph node status and NPI (HR 1.74 95% CI 0.70-4.31, P=0.230) (Table 4.5).

| Clinicopathological characteristics | Luminal B | | | |
|---|---|---|---|---|
| | Univariate analysis | | Multivariate analysis | |
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>≤50/>50 | 2.45 (0.74-8.08) | 0.143 | | |
| Histological type<br>Ductal/ Lobular/Other | 1.23 (0.51-2.95) | 0.648 | | |
| Size (mm)<br>≤20/ 21-50/ 21-50/>50 | 2.65 (1.45-4.85) | **0.002** | 2.57 (1.20-5.51) | **0.015** |
| Grade<br>I/II/III | 1.27 (0.68-2.40) | 0.455 | | |
| Lymph node status<br>Negative/ Positive | 3.97 (1.35-11.69) | **0.012** | 2.75 (0.91-8.31) | 0.073 |
| NPI<br>I (<3.5)/ II (3.5-5.5)/ III (>5.5) | 2.47 (0.72-8.51) | 0.153 | | |
| PR status<br>Negative/ Positive | 0.71 (0.43-1.48) | 0.364 | | |
| Her-2 status<br>Negative/ Positive | 1.09 (0.48-2.46) | 0.838 | | |
| Proliferation (Ki-67)<br>Low/ High | 1.36 (0.41-4.50) | 0.620 | | |
| Cytoplasmic IKKα<br>Low/High | 2.43 (1.14-5.18) | **0.022** | 1.74 (0.70-4.31) | 0.230 |
| Nuclear IKKα<br>Low/High | 0.92 (0.41-2.02) | 0.826 | | |

**Table 4.5 Univariate and multivariate analysis of IKKα and clinicopathological characteristic in luminal B tumour**

## 4.2.3 Expression of pIKKα-Ser176 in patients with ER-positive breast cancer

### 4.2.3.1 Expression of pIKKα-Ser176 and clinical outcome in patients with ER-positive breast cancer

Expression of IKKα phosphorylation at serine residue 176 (Ser176) was assessed in 188 (48%) patients form the STB-cohort (N=392). The histoscore for cytoplasmic phosphorylation of pIKKα-Ser176 ranged from 0 to 287 with a median histoscore of 60 (IQR=5.0-122.3). The histoscore for nuclear phosphorylation of pIKKα-Ser176 ranged from 0 to 287 with a median histoscore of 110 (IQR=61.3-111.3). An example of immunostaining

of low and high expression of pIKKα-Ser176 (Figure 4.12). Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of pIKKα-Ser176 are shown in Figure 4.12. There was good and moderate correlation between observes with ICCC score of 0.88 for cytoplasmic, however, there was poor correlation with ICC score of 0.73 for nuclear expression.

The ROC curve was plotted to determine the cut-off point in order to classify low and high expression of cytoplasmic and nuclear pIKKα-Ser176 (Figure 4.12). Since the AUC was 0.427 (P=0.147) and 0.54 (P=0.387) for cytoplasmic and nuclear pIKKα-Ser176, the values from ROC curves were unable to be used to determine optimal cut-off points. Therefore, the median histoscore was used as an optimal cut-off point in order to subdivide the cohort into low and high expression. Tumours with cytoplasmic histoscore over 60 and tumour with nuclear histoscore over 110 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-off values, 93 patients had high cytoplasmic expression and 95 patients had low cytoplasmic expression. 87 patients had high nuclear expression and 101 patients had low nuclear expression.

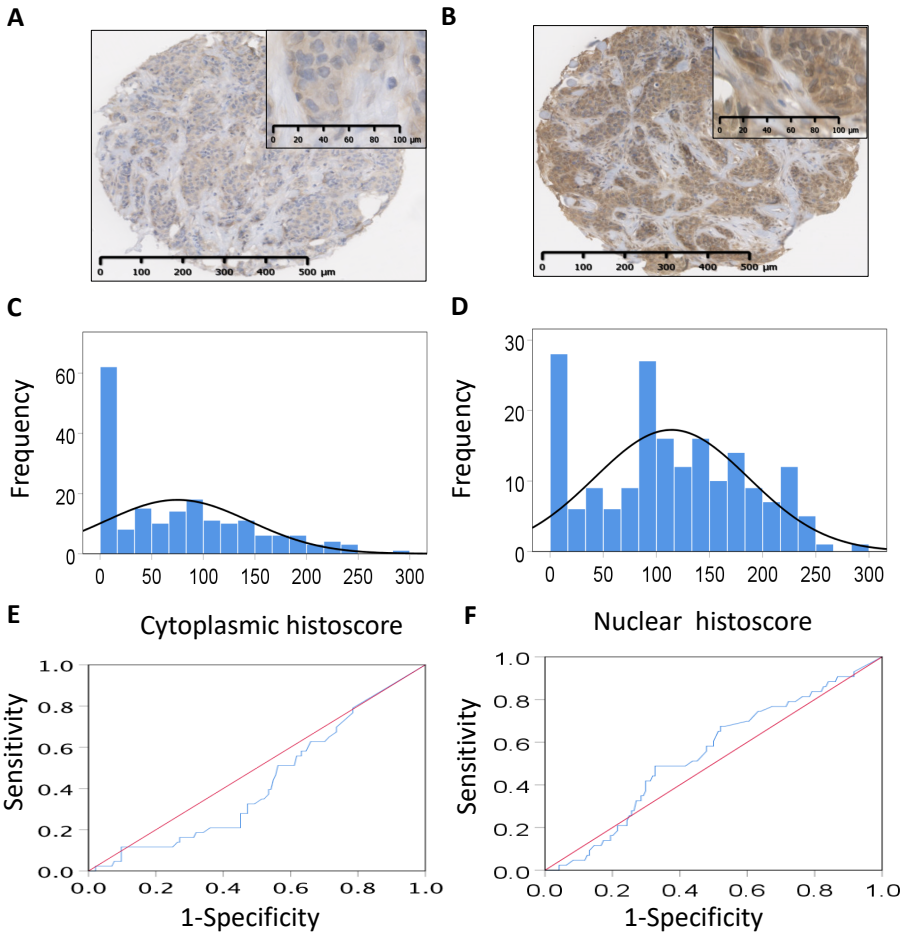


**Figure 4.12 Expression of pIKKα-Ser176.** *(A) Example of low expression and (B) of high expression of pIKKα-Ser176 (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of pIKKα-Ser176. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of pIKKα-Ser176.*

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pIKKα-Ser176 were plotted as shown in Figure 4.13. The log rank test was used to compare low and high expression. High cytoplasmic expression of pIKKα-Ser176 was associated with increased cancer specific survival (HR = 0.50 95% CI 0.27-0.92, P=0.024, Figure 4.13) but was not associated with recurrence free survival (HR = 0.636 95% CI 0.36-1.41, P=0.126, Figure 4.13) or recurrence on tamoxifen (HR = 0.61 95% CI 0.31-1.20, P=0.147, Figure 4.13). Nuclear expression of pIKKα-Ser176 was not associated with survival outcomes in terms of cancer specific survival, HR 1.16 95% CI 0.53-2.54, P=0.248 and recurrence free survival HR 1.39 95% CI 0.66-2.92, P=0.131 but was associated with decreased recurrence on tamoxifen HR 2.13 95% CI 0.80-5.65, P=0.014, Figure 4.13).

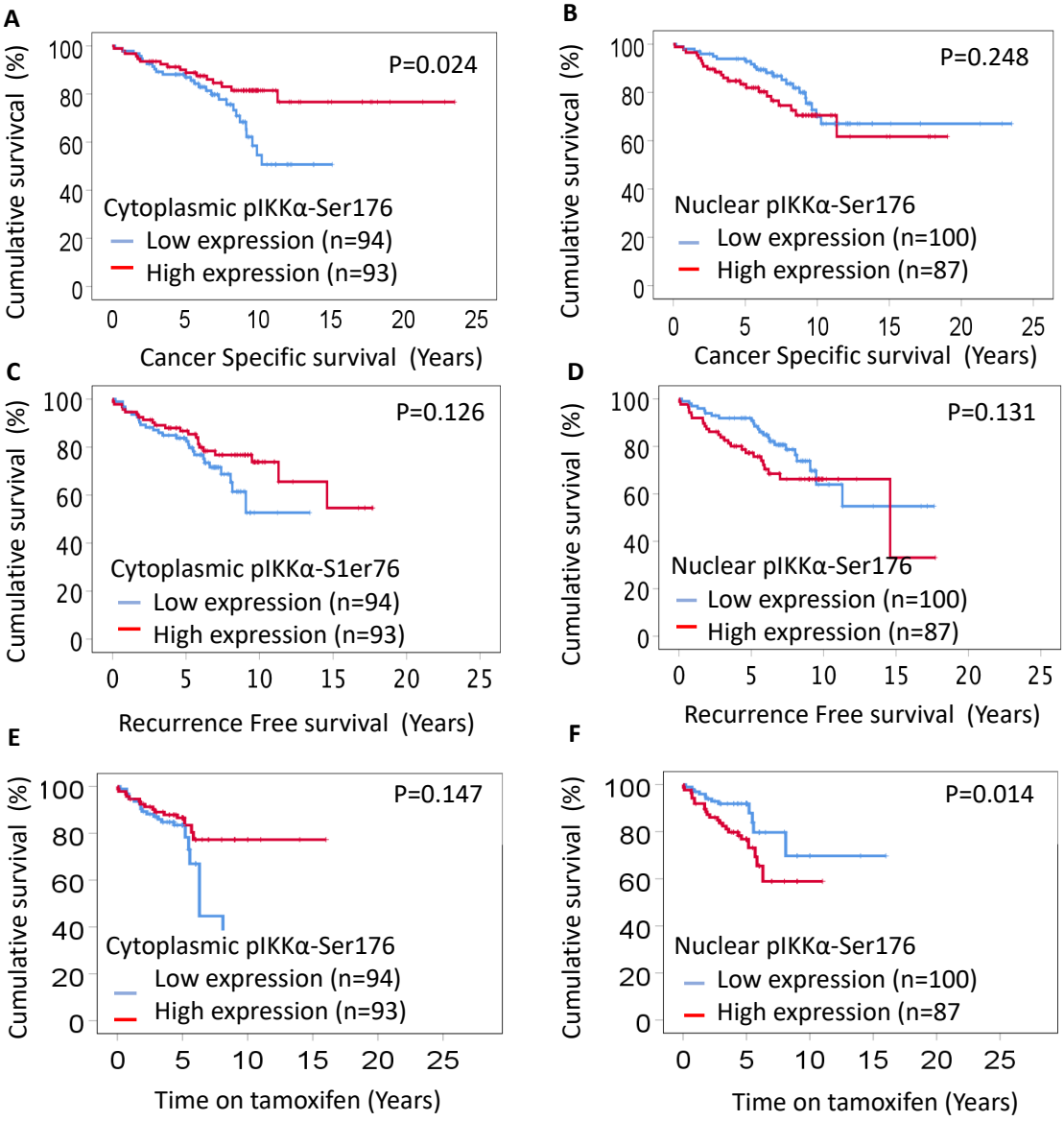


**Figure 4.13 Expression of pIKKα-Ser176 and clinical outcome in the STB-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pIKKα-Ser176 with cancer specific survival (A and B), recurrence free survival (C and D), and recurrence free survival on tamoxifen (E and F).*

No association was observed between high nuclear expression of pIKKα-Ser176 and cancer specific survival (HR = 1.42 95% CI 0.78-2.60, P=0.248, Figure 4.13) or recurrence free survival (HR = 1.54 95% CI 0.88-2.71, P=0.131, Figure 4.13). However, high expression of nuclear pIKKα-Ser176 was associated with decrease recurrence free survival in patients on tamoxifen (HR = 2.34 95% CI 1.17-4.72, P=0.014, Figure 4.13).

Multivariate analysis suggested that cytoplasmic expression of pIKKα-Ser176 was an independent prognostic marker for cancer specific survival when combined with tumour size, tumour grade, lymph node status, NPI, PR status, Ki67 and molecular subtypes, (HR 0.44 95% CI 0.21-0.92, P= 0.028) (Table 4.6).

| Clinicopathological | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>≤50>50 | 1.90 (0.99-3.70) | 0.055 | | |
| Histological type<br>Ductal/ Lobular/Other | 1.35 (0.89-2.05) | 0.159 | | |
| Size (mm)<br>≤20/ 21-50/ 21-50/>50 | 2.60 (1.88-3.61) | **<0.001** | 3.04 (1.55-5.99) | **0.001** |
| Grade<br>I/II/III | 1.87 (1.39-2.53) | **<0.001** | 1.88 (1.14-3.10) | **0.013** |
| Lymph node status<br>Negative/ Positive | 3.47 (2.15-5.60) | **<0.001** | 2.54 (1.23-5.23) | **0.012** |
| NPI<br>I (3.5)/ II (3.5-5.5)/III (>5.5) | 2.98 (2.16-4.12) | **<0.001** | 1.18 (0.40-3.54) | 0.764 |
| PR status<br>Negative/ Positive | 0.64 (0.44-0.97) | **0.033** | 0.92 (0.44-1.95) | 0.836 |
| Her-2 status<br>Negative/ Positive | 1.70 (0.82-3.51) | 0.147 | | |
| Proliferation (Ki67)<br>Low/ High | 2.05 (1.27-3.31) | **0.003** | 1.28 (0.15-10.93) | 0.819 |
| Molecular subtype<br>Luminal A/ Luminal B | 2.00 (1.26-3.17) | **0.003** | 1.16 (0.48-2.81) | 0.742 |
| Cytoplasmic pIKKα-Ser176<br>Low/High | 0.50 (0.27-0.92) | **0.027** | 0.44 (0.21-0.92) | **0.028** |
| Nuclear pIKKα-Ser176<br>Low/High | 1.42(0.78-2.59) | 0.250 | | |

**Table 4.6 Univariate and multivariate analysis of pIKKα-Ser176 and clinicopathological characteristic in the STB cohort**

#### 4.2.3.2 Associated of pIKKα-Ser176 and clinic-pathological characteristics of patients with ER-positive breast cancer

Chi-squared analysis was performed in order to determine whether expression of pIKKα-Ser176 was associated with any clinicopathological characteristic of the patients in the STB-cohort as shown in Table 4.7. Expression of cytoplasmic pIKKα-Ser176 was associated only with tumour grade (P=0.030). Whereas expression of nuclear pIKKα-Ser176 was associated with tumour size (P=0.011).

| Clinicopathological characteristics | Cytoplasmic pIKKα-Ser176 | | | Nuclear pIKKα-Ser176 | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P value | Low (%) | High (%) | P value |
| Age | | | | | | |
| ≤50 | 18 (18.9) | 19(20.4) | 0.798 | 18(17.8) | 19(21.8) | 0.490 |
| >50 years) | 77(81.1) | 74(79.6) | | 83(82.2) | 68(78.2) | |
| Histological type | | | | | | |
| Ductal | 84 (88.4) | 78(84.8) | 0.526 | 90(89.1) | 72(83.7) | 0.383 |
| Lobular | 6(6.3) | 10(10.9) | | 6(5.9) | 10(11.6) | |
| Other | 5(5.3) | 4(4.3) | | 5(5) | 4(4.7) | |
| Size (mm) | | | | | | |
| ≤20 | 47(50.5) | 38(45.2) | 0.623 | 51(52.6) | 34(42.5) | **0.011** |
| 21-50 | 42(45.2) | 40(47.6) | | 45(46.4) | 37(46.3) | |
| >50 | 4(4.3) | 6(7.1) | | 1(1) | 9(11.3) | |
| Grade | | | | | | |
| I | 25(26.9) | 23(25.0) | **0.030** | 28(28.6) | 20(23.0) | 0.134 |
| II | 52(55.9) | 38(41.3) | | 51(52) | 39(44.8) | |
| III | 16(17.2) | 31(33.7 | | 19(19.4) | 28(32.2) | |
| Lymph node status | | | | | | |
| Negative | 44(47.8) | 47(56.6) | 0.245 | 52(53.1) | 39(50.6) | 0.751 |
| Positive | 48(52.2) | 36(43.3) | | 46(46.9) | 38(49.8) | |
| NPI | | | | | | |
| I | 34(38.6) | 21(27.3) | 0.286 | 36(39.1) | 19(26.0) | 0.178 |
| II | 42(47.7) | 42(54.5) | | 44(47.8) | 40(54.8) | |
| III | 12(13.6) | 14(18.2) | | 12(13.0) | 14(19.2) | |
| PR status | | | | | | |
| Negative | 30(33.0) | 30(32.6) | 0.959 | 32(32.7) | 28(32.9) | 0.967 |
| Positive | 61(67.0) | 62(67.4) | | 66(67.3) | 57(67.) | |
| Her-2 status | | | | | | |
| Negative | 86(90.5) | 89(95.7) | 0.162 | 91(90.1) | 84(96.6%) | 0.082 |
| Positive | 9(9.5) | 4(4.3) | | 10(9.9) | 3(3.4) | |
| Proliferation (Ki67) | | | | | | |
| Low | 60(72.3) | 68(81.0) | 0.186 | 69(77.5) | 59(75.6) | 0.774 |
| High | 23(27.7) | 16(19.0) | | 20(22.) | 19(24.4) | |
| Molecular subtype | | | | | | |
| Luminal A | 57(68.7) | 65(77.4) | 0.205 | 65(73.9) | 57(72.2) | 0.803 |
| Luminal B | 26(31.3) | 19(22.6) | | 23(26.1) | 22(27.8) | |

**Table 4.7 Association of pIKKα-Ser176 with clinic-pathological characteristics of the STB-cohort.** *Chi-squared analysis explored the relationship of pIKKα-Ser176 with various characteristics of the cohort.*

#### 4.2.3.3 Expression of pIKKα-Ser176 and clinical outcome in different luminal subtypes

In order to determine whether expression of pIKKα-Ser176 was associated with clinical outcome in a particular ER-positive subtype, the cohort was subdivided into luminal A and luminal B disease. High cytoplasmic expression of pIKKα-Ser176 was not associated with any of clinical outcomes in terms of cancer specific survival (luminal A, HR 0.46 95% CI 0.21-1.04, P=0.056; luminal B, HR 0.83 95% CI 0.27-2.52, P=0.736, Figure 4.14; recurrence free survival (luminal A, HR 0.62 95% CI 0.29-1.36, P=0.231; luminal B, HR 0.70 95% CI 0.24-2.10, P=0.527, Figure 4.15) and recurrence on tamoxifen (luminal A, HR 0.66 95% CI 0.26-1.69, P=0.388; luminal B, HR 0.73 95% CI 0.25-2.18, P=0.571, Figure 4.16) in either subtype. No association was observed between nuclear expression of pIKKα-Ser176 and cancer specific survival (luminal A, HR 1.16 95% CI 0.53-2.54, P=0.719; luminal B, HR 1.86 95% CI 0.61-5.71, P=0.269, Figure 4.14;; recurrence free survival (luminal A, HR 1.39 95% CI 0.66-2.92, P=0.389; luminal B, HR 2.10 95% CI 0.70-6.27, P=0.176, Figure 4.15) and recurrence on tamoxifen (luminal A, HR 2.13 95% CI 0.80-5.65, P=0.121; luminal B, HR 2.16 95% CI 0.72-6.45, P=0.158, Figure 4.16) in either subtype.

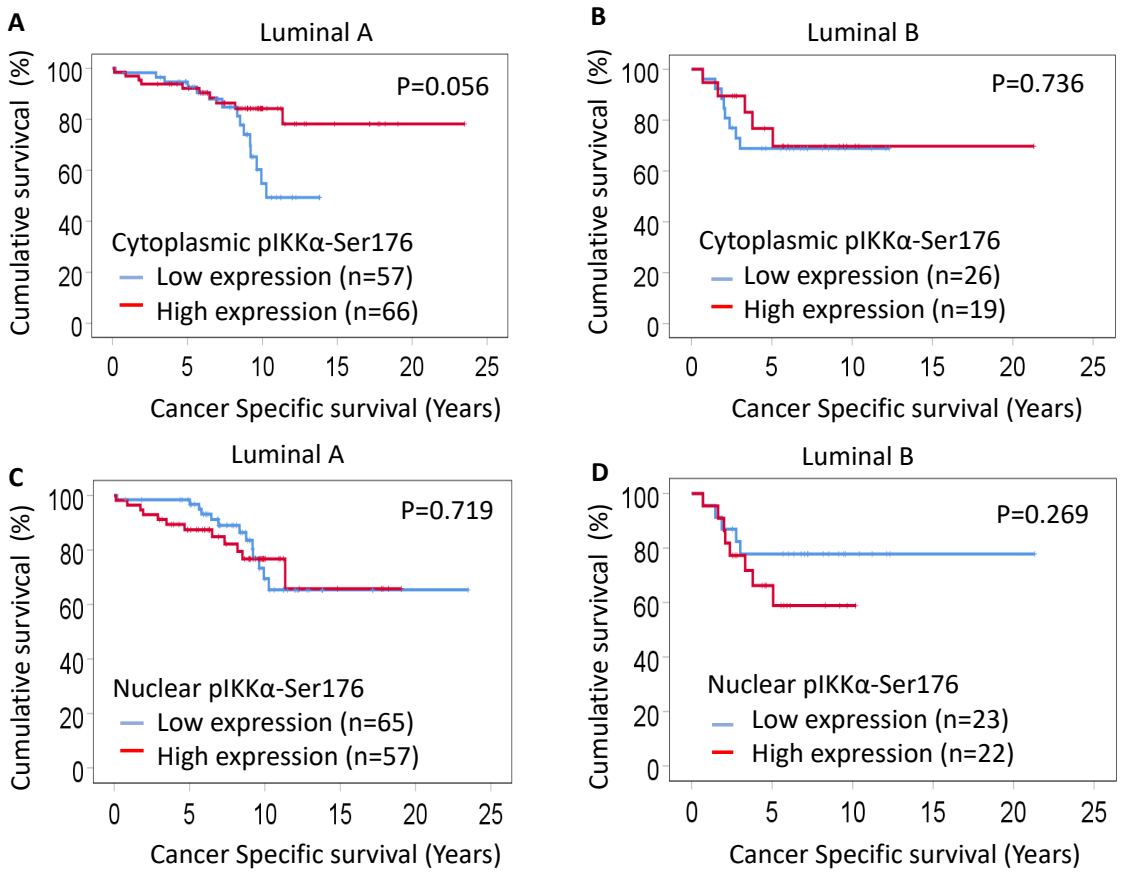


**Figure 4.14 Expression of pIKKα-Ser176 and cancer specific survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα-Ser176 and cancer specific survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pIKKα-Ser176 and cancer specific survival in luminal A tumours (C) or luminal B tumours (D).*

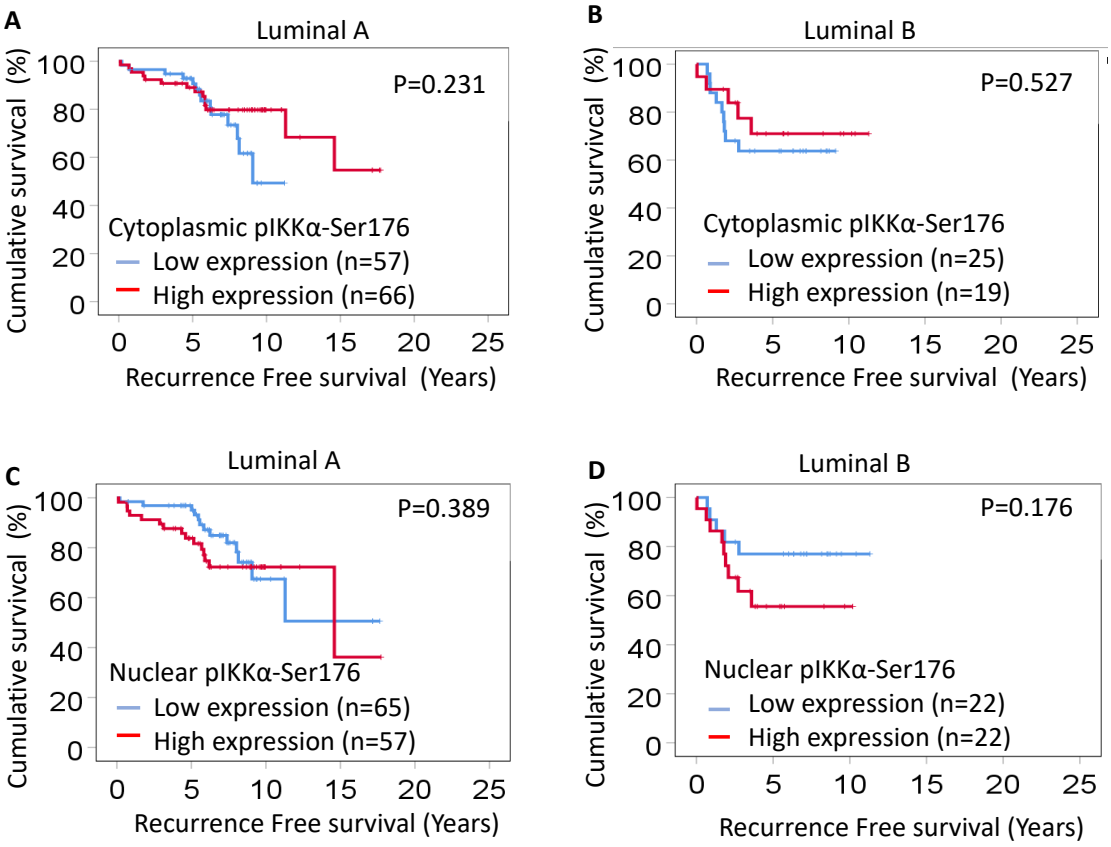


**Figure 4.15 Expression of pIKKα-Ser176 and recurrence free survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα-Ser176 and recurrence free survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pIKKα-Ser176 and recurrence free survival in luminal A tumours (C) or luminal B tumours (D).*

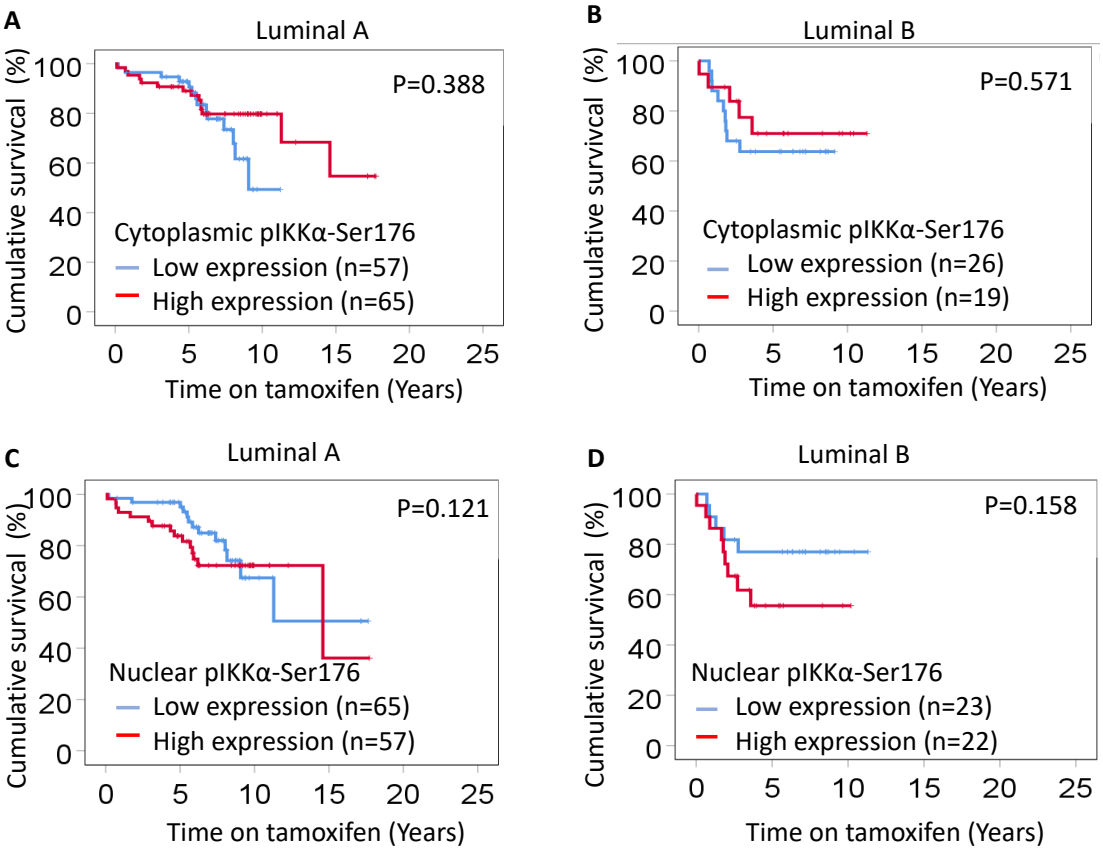


**Figure 4.16 Expression of pIKKα-Ser176 and recurrence on tamoxifen in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα-Ser176 and recurrence on tamoxifen in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pIKKα-Ser176 and recurrence on tamoxifen in luminal A tumours (C) or luminal B tumours (D).*

## 4.2.4 Expression of pIKKα-Thr23 in patients with ER-positive breast cancer

### 4.2.4.1 Expression of pIKKα- Thr23 and clinical outcome in patients with ER-positive breast cancer

Expression of IKKα phosphorylation at threonine residue 23 (Thr23) was assessed in 211 (54%) patients within the STB-cohort (N=392). The histoscore for cytoplasmic phosphorylation of pIKKα-ThrT23 ranged from 0 to 285 with a median histoscore of 135 (IQR=100-183). The histoscore for nuclear phosphorylation of pIKKα-Thr23 ranged from 0 to 300 and a median histoscore of 200 (IQR=147.3-220.0). An example of immunostaining of low and high expression of pIKKα-Thr23 is shown in Figure 4.17. Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of pIKKα-Thr23 are shown (Figure 4.17). There was moderated and good correlation between observers with ICCC score of 0.71 for cytoplasmic and 0.80 for nuclear expression, respectively.

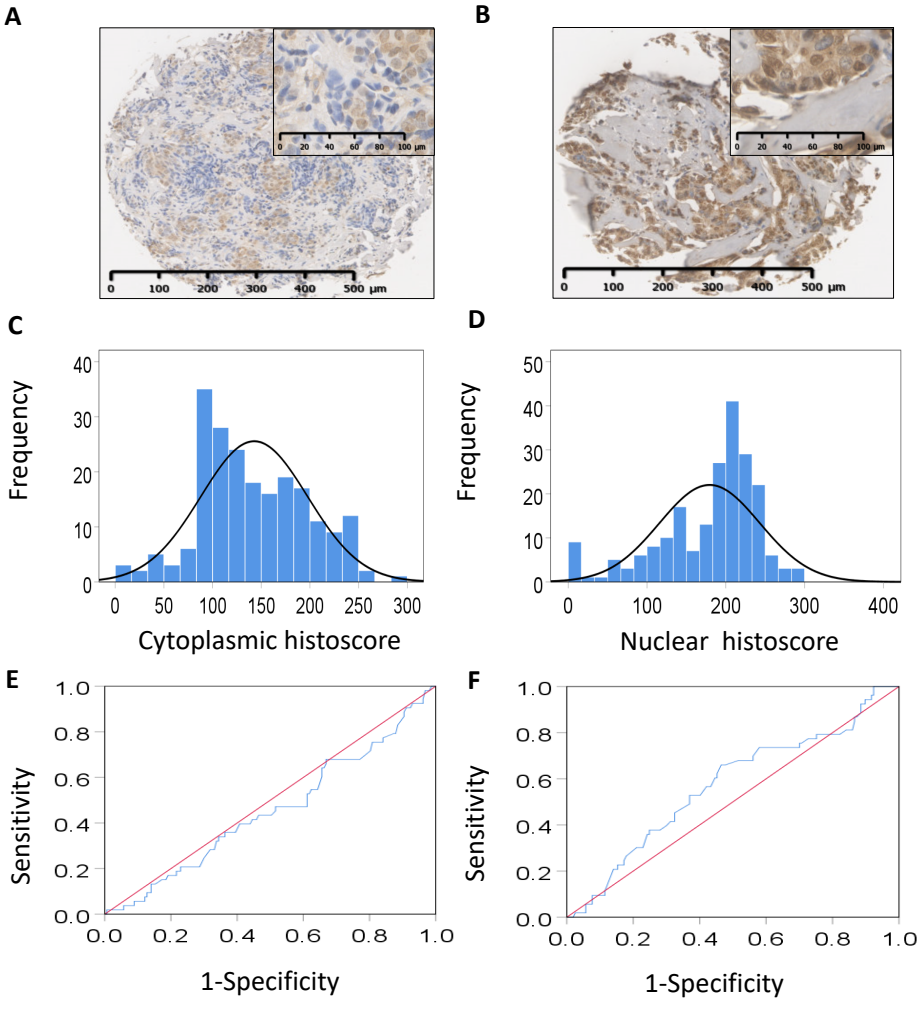


**Figure 4.17 Expression of pIKKα-Thr23.** *(A) Example of low expression and (B) of high expression of pIKKα-Thr23 (images taken at 400x magnification; scale bar represents 100 µm).*

*Histograms show distribution of histoscores for (C) cytoplasmic (D) and nuclear expression of pIKKα-Thr23. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of pIKKα-Thr23.*

The ROC curve was plotted to determine the cut-off point in order to classify low and high expression of cytoplasmic and nuclear pIKKα-Thr23 (Figure 4.17). Since the AUC was 0.455 (P=0.326) and 0.57 (P=0.122) for cytoplasmic and nuclear pIKKα-Thr23, the values from ROC curves were unable to be used to determine optimal cut-off points. Therefore, the median histoscore was used as an optimal cut-off point in order to subdivide the cohort into low and high expression. Tumours with cytoplasmic histoscore over 135 and tumour with nuclear histoscore over 200 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-off values, 102 patients had high cytoplasmic expression and 109 patients had low cytoplasmic expression. 104 patients had high nuclear expression and 107 patients had low nuclear expression.

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pIKKα-Thr23 were plotted as shown in Figure 4.18. The log rank test was used to compare low and high expression. High cytoplasmic expression of pIKKα-Thr23 was not associated with any of clinical outcome, including cancer specific survival (HR 0.80 95% CI 0.44-1.31, P=0.319, Figure 4.18), recurrence free survival (HR 0.80 95% CI 0.48-1.34, P=0.393, Figure 4.18) and time to recurrence on tamoxifen (HR 0.86 95% CI 0.49-1.50, P=0.589, Figure 4.18). Conversely, High nuclear expression of pIKKα-Thr23 was associated with shorter cancer specific survival (HR 1.89 95% CI 1.08-3.31, P=0.023, Figure 4.18.), shorter recurrence free survival (HR 11.73 95% CI 1.03-2.90, P=0.036, Figure 3.26) and shorter recurrence on adjuvant tamoxifen (HR 2.04 95% CI 1.13-3.68, P=0.015, Figure 4.18).

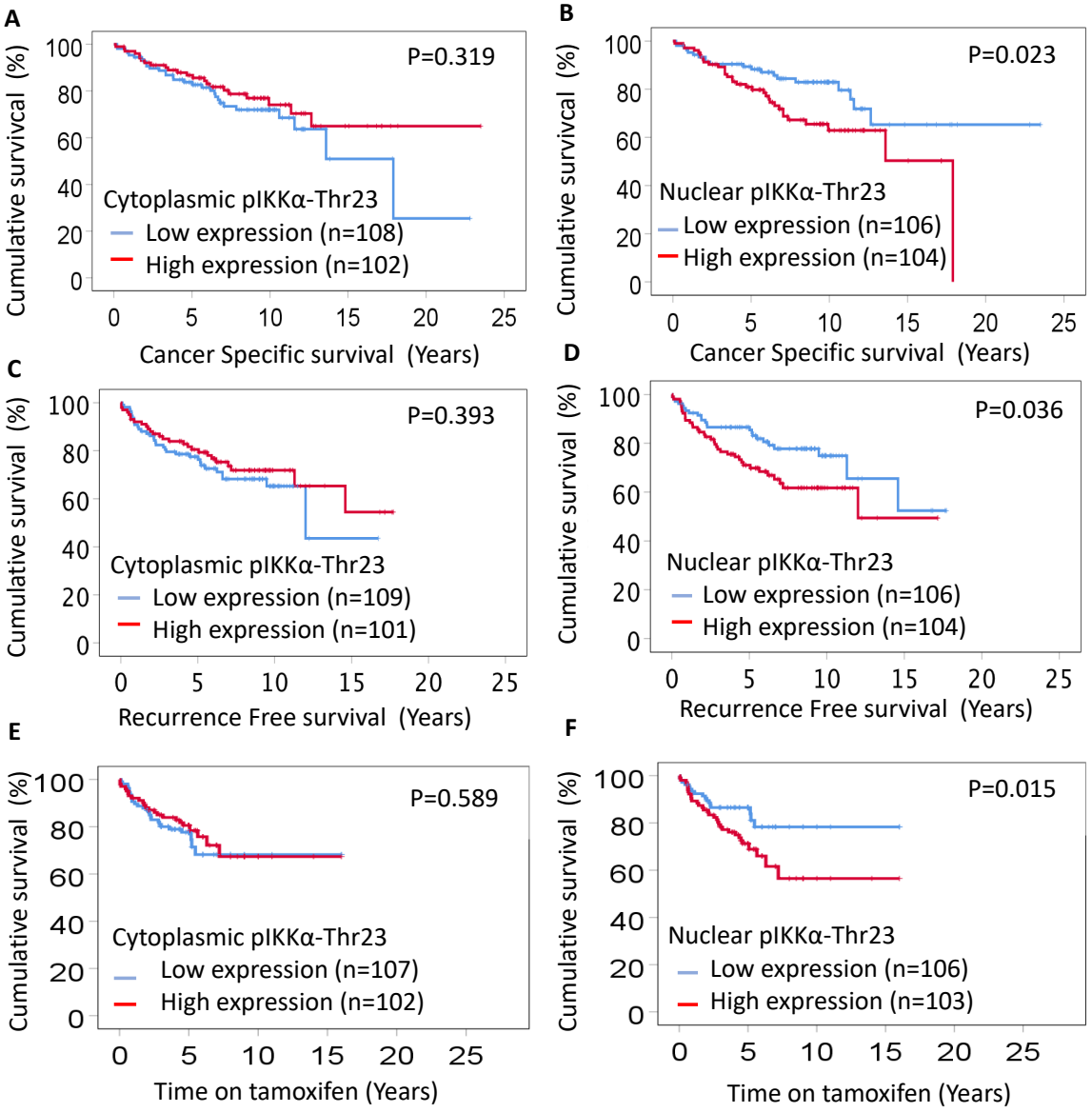


**Figure 4.18 Expression of pIKKα-Thr23 and clinical outcome in STB-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pIKKα-Thr23 with cancer specific survival (A and B), recurrence free survival (C and D), and recurrence free survival on tamoxifen (E and F).*

Multivariate analysis suggested that nuclear expression of pIKKα-Thr23 was an independent prognostic marker when combined with tumour size, tumour grade, lymph node status, NPI, PR status, Ki 67 and molecular subtypes (HR 2.01 95% CI 1.03-3.91, P=0.502 (Table 4.8).

| Clinicopathological | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>≤50>50 | 1.90 (0.99-3.70) | 0.055 | | |
| Histological type<br>Ductal/ Lobular/Other | 1.35 (0.89-2.05) | 0.159 | | |
| Size (mm)<br>≤20/ 21-50/ 21-50/>50 | 2.60 (1.88-3.61) | **<0.001** | 2.66 (1.43-4.98) | **0.002** |
| Grade<br>I/II/III | 1.87 (1.39-2.53) | **<0.001** | 1.36 (0.85-2.18) | 0.200 |
| Lymph node status<br>Negative/ Positive | 3.47 (2.15-5.60) | **<0.001** | 2.65 (1.22-5.76) | **0.014** |
| NPI<br>I (3.5)/ II (3.5-5.5)/III (>5.5) | 2.98 (2.16-4.12) | **<0.001** | 1.09 (0.44-2.70) | 0.850 |
| PR status<br>Negative/ Positive | 0.64 (0.44-0.97) | **0.033** | 0.83 (0.42-1.61) | 0.573 |
| Her-2 status<br>Negative/ Positive | 1.70 (0.82-3.51) | 0.147 | | |
| Proliferation (Ki67)<br>Low/ High | 2.05 (1.27-3.31) | **0.003** | 1.19 (0.542.62) | 0.665 |
| Molecular subtype<br>Luminal A/ Luminal B | 2.00 (1.26-3.17) | **0.003** | 1.18 (0.22-6.27) | 0.846 |
| Cytoplasmic pIKKα-Thr23<br>Low/High | 0.76 (0.44-1.31) | 0.321 | | |
| Nuclear pIKKα-Thr23<br>Low/High | 1.89 (1.08-3.31) | **0.025** | 2.01 (1.03-3.91) | **0.040** |

**Table 4.8 Univariate and multivariate analysis of pIKKα-Thr23 and clinicopathological characteristic in the STB cohort**

#### 4.2.4.2 Associations between pIKKα-Thr23 and clinicopathological characteristics of patients with ER-positive breast cancer

Chi-squared analysis was performed in order to determine whether expression of pIKKα-Thr23 was associated with any clinicopathological characteristic of the patients in the STB-cohort. Expression of cytoplasmic pIKKα-Thr23 was associated with tumour size (P=0.044). Whereas expression of nuclear pIKKα-Thr23 was associated with tumour grade (P=0.007) (Table 4.9).

| Clinicopathological characteristics | Cytoplasmic **p**IKKα-Thr23 | | | Nuclear **p**IKKα-Thr23 | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 19(17.4) | 24(23.8) | 0.256 | 23(21.5) | 20(19.4) | 0.709 |
| >50 years) | 90(82.6) | 77(76.2) | | 84(78.5) | 83(80.6) | |
| Histological type | | | | | | |
| Ductal | 97(89.8) | 85(85.9) | 0.678 | 91(85.8) | 91(90.1) | 0.611 |
| Lobular | 6(5.6) | 8(8.1) | | 8(7.5) | 6(5.9) | |
| Other | 5(4.6) | 6(6.1) | | 7(6.6) | 4(4) | |
| Size (mm) | | | | | | |
| ≤20 | 56(53.8) | 34(36.2) | **0.044** | 51(50.5) | 39(40.2) | 0.273 |
| 21-50 | 43(41.3) | 54(57.4) | | 46(45.5) | 51(52.6) | |
| >50 | 5(4.8) | 6(6.4) | | 4(4) | 7(7.2) | |
| Grade | | | | | | |
| I | 25(24.3) | 18(18.0) | 0.259 | 31(30.1) | 12(12) | **0.007** |
| II | 43(41.7) | 53(53.0) | | 44(42.7) | 52(52) | |
| III | 35(34.0) | 29(29.0) | | 28(27.2) | 36(36) | |
| Lymph node status | | | | | | |
| Negative | 47(45.6) | 42(51.2) | 0.450 | 49(52.1) | 40(44) | 0.266 |
| Positive | 56(54.4) | 40(48.8) | | 45(47.9) | 51(56) | |
| NPI | | | | | | |
| I | 29(31.2) | 18(23.4) | 0.292 | 29(33.3) | 18(21.7) | 0.094 |
| II | 42(45.2) | 44(57.1) | | 44(50.6) | 42(50.6) | |
| III | 22(23.7) | 15(19.5) | | 14(16.1) | 23(27.7) | |
| PR status | | | | | | |
| Negative | 39(37.5) | 47(46.5) | 0.190 | 41(40.2) | 45(43.7) | 0.612 |
| Positive | 65(62.5) | 54(53.5) | | 61(59.8) | 58(56.3) | |
| Her-2 status | | | | | | |
| Negative | 101(94.4) | 96(94.1) | 0.932 | 97(92.4) | 100(96.2) | 0.241 |
| Positive | 6(5.6) | 6(5.9) | | 8(7.6) | 4(3.8) | |
| Proliferation (Ki-67) | | | | | | |
| Low | 72(73.5) | 72(79.1 | 0.362 | 75(79.8) | 69(72.6) | 0.248 |
| High | 26(26.5) | 19(20.9) | | 19(20.2) | 26(27.4) | |
| Molecular subtype | | | | | | |
| Luminal A | 69(70.4) | 68(74.7) | 0.507 | 70(74.5) | 67(70.5) | 0.544 |
| Luminal B | 29(29.6) | 23(25.3) | | 24(25.5) | 287(29.5) | |

**Table 4.9 Association of pIKKα-Thr23 with clinic-pathological characteristics of the STB-cohort.** *Chi-squared analysis explored the relationship of pIKKα-Thr23 with various characteristics of the cohort*

#### 4.2.4.3 Expression of pIKKα- Thr23 and clinical outcome in different luminal subtypes

In order to determine whether expression of pIKKα-Thr23 was associated with clinical outcome in a particular ER-positive subtype, the cohort was subdivided into luminal A and luminal B disease. High cytoplasmic expression of pIKKα-Thr23 was not associated with clinical outcome in terms of cancer specific survival (luminal A, HR 0.71 95% CI 0.35-1.43), P=0.056; luminal B, HR 1.07 95% CI 0.42-2.72, P=0.736, Figure 4.19), recurrence free survival (luminal A, HR 0.65 95% CI 0.33-1.31, P=0.204; luminal B, HR 1.52 95% CI 0.63-3.66, P=0.347, Figure 4.20) and recurrence on tamoxifen (luminal A, HR 0.74 95% CI 0.34-1.60, P=0.420; luminal B, HR 1.46 95% CI 0.61-3.52, P=0.399, Figure 4.21) in either subtype.

In luminal A tumour, high nuclear expression of pIKKα-Thr23 was associated with decreased cancer specific survival (HR 2.60 95% CI 1.23-5.50, P=0.010, Figure 4.19), decreased recurrence free survival (HR 2.14 95% CI 1.06-4.32, P=0.026, Figure 3.28) and decreased recurrence on tamoxifen (HR 3.04 95% CI 1.28-7.23, P=0.007, Figure 4.21). Converstly, in luminal B tumour, high nuclear expresion of p-IKKα-Thr23 was not associated with cancer specific survival (HR 1.12 95% CI 0.44-2.84, P=0.825, Figure 4.19), recurrence free survival (HR 1.19 95% CI 0.49-2.91, P=0.702, Figure 3.28) and recurrence on tamoxifen (HR 1.20 95% CI 0.49-2.95, P=0.686, Figure 4.21).

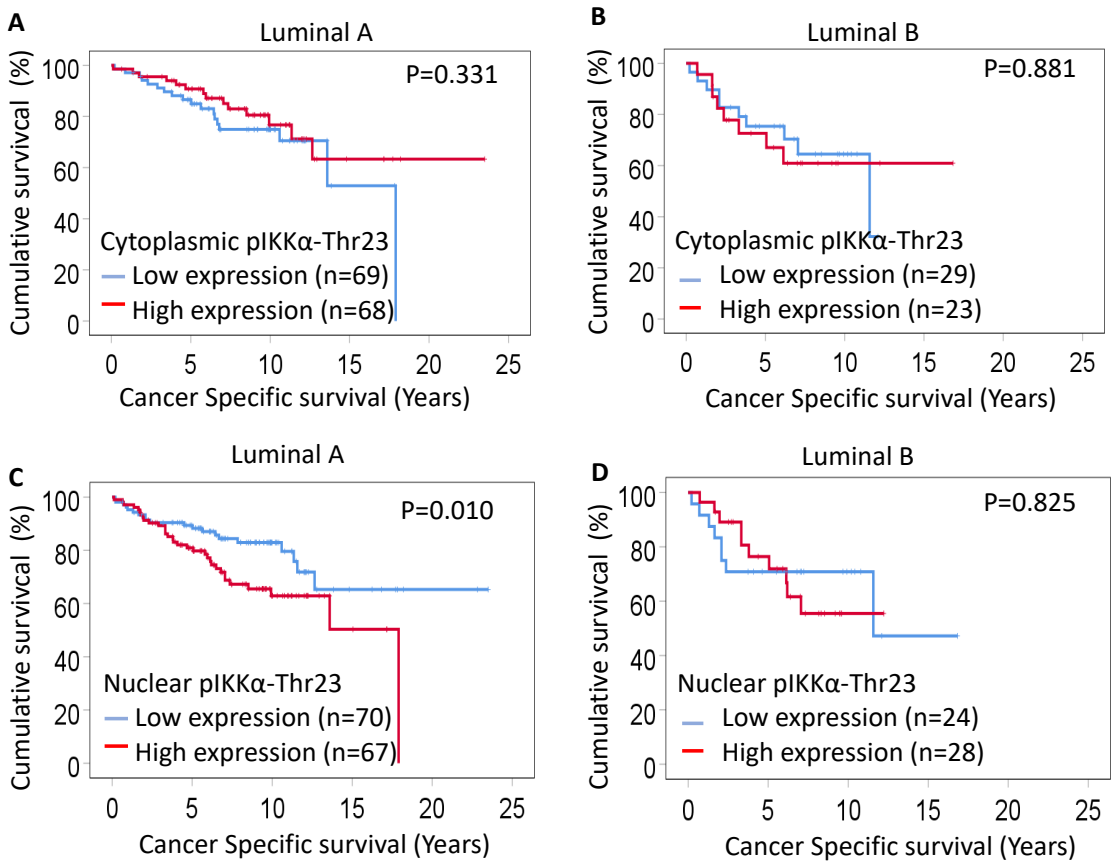


**Figure 4.19 Expression of pIKKα-Thr23 and cancer specific survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα -Thr23 and cancer specific survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pIKKα -Thr23 and cancer specific survival in luminal A tumours (C) or luminal B tumours (D).*

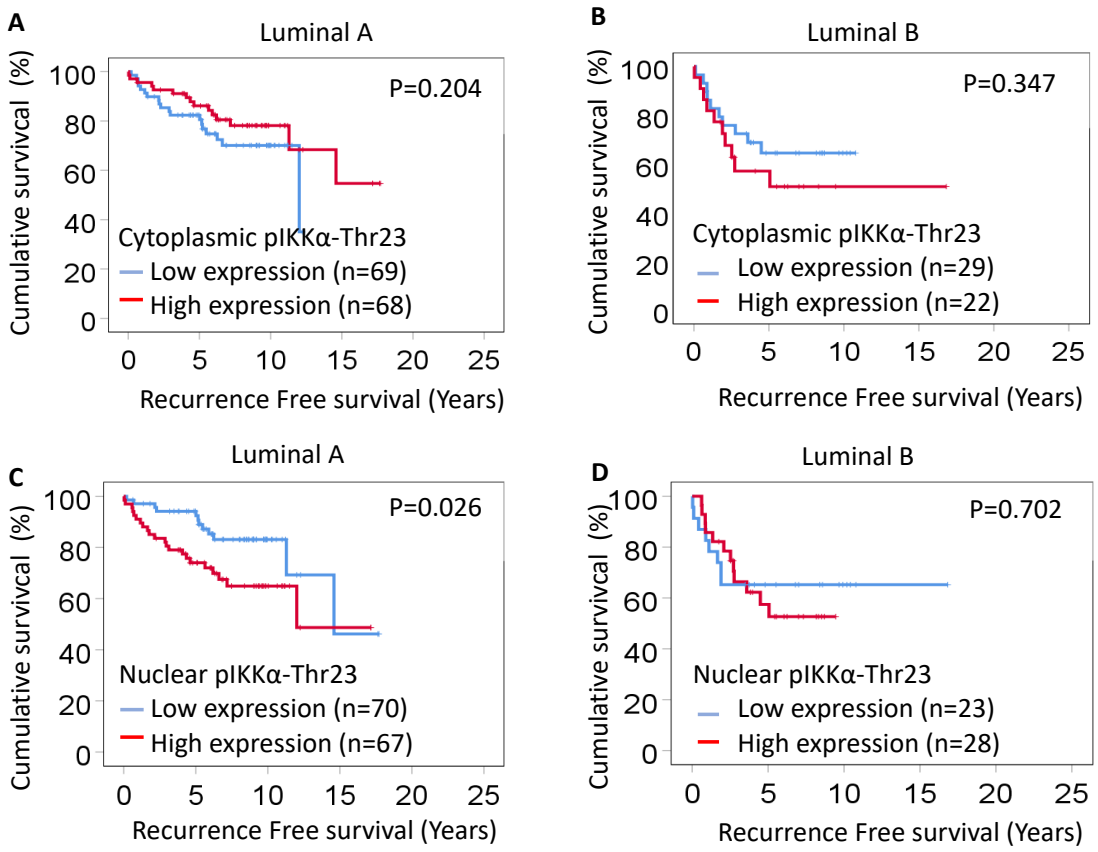


**Figure 4.20 Expression of pIKKα-Thr23 and recurrence free survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα -Thr23 and recurrence free survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pIKKα -Thr23 and recurrence free survival in luminal A tumours (C) or luminal B tumours (D).*

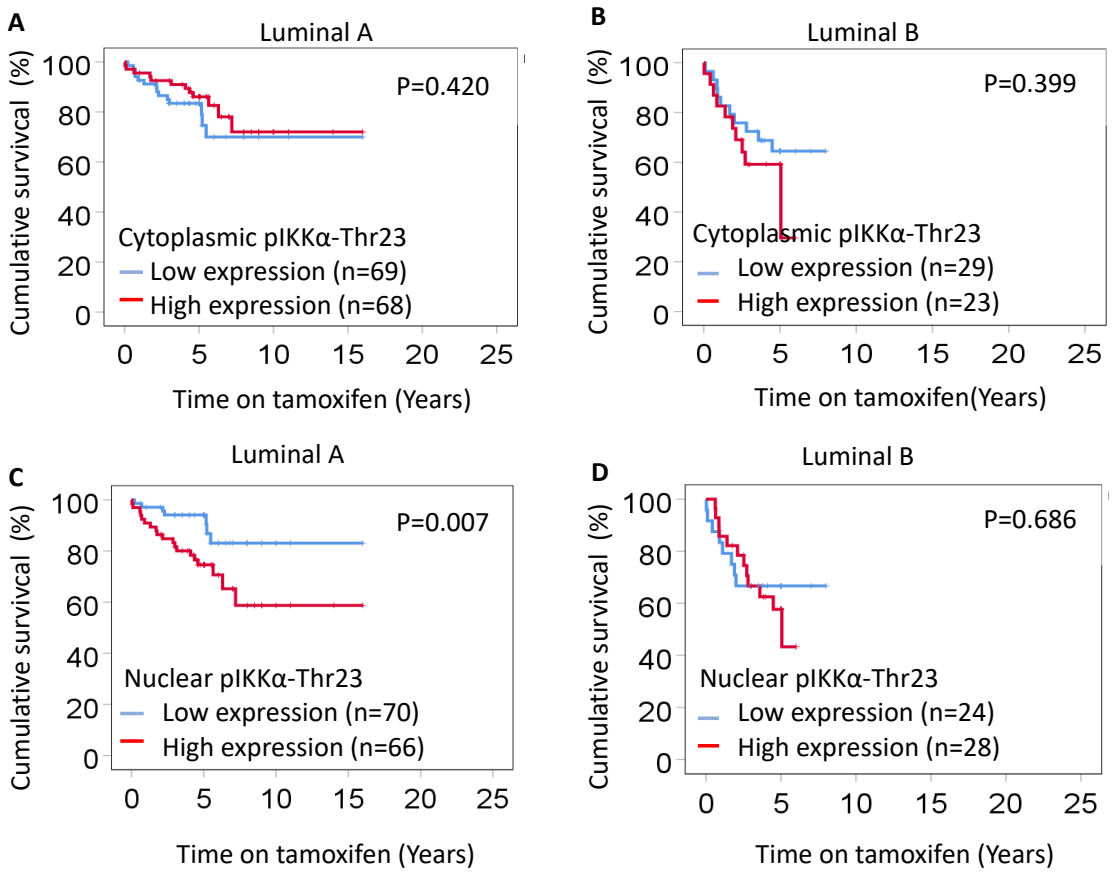


**Figure 4.21 Expression of pIKKα-Thr23 and recurrence on tamoxifen in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pIKKα -Thr23 and recurrence on tamoxifen in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pIKKα -Thr23 and recurrence on tamoxifen in luminal A tumours (C) or luminal B tumours (D).*

Multivariate analysis suggested that nuclear pIKKα-Thr23 was an independent prognostic marker when combined with tumour size, lymph node status and NPI (HR 2.77 95% CI 1.21-6.33, P=0.016) (Table 4.10).

| Clinicopathological characteristics | Luminal A | | | |
|---|---|---|---|---|
| | Univariate analysis | | Multivariate analysis | |
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>≤50/>50 | 2.28 (0.82-6.33) | 0.116 | | |
| Histological type<br>Ductal/ Lobular/Other | 1.42 (0.76-2.64) | 0.275 | | |
| Size (mm)<br>≤20/ 21-50/ 21-50/>50 | 2.38 (1.46-3.90) | **0.001** | 2.12 (0.89-5.08) | 0.091 |
| Grade<br>I/II/III | 1.50 (0.98-2.31) | 0.065 | | |
| Lymph node status<br>Negative/ Positive | 3.28 (1.71-6.26) | **<0.001** | 2.99 (1.23-7.28) | **0.016** |
| NPI<br>I (<3.5)/ II (3.5-5.5)/ III (>5.5) | 8.32 (2.87-24.11) | **<0.001** | 1.32 (0.56-3.15) | 0.525 |
| PR status<br>Negative/ Positive | 0.72 (0.41-1.28) | 0.269 | | |
| Cytoplasmic p- KKα Thr23<br>Low/High | 0.71 (0.35-1.43) | 0.333 | | |
| Nuclear p- KKα Thr23<br>Low/High | 2.60 (1.23-5.50) | **0.012** | 2.77 (1.21-6.33) | **0.016** |

**Table 4.10 Univariate and multivariate analysis of pIKKα-Thr23 and clinicopathological characteristic in luminal A tumour.**

### 4.2.5 Expression of pAKT in a cohort of patients with ER-positive breast cancer

As pAKT has been demonstrated as one of the upstream regulators that influence IKKα activation, expression of pAKT was also assessed.

#### 4.2.5.1 Association of pAKT with clinical outcome in a cohort of patients with ER-positive breast cancer

Expression of pAKT, a kinase upstream of IKKα, was already available in the STB cohort from a previous study by Dr Tove Kirkegaard (211). The study demonstrated that patients with ER-positive breast cancer, whose tumour had high activation of AKT at serine 473 associated with decrease overall survival (P=0.041) (211). The authors also showed that phospho-AKT (pAKT) was linked to activation of both HER2 and ER, indicating its role in enhancing endocrine resistance (211). Correspondingly, in the present study, we hypothesised that IKKα might associate with AKT linking NF-κB and PI3K/AKT/mTOR pathway to enhance endocrine resistance in ER positive breast cancer. Therefore, the association between phospho-AKT at serine 473, IKKα and clinical outcomes was investigated in the full cohort. In addition, when the staining for pAKT was done, the molecular subtypes were not available for this cohort. Therefore, this data was firstly used in the present study to investigate prognostic role of pAKT in different breast cancer subtypes.

The expression of pAKT was analysed in 382 (94.6%) patients (N=392). The histoscore for cytoplasmic expression of pAKT ranged from 0 to 200 with a median histoscore of 75 (IQR=40-110) and the histoscore for nuclear expression ranged from 0-180 with a median histoscore of 28 (IQR=13-54). An example of immunostaining for low and high expression of pAKT was shown in Figure 4.22. Distribution of histoscores for cytoplasmic and nuclear expression of pAKT are also shown in Figure 4.22. There was good correlation between observers with an ICCC score of 0.83 for cytoplasmic and 0.80 for nuclear expression.

The receiver operating characteristic (ROC) curve was then plotted to determine the cut-off point in order to classify low and high expression of cytoplasmic pAKT and nuclear p-AKT (Figure 4.22). Since the AUC was 0.51 (P=0.797) and 0.487 (P=0.701), the values from the ROC curves were unable to be used to determine the optimal cut-off points. The cohort was therefore subdivided into low and high expression by using the median histoscore as a cut-

off point. Tumours with cytoplasmic histoscore over 75 and tumour with nuclear histoscore over 30 were classified as high cytoplasmic and high nuclear expression, respectively. Using these cut-off values, 183 patients had high cytoplasmic expression and 188 patients had low cytoplasmic expression. 169 patients had high nuclear expression and 202 patients had low nuclear expression.

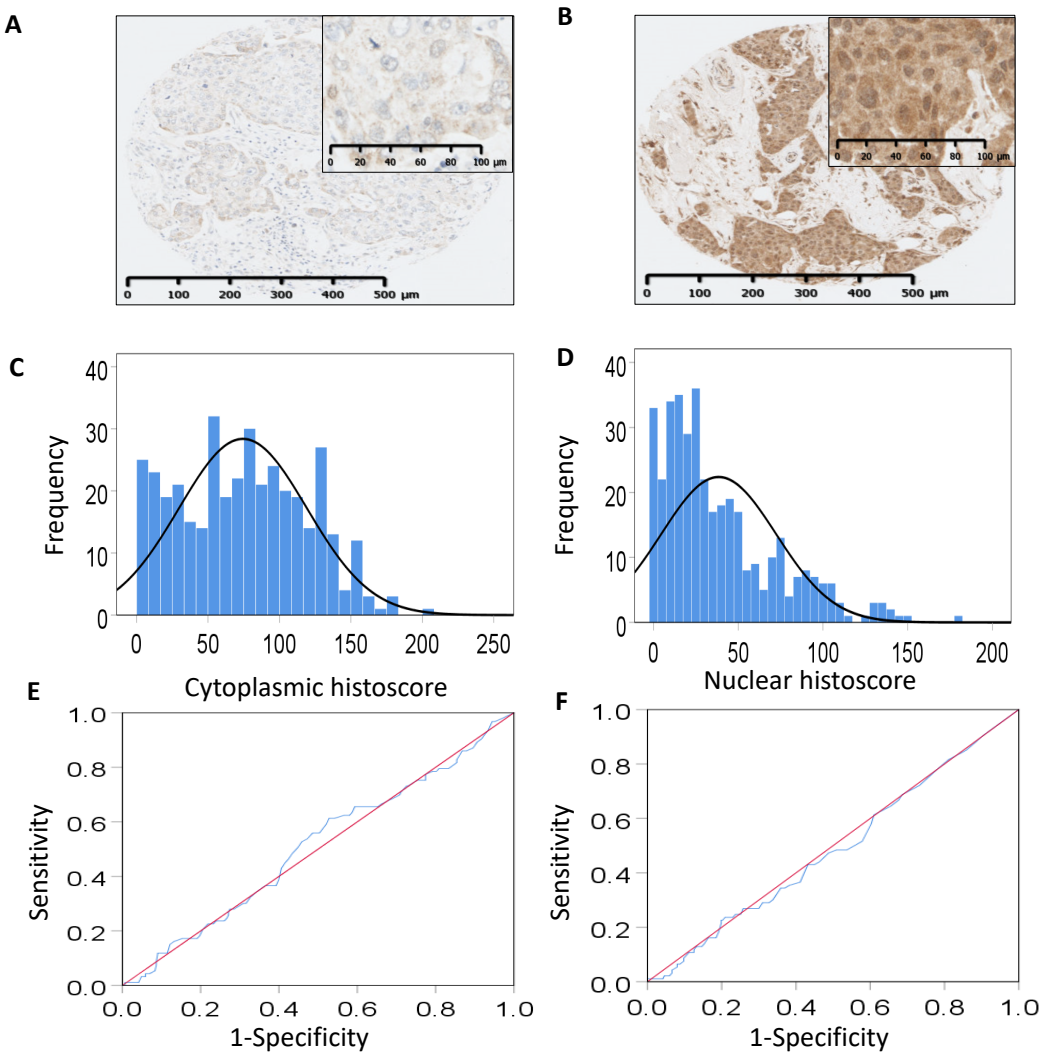


**Figure 4.22 Expression of pAKT.** *(A) Example of low expression and (B) of high expression of pAKT (images taken at 400x magnification; scale bar represents 100 µm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of pAKT. Receiver Operating Characteristics (ROC) Curve represent area under the curve for cytoplasmic (E) and nuclear (F) expression of pAKT.*

In order to determine whether pAKT expression was associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pAKT were plotted (Figure 4.22). Low and high expression were compared using the log rank test. Cytoplasmic

expression of pAKT was not associated with any clinical outcomes, in terms of cancer specific survival (HR 1.290 95% CI 0.85-1.96, P=0.234), recurrence free survival (HR 1.37 95% CI 0.91-2.08, P=0.134) and recurrence on tamoxifen (HR 1.38 95% CI 0.86-2.32, P=0.183). Similarly, nuclear expression of pAKT was not associated with clinical outcomes, including cancer specific survival (HR 0.89 95% CI 0.58-1.37, P=0.601), recurrence free survival (HR 1.08 95% CI 0.71-1.64, P=0.709) and recurrence on tamoxifen (HR 1.02 95% CI 0.63-1.65, P=0.992).

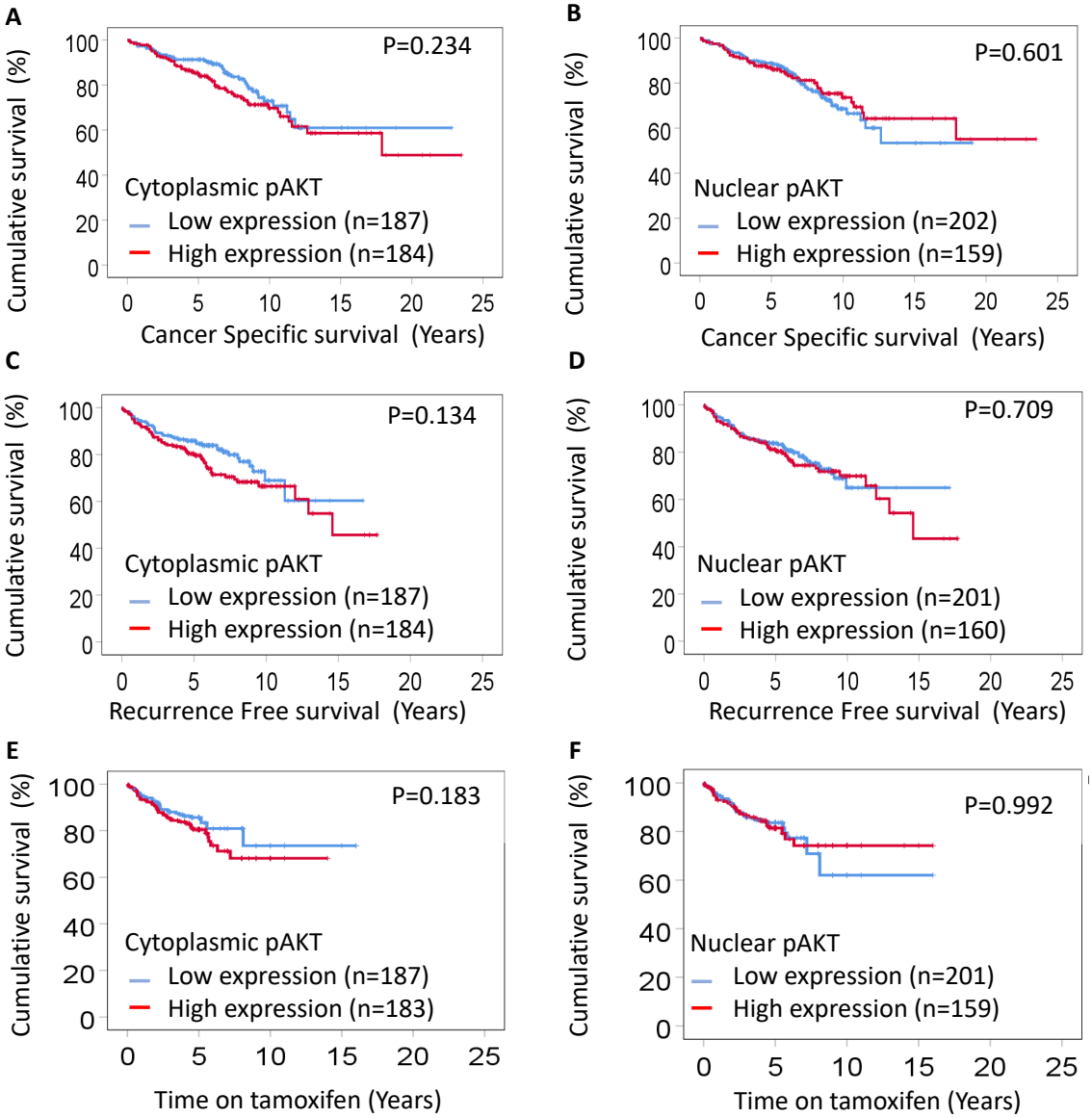


**Figure 4.23 Expression of pAKT and clinical outcome in STB-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pAKT with cancer specific survival (A and B), recurrence free survival (C and D), and recurrence free survival on tamoxifen (E and F).*

#### 4.2.5.2 Associations between pAKT and clinicopathological characteristics of patients with ER-positive breast cancer

Chi-squared analysis was performed to determine whether expression of pAKT was associated with any clinicopathological characteristic of ER-positive patients within in the STB-cohort as shown in Table 4.11. Expression of cytoplasmic pAKT was associated with tumour grade (P=0.013), lymph node status (P=0.025) and NPI (P<0.001). Whereas expression of nuclear pAKT was associated with histological type (P=0.003) and HER2 status (P=0.017).

| Clinicopathological characteristics | Cytoplasmic pAKT | | | Nuclear pAKT | | |
|---|---|---|---|---|---|---|
| | Low (%) | High (%) | P | Low (%) | High (%) | P |
| Age | | | | | | |
| ≤50 | 29 (15.4) | 38(20.8) | 0.181 | 31(15.3) | 34(21.4) | 0.138 |
| >50 years) | 159(84.6) | 145(79.2) | | 171(84.7) | 125(78.6) | |
| Histological type | | | | | | |
| Ductal | 147 (79.5) | 152(84.0) | 0.147 | 171(86.4) | 119(75.3) | **0.003** |
| Lobular | 27(14.6) | 15(8.3) | | 21(10.6) | 20(12.7) | |
| Other | 11(5.9) | 14(7.7) | | 6(3.0) | 19(12.0) | |
| Size (mm) | | | | | | |
| ≤20 | 102 (57.0) | 78(45.1) | 0.063 | 94(48.2) | 81(54.4) | 0.519 |
| 21-50 | 70(39.1) | 83(48.0) | | 90(40.6) | 60(40.3) | |
| >50 | 7(3.9) | 12(6.9) | | 11(5.6) | 8(5.4) | |
| Grade | | | | | | |
| I | 58(31.4) | 35(19.7) | **0.013** | 51(25.6) | 40(25.8) | 0.745 |
| II | 90(48.6) | 89(50.0) | | 95(45.6) | 79(51.0) | |
| III | 37(20.0) | 54(30.0) | | 53(26.6) | 36(23.2) | |
| Lymph node status | | | | | | |
| Negative | 104(58.8) | 77(46.7) | **0.025** | 100(53.2) | 75(52.1) | 0.841 |
| Positive | 73(41.2) | 88(53.0) | | 88(46.8) | 69(47.9) | |
| NPI | | | | | | |
| I | 77(46.4) | 45(29.0) | **<0.001** | 65(35.9) | 55(41.1) | 0.616 |
| II | 72(43.4) | 73(47.1) | | 84(46.4) | 56(42.1) | |
| III | 17(10.2) | 37(23.9) | | 32(17.7) | 22(16.5) | |
| PR status | | | | | | |
| Negative | 63(34.8) | 69(38.3) | 0.487 | 72(36.7) | 59(38.1) | 0.798 |
| Positive | 118(65.2) | 111(61.7) | | 124(63.3) | 96(61.9) | |
| Her-2 status | | | | | | |
| Negative | 171(91.4) | 175(95.1) | 0.159 | 182(90.5) | 155(96.9) | **0.017** |
| Positive | 16(8.6) | 9(4.9) | | 19(9.5) | 5(3.1) | |
| Proliferation (Ki67) | | | | | | |
| Low | 111(77.6) | 117(76.0) | 0.737 | 128(77.1) | 91(25.2) | 0.708 |
| High | 32(22.4) | 37(24.0) | | 38(22.9) | 30(24.8) | |
| Molecular subtype | | | | | | |
| Luminal A | 101(70.1) | 114(73.1) | 0.573 | 119(70.4) | 88(72.7) | 0.667 |
| Luminal B | 43(29.9) | 42(26.9) | | 50(29.6) | 50(29.6) | |

**Table 4.11 Association of pAKT with clinic-pathological characteristics of the STB-cohort.**
*Chi-squared analysis exploring associations between pAKT with clinicopathological characteristics of the cohort.*

#### 4.2.5.3 Expression of pAKT and clinical outcome in different luminal subtypes

In order to determine whether expression of pAKT was associated with clinical outcome in a particular ER-positive subtype, the cohort was subdivided into luminal A and luminal B tumours. In luminal A tumour, expression of pAKT was not associated with cancer specific survival (cytoplasmic, HR 1.17 95% CI 0.65-2.12, P=0.601; nuclear, HR 1.10 95% CI 0.61-1.99, P=0.741, Figure 4.24), recurrence free survival (cytoplasmic, HR 1.61 95% CI 0.87-2.98, P=0.148; nuclear, HR 1.65 95% CI 0.91-2.99, P=0.114, Figure 4.25) and recurrence on tamoxifen (cytoplasmic, HR 1.59 95% CI 0.77-3.30, P=0.225; nuclear, HR 1.24 95% CI 0.62-2.50, P=0.571, Figure 4.26).

In luminal B tumour, cytoplasmic expression of pAKT was associated with decreased cancer specific survival (HR 2.27 95% CI 1.02-5.08, P=0.040, Figure 4.24) but was not associated with recurrence free survival (HR 1.65  95% CI 0.79-3.43, P=0.176, Figure 4.25) or recurrence on tamoxifen (HR 1.87 95% CI 0.88-3.96, p=0.097, Figure 4.26). Nuclear expression of pAKT was not associated with any clinical outcomes, including cancer specific survival (HR 1.16 95% CI 0.54-2.51, P=0.699, Figure 4.24; recurrence free survival (HR 1.03 95% CI 0.50-2.15, P=0.930, Figure 4.25) and recurrence on tamoxifen (HR 1.1195% CI 0.53-2.33, P=0.781, Figure 4.26).

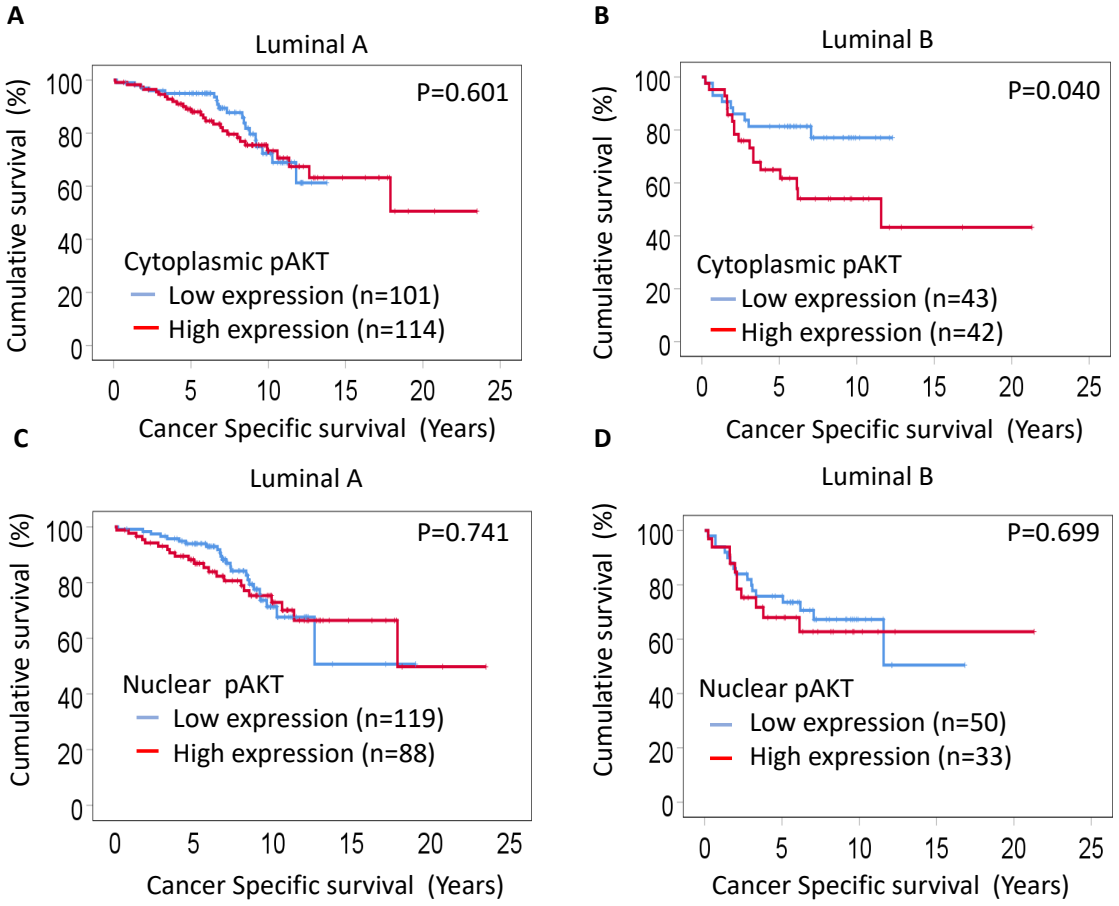


**Figure 4.24 Expression of pAKT and cancer specific survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pAKT and cancer specific survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pAKT and cancer specific survival in luminal A tumours (C) or luminal B tumours (D).*

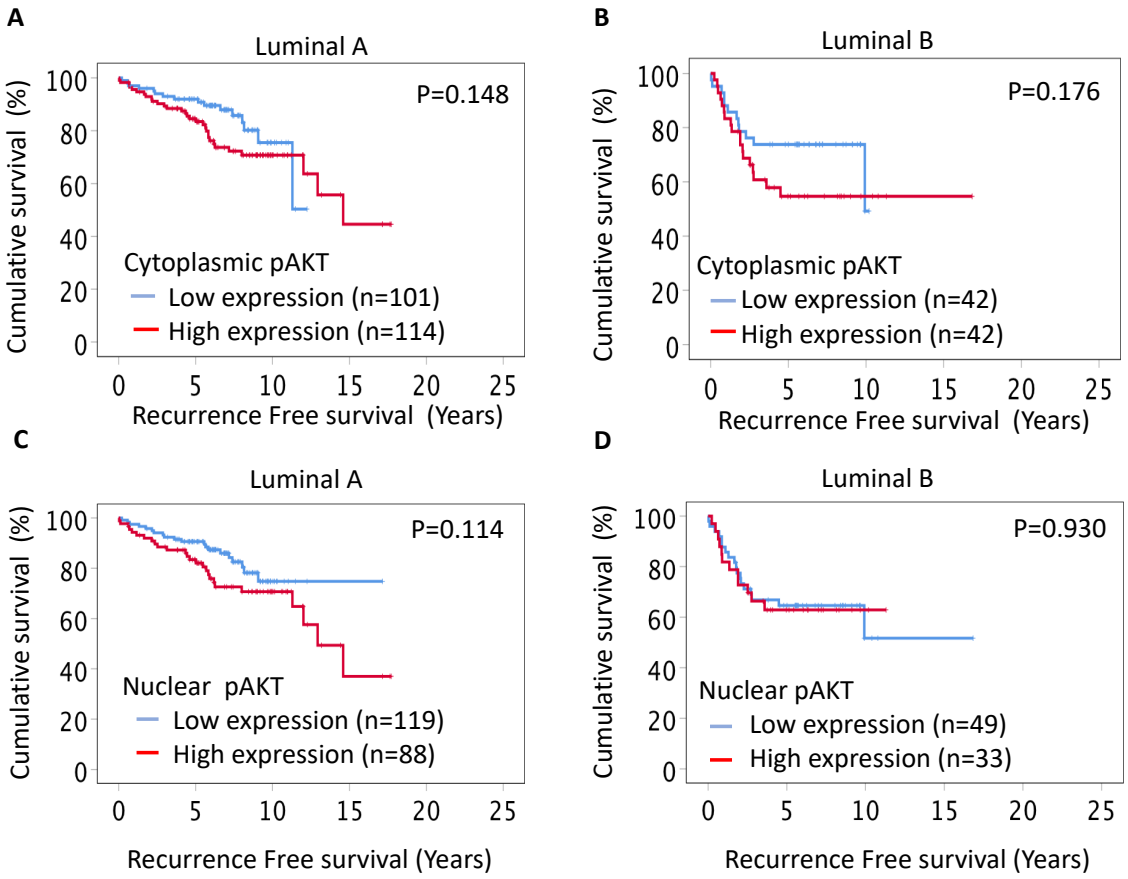


**Figure 4.25 Expression of pAKT and recurrence free survival in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pAKT and recurrence survival in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pAKT and recurrence survival in luminal A tumours (C) or luminal B tumours (D).*

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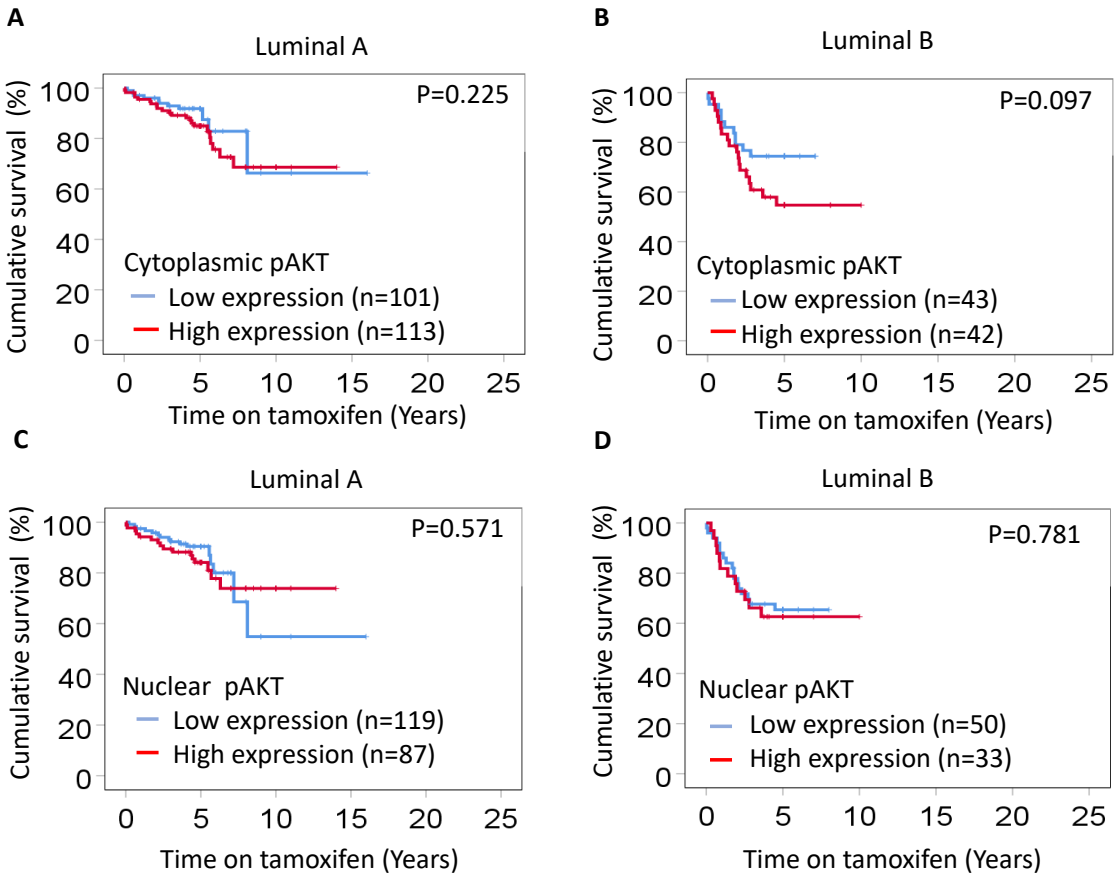


**Figure 4.26 Expression of pAKT and recurrence on tamoxifen in different ER-positive breast cancer subtypes.** *Kaplan Meier curves showing associations between cytoplasmic pAKT and recurrence on tamoxifen in luminal A tumours (A) or luminal B tumours (B). Kaplan Meier curves showing associations between nuclear expression of pAKT and recurrence on tamoxifen in luminal A tumours (C) or luminal B tumours (D)*

Multivariate analysis suggested that cytoplasmic pAKT was not an independent prognostic marker when combined with tumour size, lymph node status and NPI (HR 1.16 95% CI 0.45-3.00), P=0.758) (Table 4.12).

| Clinicopathological characteristics | Luminal B | | | |
|---|---|---|---|---|
| | Univariate analysis | | Multivariate analysis | |
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>≤50/>50 | 2.45 (0.74-8.08) | 0.143 | | |
| Histological type<br>Ductal/Lobular/Other | 1.23 (0.51-2.95) | 0.648 | | |
| Size (mm)<br>≤20/ 21-50/ 21-50/>50 | 2.65 (1.45-4.85) | **0.002** | 2.67 (1.23-5.53) | **0.012** |
| Grade<br>I/II/III | 1.27 (0.68-2.40) | 0.455 | | |
| Lymph node status<br>Negative/ Positive | 3.97 (1.35-11.69) | **0.012** | 2.91 (0.97-8.74) | 0.057 |
| NPI<br>I (<3.5)/ II (3.5-5.5)/ III (>5.5) | 2.47 (0.72-8.51) | 0.153 | | |
| PR status<br>Negative/ Positive | 0.71 (0.43-1.48) | 0.364 | | |
| Her-2 status<br>Negative/ Positive | 1.09 (0.48-2.46) | 0.838 | | |
| Proliferation (Ki-67)<br>Low/ High | 1.36 (0.41-4.50) | 0.620 | | |
| Cytoplasmic pAKT<br>Low/High | 2.27 (1.02-5.08) | **0.046** | 1.16 (0.45-3.00) | 0.758 |
| Nuclear pAKT<br>Low/High | 1.16 (0.54-2.51) | 0.700 | | |

**Table 4.12 Univariate and multivariate analysis of pAKT and clinicopathological characteristic in luminal A tumour.**

### 4.2.6 Correlation between expression of NIK, IKKα, pIKKα-S176 and pIKKα-Thr23

Correlation between NIK, IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (199). There was no correlation between expression of cytoplasmic NIK and expression of IKKα (Cytoplasmic Spearman's rho=-0.084, P=0.156; nuclear IKKα; Spearman's rho=-0.094, P=0.039). There was a statistically significant, moderate negative correlation between cytoplasmic NIK and cytoplasmic pIKKα-Ser176 (Spearman's rho=0.403, P<0.001) and a statistically significant,

weak negative correlation between cytoplasmic NIK and nuclear pIKKα-Ser176 (Spearman's rho=-0.347, P<0.001). There was a statistically significant, weak negative correlation between expression of cytoplasmic NIK and expression of cytoplasmic pIKKα-Thr23 (Spearman's rho-=0.192, p=0.0041) and a statistically significant, weak positive correlation between expression of cytoplasmic NIK and expression of nuclear pIKKα-Thr23 (Spearman's rho-=0.136, p=0.042).

#### 4.2.6.1 Cytoplasmic NIK, IKKα and phospho-IKKα activity (pIKKα-Ser176, pIKKα-Thr23) as a combined biological marker

Although cytoplasmic NIK alone did not show significant association with poor clinical outcomes, as an upstream kinase for IKKα activation in non-canonical NF-κB pathway, we hypothesised that a combined high expression of both cytoplasmic NIK and IKKα may associated with poor clinical outcomes, and we classified this group of patients as a 'high risk'. A combination of at least one high was classified as 'intermediate risk' and a combination of both low was classified as a 'low risk' group. We also hypothesised that a combined high expression of cytoplasmic NIK and low cytoplasmic pIKKα-Ser176 may associated with poor clinical outcomes and the patients who had high cytoplasmic NIK and low cytoplasmic pIKKα was classified as a 'high risk' group. The patients who had one high cytoplasmic NIK or one low cytoplasmic pIKKα-Ser176 was classified as 'intermediate risk' group and the patients who had low cytoplasmic NIK and high cytoplasmic pIKKα-Ser176 was classified as a 'low risk' group. In addition, patients who had both high cytoplasmic NIK and nuclear pIKKα-Thr23 were classified as a 'high risk' group. Patients who had at least one high were classified as an 'intermediate risk' group. The patients who had both low were classified as a 'low risk' group.

A combined expression of cytoplasmic NIK and IKKα was not associated with decreased cancer specific survival (HR 1.30 95% CI 0.93-1.83, P=0.297), recurrence free survival (HR 1.31 95% CI 0.94-1.83, P=0.257) and recurrence on tamoxifen (HR 1.30 95% CI 0.90-1.89, P=0.380), as shown in Figure 4.27.

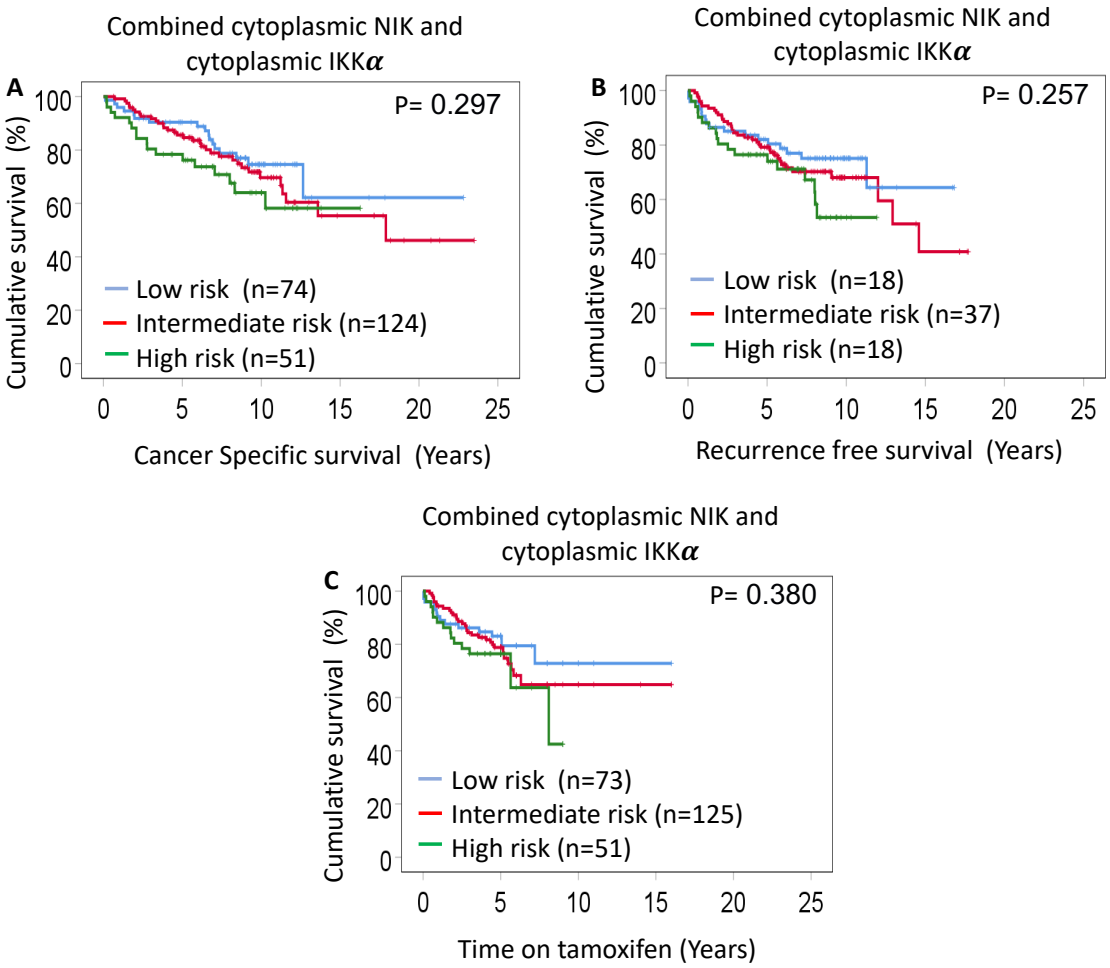


**Figure 4.27 Combined expression of cytoplasmic NIK and IKKα.** *Kaplan Meier curves showing associations between combined cytoplasmic NIK and IKKα and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

Similarly, a combined expression of cytoplasmic NIK and cytoplasmic pIKKα-Ser176 was not associated with decreased cancer specific survival (HR 1.41 95% CI 0.97-2.07, P=0.135), recurrence free survival (HR 1.36 95% CI 0.94-1.95, P=0.092) and recurrence on tamoxifen (HR 1.43 95% CI 0.93-2.20, P=0.113, as shown in Figure 4.28.

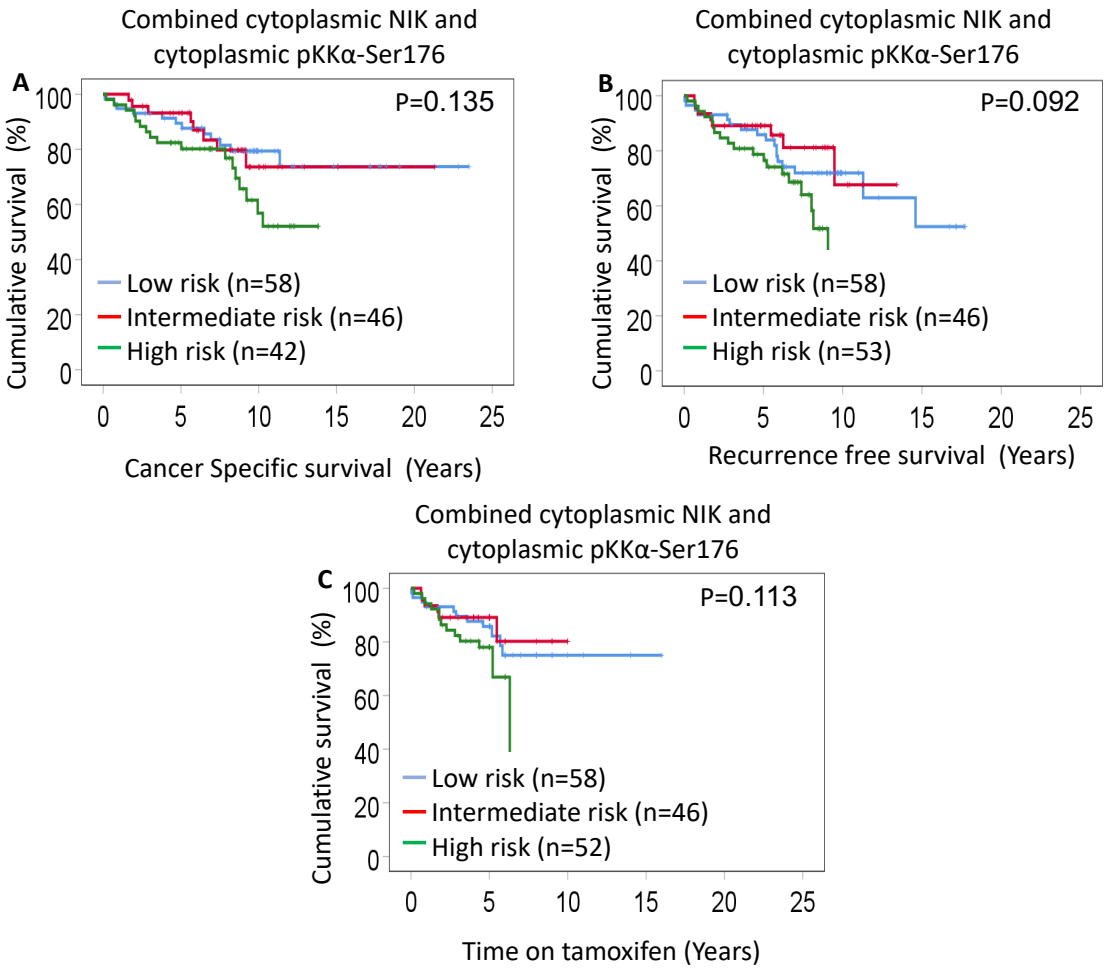


**Figure 4.28 Combined expression of cytoplasmic NIK and pIKKα-Ser176.** *Kaplan Meier curves showing associations between combined cytoplasmic NIK and pIKKα-Ser176 and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

No significant association was found between a combined expression of cytoplasmic NIK and nuclear pIKKα-Thr23, and clinical outcomes, in terms of cancer specific survival (HR 1 1.31 95% CI 0.90-1.90, P=0.135), recurrence free survival (HR 1.36 95% CI 0.94-1.95, P=0.092) and recurrence on tamoxifen (HR 1.43 95% CI 0.93-2.20, P=0.113, as shown in Figure 4.29.

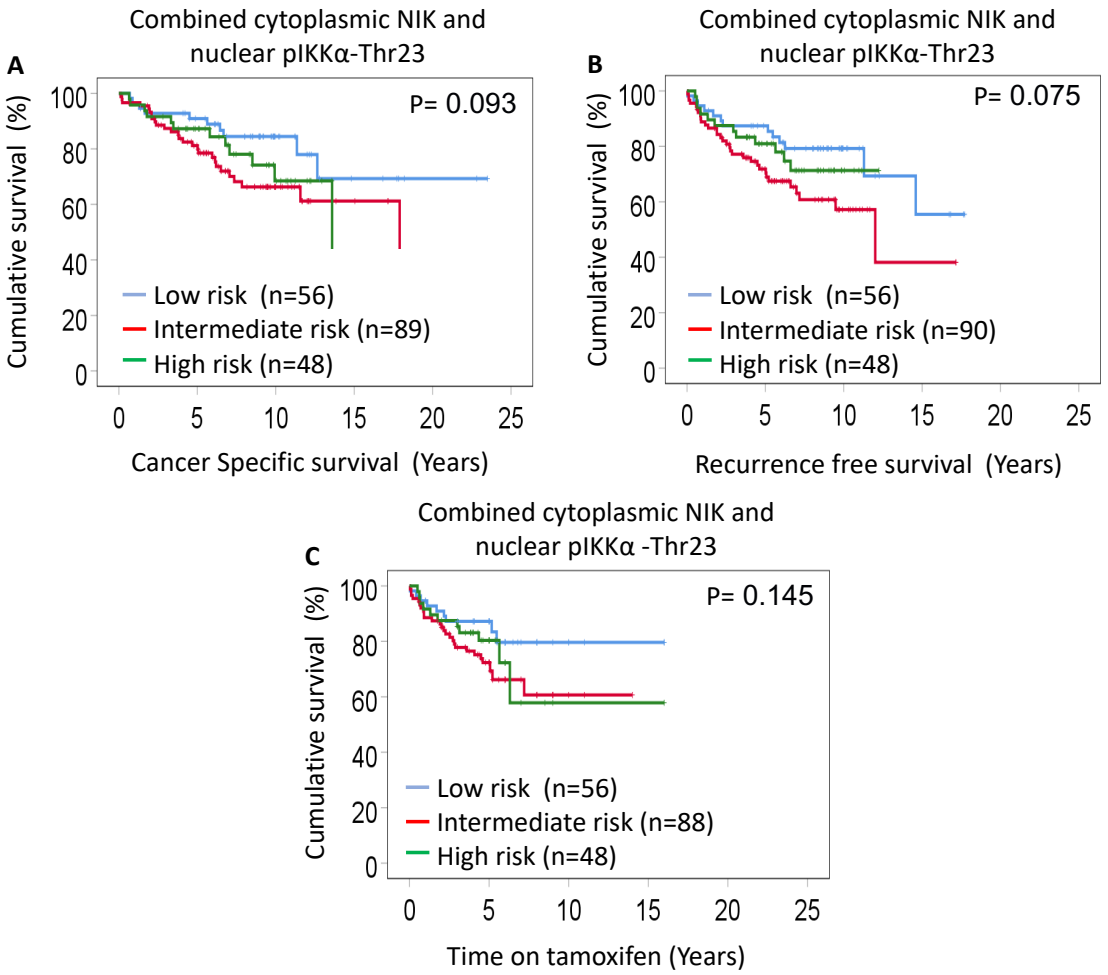


**Figure 4.29 Combined expression of cytoplasmic NIK and pIKKα-Thr23.** *Kaplan Meier curves showing associations between combined cytoplasmic NIK and pIKKα-Thr23 and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

### 4.2.7 Correlation between expression of pAKT, IKKα, pIKKα-Ser176 and pIKKα-Thr23

Correlation between pAKT, IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (199). There was a statistically significant, weak positive correlation between expression of cytoplasmic pAKT and expression of cytoplasmic IKKα (Spearman's rho=-0.175, P=0.0.001) but no correlation between cytoplasmic pAKT and nuclear IKKα (Spearman's rho=-0.094, P=0.088). There was a statistically significant, weak positive correlation between cytoplasmic pAKT and expression of pIKKα-Ser176 (Cytoplasmic, Spearman's rho=0.299, P<0.001); nuclear, Spearman's rho=0.398, P<0.001). There was a statistically significant, weak positive correlation between expression of cytoplasmic pAKT and expression of cytoplasmic pIKKα-

Thr23 (Spearman's rho=0.195, p=0.003) but no correlation between cytoplasmic pAKT and expression of nuclear pIKKα-Thr23 (Spearman's rho=-0.002, P=0.977).

#### 4.2.7.1 Cytoplasmic pAKT, IKKα and phospho-IKKα activity (pIKKα-Ser176, pIKKα-Thr23) as a combined biological marker of clinical outcomes

Since the significant correlation was observed between pAKT and IKKα, pIKKα-Ser176 and pIKKα-Thr23, we hypothesised that a combined high expression of both cytoplasmic pAKT and IKKα may be associated with poor clinical outcomes, and we classified this group of patients as a 'high risk'. A combination of at least one high was classified as 'intermediate risk' and a combination of both low was classified as a 'low risk' group. We also hypothesised that a combined high expression of cytoplasmic pAKT and low cytoplasmic pIKKα-Ser176 may associated with poor clinical outcomes. The patients who had one high cytoplasmic pAKT or one low cytoplasmic pIKKα-Ser176 were classified as 'intermediate risk' group and the patients who had low cytoplasmic pAKT and high cytoplasmic pIKKα-Ser176 were classified as a 'low risk' group. In addition, patients who had both high cytoplasmic pAKT and nuclear pIKKα-Thr23 were classified as a 'high risk' group. Patients who had at least one high were classified as an 'intermediate risk' group. The patients who had both low were classified as a 'low risk' group.

A combined expression of cytoplasmic pAKT and IKKα was associated with decreased cancer specific survival (HR 1.53 95% CI 1.13-2.08, P=0.022), recurrence free survival (HR 1.63 95% CI 1.21-2.12, P=0.006) and recurrence on tamoxifen (HR 1.66 95% CI 1.18-2.33, P=0.013), as shown in Figure 4.30.

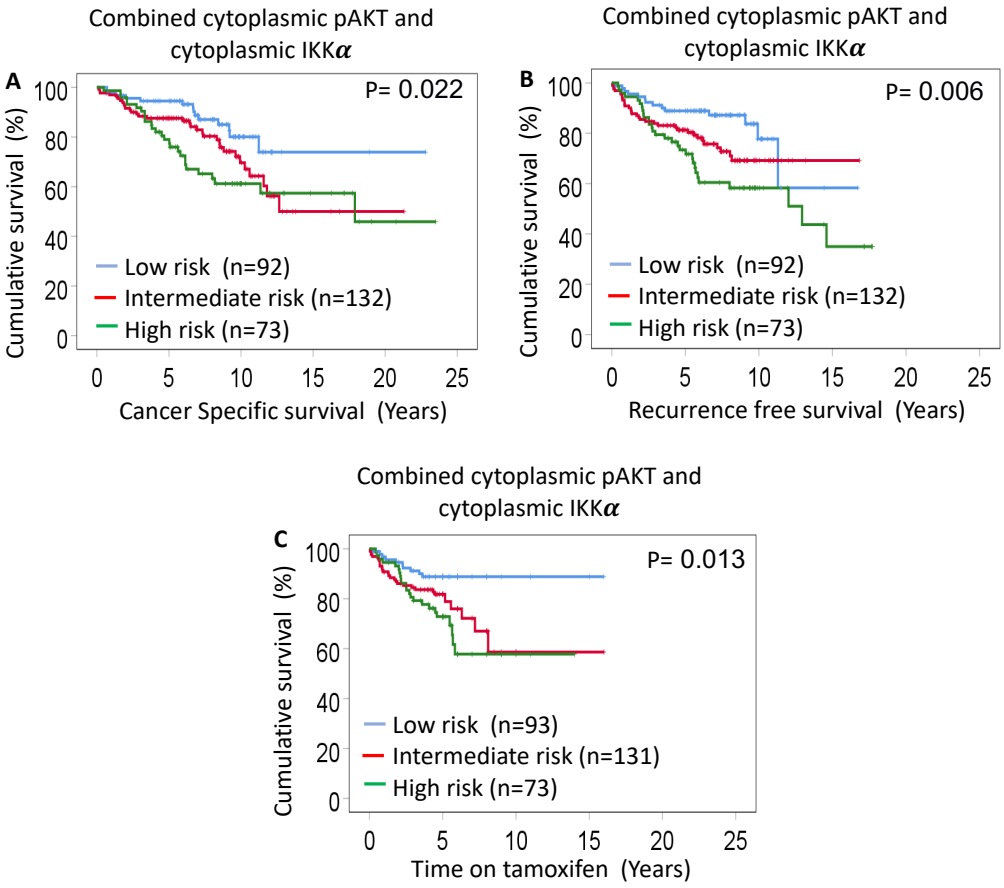


**Figure 4.30 Combined expression of cytoplasmic pAKT and IKKα.** *Kaplan Meier curves showing associations between combined cytoplasmic pAKT and IKKα and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

A combined expression of cytoplasmic pAKT and pIKKα-Ser176 was associated with decreased cancer specific survival (HR 2.29 95% CI 1.33-3.94, P=0.011). However, the correlation was not observed with recurrence free survival (HR 1.86 95% CI 1.11-3.11, P=0.054) and recurrence on tamoxifen (HR 2.01 95% CI 1.08-3.73, P=0.072), as shown in Figure 4.31.

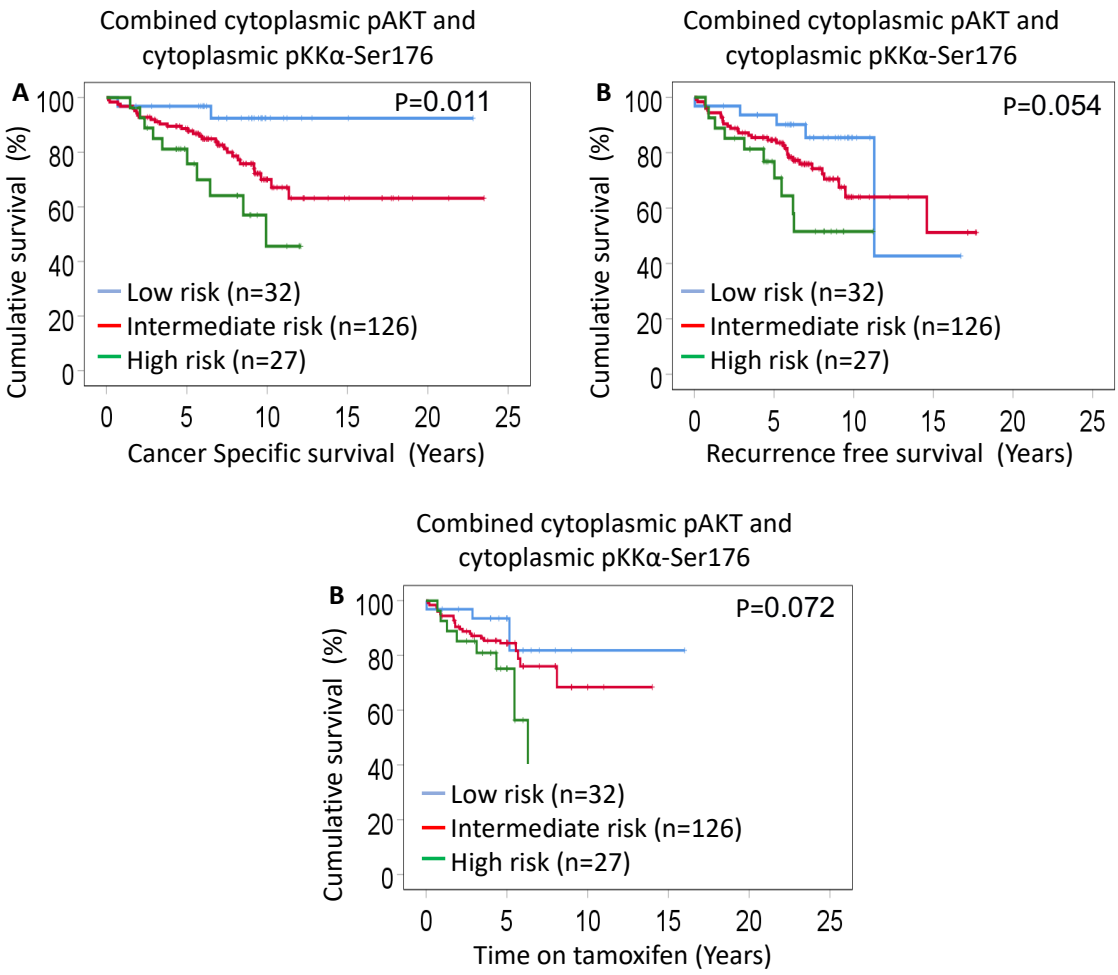


**Figure 4.31 Combined expression of cytoplasmic pAKT and pIKKα-Ser176.** *Kaplan Meier curves showing associations between combined cytoplasmic pAKT and pIKKα-Ser176 and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

A combined expression of cytoplasmic pAKT and pIKKα-ThrT23 was associated with decreased cancer specific survival (HR 1.83 95% CI 1.19-2.83, P=0.008), recurrence free survival (HR 1.54 95% CI 1.03-2.29, P=0.006) and recurrence on tamoxifen (HR 1.74 95% CI 1.11-2.74, P=0.001) as shown in Figure 4.32.

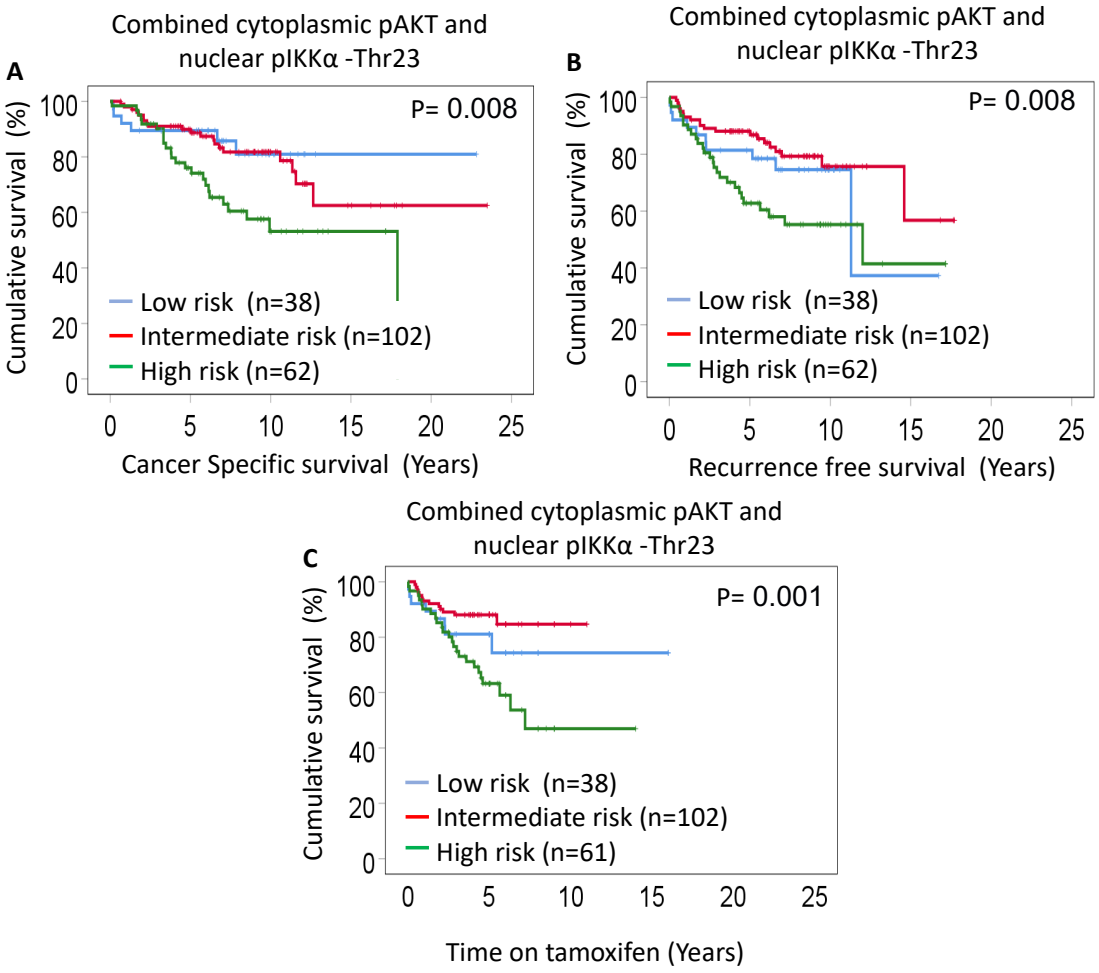


**Figure 4.32 A combined expression of cytoplasmic pAKT and pIKKα-Thr23.** *Meier curves showing associations between combined cytoplasmic pAKT and pIKKα-Thr23 and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

## 4.2.8 Correlation between IKKα, pIKKα-Ser176, and pIKKα-Thr23

Correlation between IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (199). There was no correlation between cytoplasmic IKKα and expression of pIKKα-Ser176 (Cytoplasmic, Spearman's rho=0.045, P=0.547); nuclear, Spearman's rho=0.069, P=0.352). There was a statistically significant, weak positive correlation between nuclear IKKα and expression of cytoplasmic

pIKKα-Ser176 (Spearman's rho=0.178, P=0.015) but no correlation between nuclear IKKα and expression of nuclear pIKKα-Ser176 (Spearman's rho=0.072, P=0.019). No correlation was observed between total IKKα and pIKKα-Ser176, this implies that overall protein levels does not influence the activation status, more protein there does not mean it will be phosphorylase and activated we require the upstream kinase to result in activation.

There was a statistic significant, weak positive correlation between expression of cytoplasmic IKKα and expression of cytoplasmic pIKKα-Thr23 (Spearman's rho=0.174, p=0.010) but no correlation between cytoplasmic IKKα and expression of nuclear pIKKα-Thr23 (Spearman's rho=0.115, P=0.089). There was no correlation between expression of cytoplasmic IKKα and expression of pIKKα-Thr23 (Cytoplasmic Spearman's rho=0.039, p=0.562; nuclear, Spearman's rho=0.003, P=0.968).

#### 4.2.8.1 Cytoplasmic IKKα and phospho-IKKα activity (pIKKα-Ser176, pIKKα-Thr23) as a combined biological marker

There was a significant correlation was observed between IKKα, pIKKα-Ser176 and pIKKα-Thr23 and each of these NF-κB members were previously demonstrated as a prognostic biomarker in the earlier part of this chapter. Therefore, the present study hypothesised that a combined high cytoplasmic IKKα and low cytoplasmic IKKα-Ser176 may associated with poor clinical outcomes, and we classified this group of patients as a 'high risk'. A combination of at least one high cytoplasmic IKKα or one low cytoplasmic IKKα-Ser176 was classified as 'intermediate risk' and a combination of low cytoplasmic IKKα and high cytoplasmic IKKα-Ser176 was classified as a 'low risk' group. In addition, patients who had both high cytoplasmic IKKα and nuclear pIKKα-Thr23 were classified as a 'high risk' group. Patients who had at least one high were classified as an 'intermediate risk' group and patients who had both low were classified as a 'low risk' group.

A combined expression of cytoplasmic IKKα and pIKKα-Ser176 was associated with decreased cancer specific survival (HR 1.50 95% CI 0.94-2.40, P=0.016), recurrence free survival (HR 1.50 95% CI 0.97-2.34, P=0.121) and recurrence on tamoxifen (HR 1.49 95% CI 0.90-2.45, P=0.129), as shown in Figure 4.33.

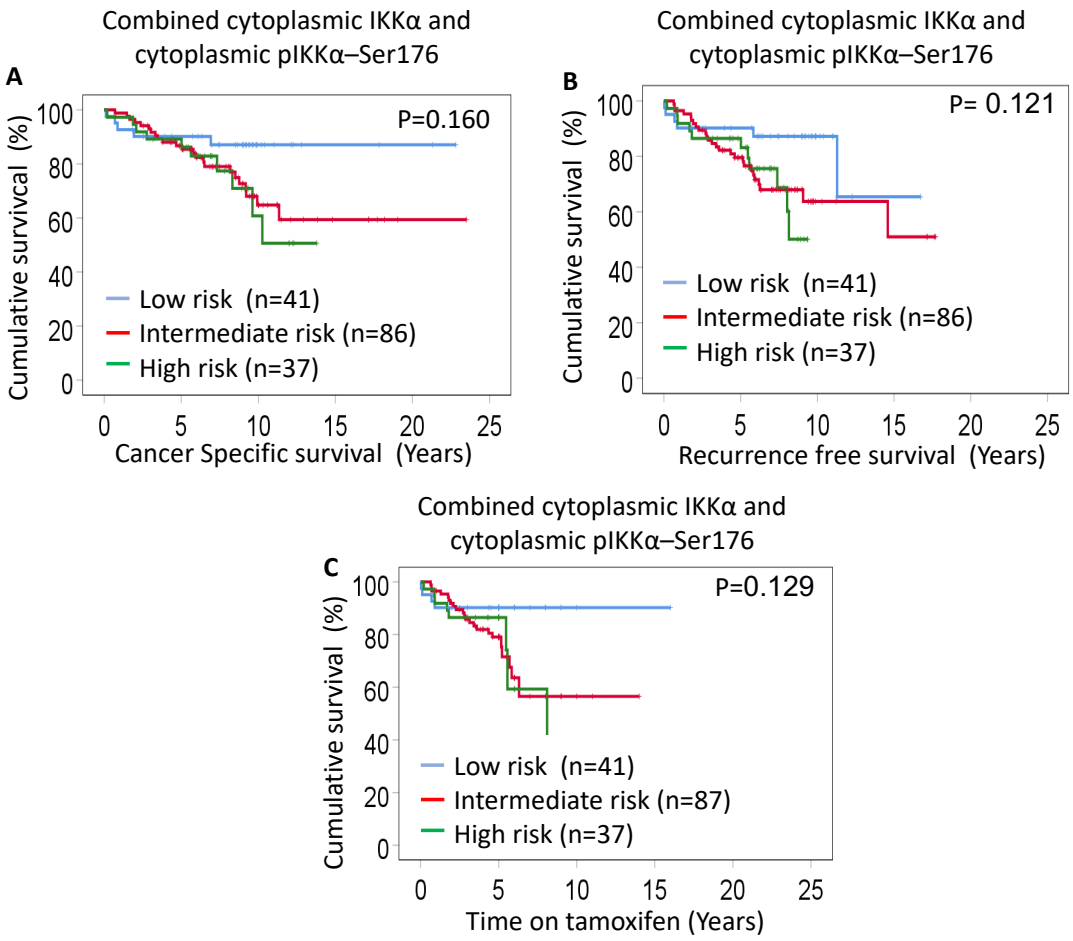


**Figure 4.33 A combined expression of cytoplasmic IKKα and pIKKα-Ser176.** *Kaplan Meier curves showing associations between combined cytoplasmic IKKα and pIKKα-Ser176 and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

A combined expression of cytoplasmic IKKα and nuclear pIKKα-Thr23 was associated with decreased cancer specific survival (HR 1 1.83 95% CI 1.24-2.69, P=0.004) recurrence free survival (HR 1.72 95% CI 1.20-2.49, P=0.012) and recurrence on tamoxifen (HR, 1.88 95% CI 1.26-2.80, P=0.013), as shown in Figure 4.34.

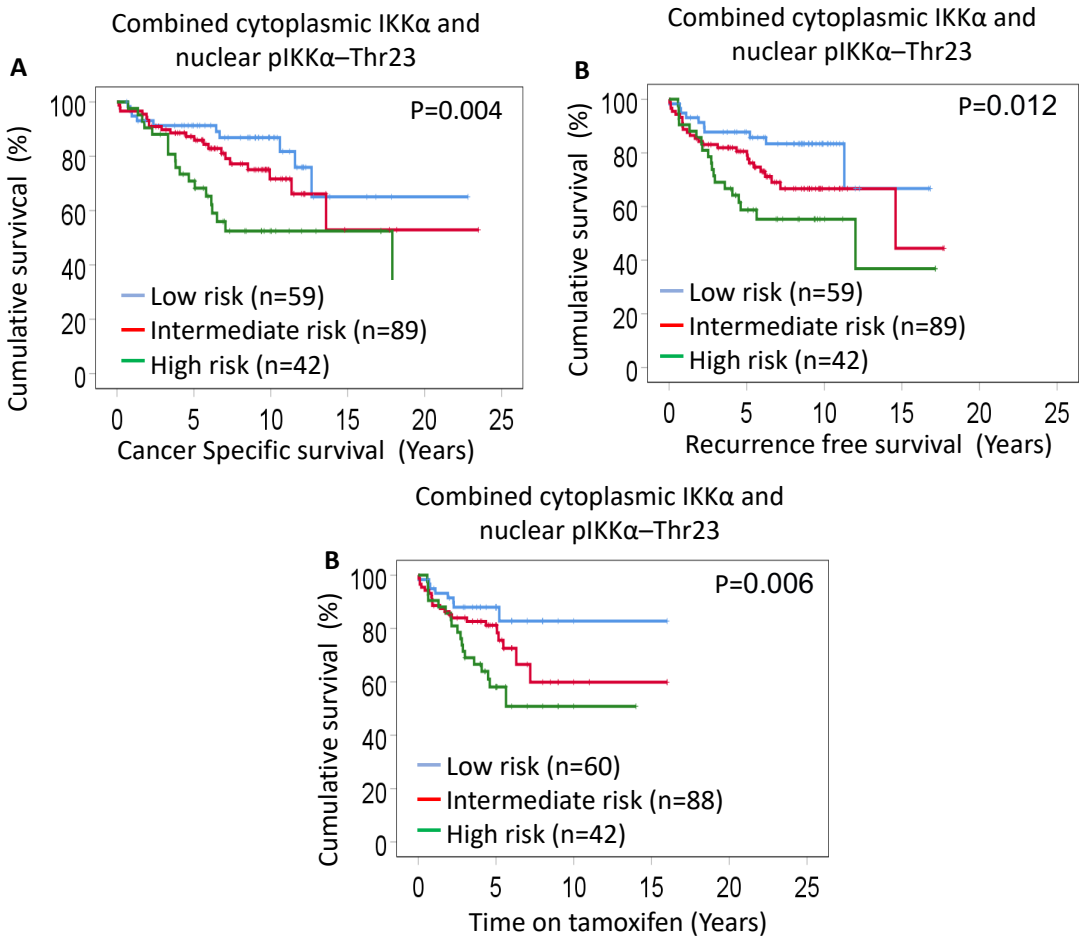


**Figure 4.34 A combined expression of cytoplasmic IKKα and nuclear pIKKα-Thr23.** *Kaplan Meier curves showing associations between combined cytoplasmic IKKα, nuclear pIKKα-T23 and cancer specific survival (A), recurrence free survival (B) and recurrence on tamoxifen (C).*

## 4.3 Discussion

It was observed in the previous chapter that high expression of cytoplasmic pIKKα-Ser176 was a favourable biomarker for the entire cohort of mixed breast cancer subtype and in luminal B tumour. High expression of both nuclear and cytoplasmic pIKKα-Thr23 was an unfavourable prognostic marker for TNBC tumour but was a favourable prognostic marker for HER2 disease. Nuclear expression of NIK was a favourable prognostic marker in the entire cohort but expression of cytoplasmic pAKT was an unfavourable prognostic marker for the entire cohort. Therefore, these biomarkers along with their upstream kinases NIK and pAKT were investigated in ER positive cohort. The median values from the previous chapter were not applied in this cohort as it might not represent the appropriate value for this cohort, as this cohort consist of ER positive patients only.

Although NF-κB activation is shown predominantly in ER-negative breast cancer, the result from the current study suggests a role of a key members of the NF-κB pathway in ER-positive breast cancer. Corresponding to several studies demonstrated high NF-κB activity in ER-positive breast cancer (213-215). Abnormal constitutive expression of both canonical and non-canonical NF-κB subunits has been reported in ER-positive breast cancer (171, 215, 216). Increased p50 and p65 DNA-binding activities were observe in ER-positive breast cancer patients and high level of p50 was associated with reduced disease-free survival (216). The study also showed that tamoxifen-resistant BT474 and MCF7/HER2 cell lines exhibited intermediate level of p50 DNA-binding activities (216). In addition, expression of both p52 and RelB were shown to associate with worse disease-free survival in patients with ER-positive breast cancer (171). There are a limited number of studies that investigate the role of IKKα in ER-positive breast cancer, the current chapter aimed to investigate the role of IKKα, its phosphorylation and its upstream kinases in this specific subgroup of patients.

High cytoplasmic expression of IKKα was associated with reduced cancer specific survival, recurrence free survival and recurrence on tamoxifen in the full cohort and in luminal B tumour. This result corresponds to the previous study that demonstrated high expression of cytoplasmic IKKα was associated with reduced cancer specific survival and time to recurrence in ER-positive breast cancer (204).

High cytoplasmic expression of pIKKα-Ser176 was associated with increased cancer specific survival in the full cohort. However, the association was not found in a particular

molecular subtype. High nuclear expression of pIKKα-Thr23 was associated with shorter cancer specific survival, recurrence free survival and recurrence on tamoxifen in the full cohort and was potentiated in luminal A tumours. Patients were also classified as at risk of cancer death and recurrence. This included those had high cytoplasmic IKKα and low cytoplasmic pIKKα-Ser176; those who had high cytoplasmic IKKα and high nuclear pIKKα-Thr23. Interestingly, the significant decrease in cancer specific survival, recurrence free survival and recurrence on tamoxifen was found in high-risk group of those who had high cytoplasmic IKKα and high nuclear pIKKα-Thr23, suggesting additional prognostic power of pIKKα-Thr23 to cytoplasmic IKKα.

Moreover, poor clinical outcomes were observed in the high-risk group of patients when combined with pAKT. Decreased cancer specific survival was found in high-risk groups of patents who had high cytoplasmic pAKT and low cytoplasmic pIKKα-Ser176. Decreased cancer specific survival, recurrence free survival and recurrence on tamoxifen was observed in patients who had both high cytoplasmic pAKT and cytoplasmic IKKα. Similarly, patients who had both high cytoplasmic pAKT and nuclear pIKKα-Thr23 had a shorter survival outcome, in terms of cancer specific survival, recurrence free survival or recurrence on tamoxifen. These finding suggest pAKT has a significant role in activation of IKKα at both phosphorylation sites in ER-positive breast cancer.

Although NIK is a central signalling component of the non-canonical NF-κB, patients with high expression of NIK did not show association with any of clinical outcomes in the present chapter. This result is different from another study that demonstrated expression of NIK was associated with better recurrence free survival in all patients regardless of their ER status.

In this chapter, the expression of pAKT was not associated with any outcomes for the entire cohort. This finding does not support the previous study that found cytoplasmic pAKT was associated with decreased overall survival in ER-positive breast cancer patients treated with tamoxifen (211). A possible explanation for this finding might be that the previous study used the overall survival to measure clinical outcomes. Still, the current chapter used cancer-specific survival as a clinical outcome. In evaluating outcomes by overall survival, a significant amount of non-cancer death might cause an overestimation of mortality in older patients. A majority of patients in this cohort are older than 50 years, and they might have multiple comorbidities that leads to non-cancer death (211).

As a result of this chapter, it might suggest that IKKα has a different mechanism to control breast cancer progression. High cytoplasmic distribution of classical phosphorylation at serine 176 has protective roles in ER-positive breast cancer regardless of the upstream activation pathway. However, nuclear distribution of phosphorylation at threonine 23 acts as a negative prognostic factor in ER-positive breast cancer in regard to the upstream activation form pAKT. This result can be explained by the study that demonstrated a nuclear role of IKKα to promote endocrine resistance through several ways, including binding to the oestrogen-responsive elements (EREs)-containing promotors and mediating phosphorylation of histone H3, ER, and coactivators AIB1/SRC-3, leading to the increase in expression of cyclin D1 and Myc expression (12). In addition, it has been reported that IKKα siRNA treatment in MCF7 cells leads to a dramatic decrease in the expression of E2F1-regulated genes that are crucial for S phase entry, including thymidine kinase (1TK1), proliferating cell nuclear antigen (PCNA), cell division cycle 25 homolog A (CDC25A) and CYCLIN E (13).

In conclusion, the results from this chapter support the hypothesis that high IKKα was associated with poor clinical outcome, indicating a negative prognostic role for IKKα. In addition, more prognostic power was seen in the high group of patients those who express both IKKα and its phosphorylation site. However, these activities are dependent of phosphorylation site and cellular distribution as cytoplasmic pIKKα-Ser176 has a favourable prognostic value but nuclear pIKKα-Thr23 demonstrating an unfavourable prognostic value. Therefore, further study in an independent cohort combined with mechanistic studies is required to investigate the role of IKKα and its phosphorylation sites in the progression of breast cancer. However, these results highlight the heterogeneity of ER-positive breast cancer and the complexity of signalling pathways that crosstalk with the NF-κB pathway to regulate disease progression.

# Chapter 5 : Expression of IKKα, pIKKα-Ser176 and pIKKα-Thr 23 in an independent cohort of TNBC tumours

## 5.1 Introduction

It was previously demonstrated in chapter 3 that pIKKα-Thr23 was associated with reduced cancer specific survival and recurrence free survival in the TNBC subgroup of the 1800 cohort. These findings require validation in an independent cohort of TNBC or the Belfast cohort. Although IKKα and pIKKα-Ser176 were not associated with the outcomes in the TNBC subgroup of the 1800 cohort, these two proteins also assess in the Belfast cohort. This was because previous evidence has shown that constitutive activation of NF-κB is frequently found in TNBC (208). This activation enhances the transcription of genes that involve in cell proliferation, suppresses apoptosis, promotes metastasis and induces treatment resistance (217, 218). Importantly, recent evidence suggests that Notch1 activates IKKα to induces two parallel pathways, including mTORC2-dependent AKT activation and NF-κB pathway, which enhance Notch-dependent cancer stem-like cells (CSCs) in TNBC (206). A complex of Nothc1, IKKα and NF-κB was also required for transcription of anti-apoptotic gene cIAP-2 in TNBC (206). Therefore, IKKα and pIKKα-Ser176 also assess in this TNBC cohort (Belfast cohort).

## 5.2 Expression and clinical outcome of members of non-canonical NF-κB pathway in a cohort of patients with TNBC (the Belfast-cohort)

The Belfast cohort included 109 patients presenting with invasive breast cancer between 2000 and 2013. A data base of clinicopathological features was available, including patient age, tumour size, grade, lymph node status and AR status. Patients with TMA core missing or if inadequate quality for scoring were excluded (Figure 5.1).

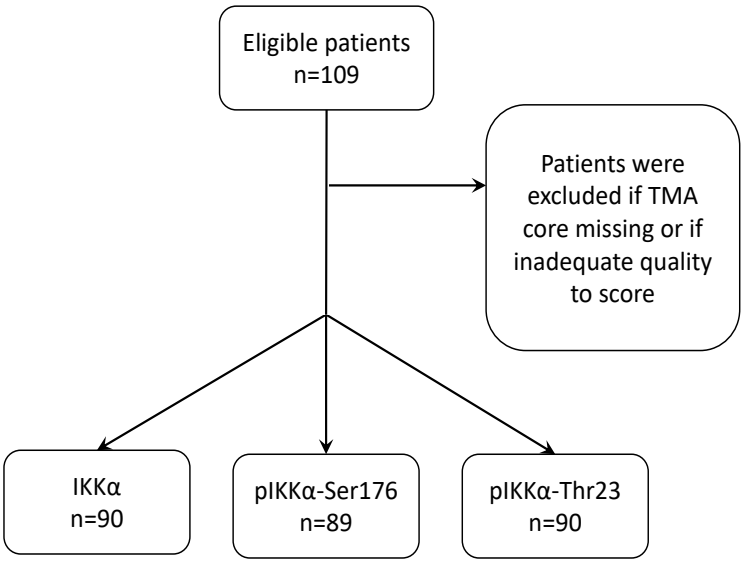


**Figure 5.1 Flow diagram demonstrating patient exclusions.** *Patients with missing cores or insufficient tumour for analysis were excluded*

The majority of patients were over 50 years old (56.9%), 53 (48.6%) had tumour size between 21-50mm, 96 (88.1%) had tumour grade III, 63 (57.8%) had lymph negative disease. Information for chemotherapy was available in 91 patients, of these 75 (68.8%) received chemotherapy (information on neoadjuvant or adjuvant treatment not available). Among 109 patients, 4 patients (3.7%) had distance metastatic disease at the time of presentation (Table 5.1).

| **Clinicopathological characteristics (n=109)** | **Patients number (%)** |
|---|---|
| Age | |
| ≤50 | 47 (43.1) |
| >50 | 62 (56.9) |
| Size (mm) | |
| ≤20 | 31 (28.4) |
| 21-50 | 53 (48.6) |
| >50 | 25 (22.9) |
| Grade | |
| I | 0 (0) |
| II | 13 (11.9) |
| III | 96 (88.1) |
| Lymph node status | |

| | |
|---|---|
| Negative | 63 (57.8) |
| Positive | 45 (41.3) |
| Chemotherapy | |
| No | 16 (14.7) |
| Yes | 75 (68.8) |
| Missing | 18 (16.5) |
| Survival status | |
| Alive | 67 (61.5 |
| Death | 4 (37.6) |
| Missing | 1 (0.9) |
| Recurrence status | |
| No recurrence | 53 (48.6 |
| Recurrence | 55 (40.5) |
| Missing | 1 (0.9) |

**Table 5.1 Clinicopathological characteristics of the Belfast cohort**

## 5.2.1 Expression of IKKα in the Belfast cohort

### 5.2.1.1 Association of IKKα with clinical outcome in the Belfast cohort

Expression of IKKα was assessed in 90 patients from the Belfast cohort. The histoscore for cytoplasmic expression of IKKα ranged from 0 to 300 with a median histoscore of 150 (IQR=111.8-218.8). The histoscore for nuclear expression of IKKα ranged from 52 to 300 with a median histoscore of 170 (IQR=130.0-208.33). The median histoscore from the previous cohort (The 1800-cohort) was 120 for cytoplasmic and 100 for nuclear expression, which was employed as the cut off to determine high and low expression. However, in this cohort only 7 patients had a histoscore of 100 or less for nuclear expression, therefore, the previously established cut-off could not be applied for analysis due to insufficient numbers with low nuclear expression. The median histoscore of 150 and 170 for cytoplasmic and nuclear IKKα observed in this cohort was therefore used to subdivide patients into those with low and high expression. 43 patients were found to have low expression of cytoplasmic IKKα, and 47 patients were classified as having high expression. 44 patients were found to have low expression of nuclear IKKα, and 46 patients were classified as having high expression. An example of immunostaining of low and high expression of IKKα and Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of IKKα are shown in Figure 5.2. There was good correlation between observers with ICCC score of 0.86 for cytoplasmic and 0.81for nuclear expression.

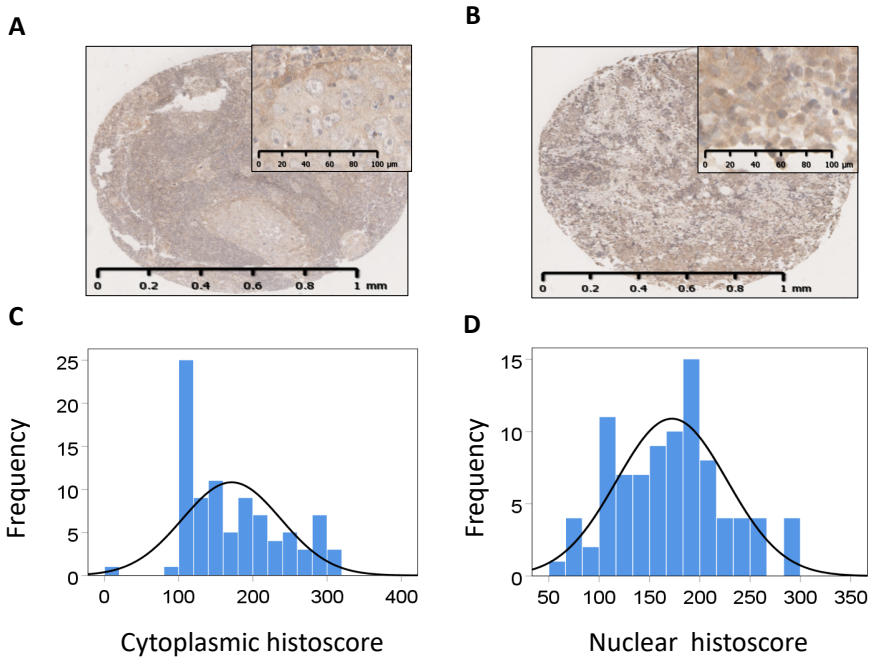


**Figure 5.2 Expression of IKKα.** *(A) Example of low expression and (B) of high expression of IKKα (images taken at 400x magnification; scale bar represents 100μm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of IKKα*

To determine whether IKKα expression was associated with clinical outcome, Kaplan-Meier survival curves for cytoplasmic and nuclear expression of IKKα were plotted as shown in Figure 5.3. Low and high expression were compared by using the log rank test. For this cohort, overall survival and recurrence free survival were analysed. High expression of cytoplasmic IKKα was associated with increased overall survival (HR 0.50 95% CI 0.25-0.99, P=0.044, Figure 5.3. However, no association was found between cytoplasmic IKKα and recurrence free survival (HR 0.76 95% CI 0.43-1.36, P=0.845, Figure 5.3). Expression of nuclear IKKα was not associated with overall survival (HR 0.68 95% CI 0.34-1.33, P=0.252) or recurrence free survival (HR 0.68 95% CI 0.38-1.21, P=0.183, Figure 5.3).

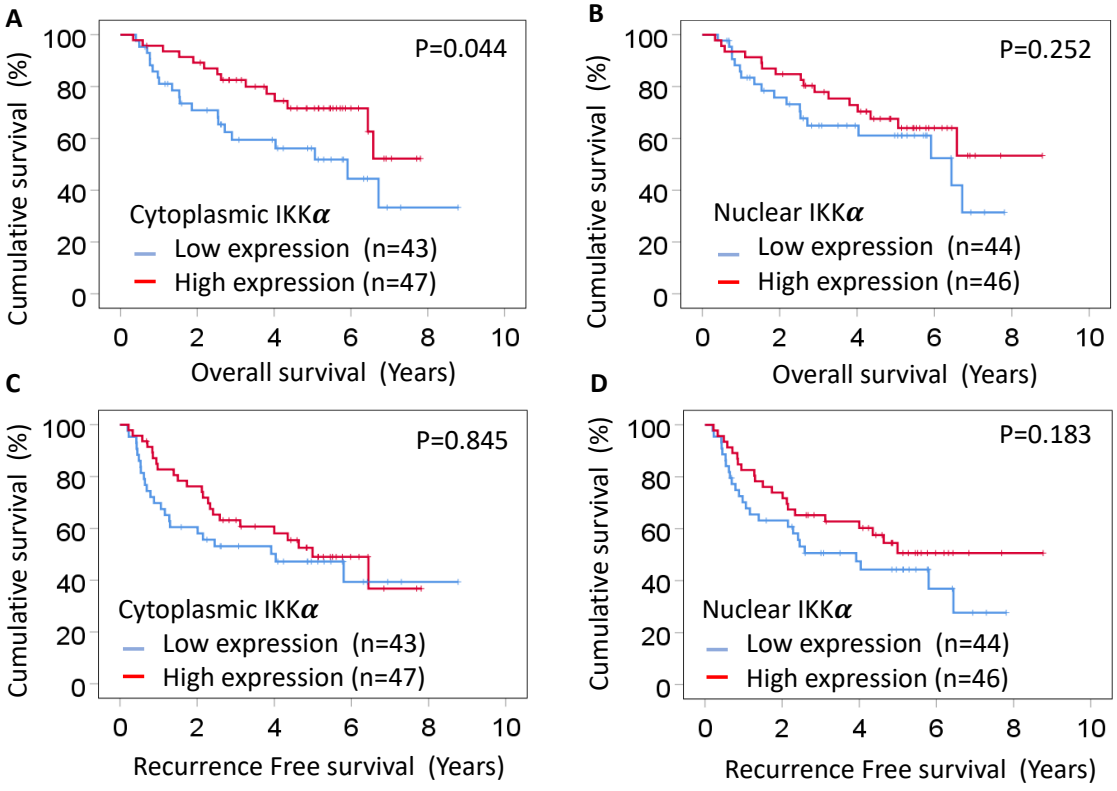


**Figure 5.3 Expression of IKKα and clinical outcome in the Belfast cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear IKKα with overall survival (A and B) and recurrence free survival (C and D).*

Multivariate analysis suggested that cytoplasmic expression of IKKα was not an independent prognostic marker for overall survival when combined with tumour size and lymph node status (HR 0.64 95% CI 0.31-1.33), P= 0.231) (Table 5.2).

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 2.72 (0.36-20.87) | 0.336 | | |
| Size (mm)<br>20/ 21-50/>50 | 1.99 (1.27-3.10) | **0.003** | 1.89 (1.11-3.22) | 0.019 |
| Grade<br>I/II/III | 1.59 (0.56-4.53) | 0.383 | | |
| Lymph node status<br>Negative/ Positive | 4.10 (2.11-7.96) | **<0.001** | 4.05 (1.92-8.55) | **<0.001** |
| Cytoplasmic IKKα<br>Low/High | 0.50 (0.25-0.99) | 0.049 | 0.64 (0.31-1.33) | 0.231 |
| Nuclear IKKα<br>Low/High | 0.68 (0.34-1.33) | 0.255 | | |

**Table 5.2 Univariate and multivariate analysis of IKKα and clinicopathological characteristic in the Belfast cohort**

#### 5.2.1.2 Associated of IKKα and clinic-pathological characteristics

Chi-squared analysis was performed in order to determine whether expression of IKKα was associated with any clinicopathological characteristic of the patients in the Belfast cohort as shown in Table 5.3. Expression of cytoplasmic IKKα was associated only with tumour grade (P=0.006) and lymph node status (P=0.027).

| Clinicopathological characteristics | Cytoplasmic IKKα | | |
|---|---|---|---|
| | Low | High | P |
| Age | | | |
| ≤50 | 19 (44.2) | 22 (46.8) | 0.803 |
| >50 years) | 24 (55.8) | 25 (53.2) | |
| Size (mm) | | | |
| ≤20 | 10 (23.3) | 15 (31.9) | 0.511 |
| 21-50 | 21 (48.8) | 23 (48.9) | |
| >50 | 12 (27.9) | 9 (19.1) | |
| Grade | | | |
| II | 9 (20.9) | 1 (2.1) | **0.006** |
| III | 34 (79.1) | 45 (97.9) | |
| Lymph node status | | | |
| Negative | 20 (46.5) | 32 (69.6) | **0.027** |
| Positive | 23 (53.5) | 14 (30.4) | |

**Table 5.3 Association of IKKα with clinicopathological characteristics of the Belfast cohort.** *Chi-squared analysis explored the relationship of IKKα with clinicopathological characteristics of the cohort.*

## 5.2.2 Expression of pIKKα-Ser176 in the Belfast cohort

Expression of pIKKα-Ser176 was assessed in 89 patients. The histoscore for cytoplasmic pIKKα-Ser176 ranged from 100 to 300 with a median histoscore of 170 (IQR=126.6-220.8). The histoscore for nuclear pIKKα-Ser176 ranged from 45 to 300 with a median histoscore of 140 (IQR=104.5-176.3). Again, the median histoscore from the previous cohort (The 1800-cohort) was 100 for cytoplasmic and 70 for nuclear expression, these were the values employed as cut-offs to divide patients into high and low expression. However, only 1 patient had a histoscore of 100 or less for cytoplasmic and only 4 patients had a histoscore of 70 or less for nuclear expression. Therefore, the previously established cut-off could not be applied for analysis due to insufficient number. The median histoscore of 160 and 140

for cytoplasmic and nuclear pIKKα-Ser176 observed in this cohort was therefore used to subdivide patients into those with low and high expression. 43 patients were found to have low expression of cytoplasmic pIKKα-Ser176, and 46 patients were classified as having high expression. 48 patients were found to have low expression of nuclear pIKKα-Ser176, and 41 patients were classified as having high expression. An example of immunostaining of low and high expression of pIKKα-S176 is shown in Figure 5.4  Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of pIKKα-S176 are shown in Figure 5.4. There was good correlation between observers with ICCC score of 0.80 for cytoplasmic and 0.81for nuclear expression.

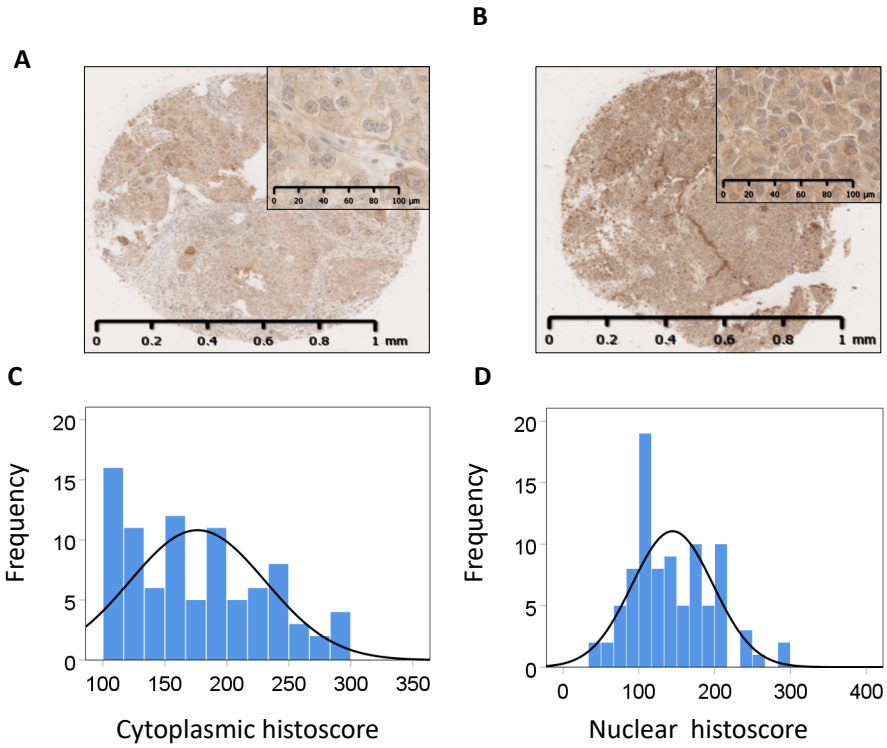


**Figure 5.4 Expression of pIKKα-Ser176.** *(A) Example of low expression and (B) of high expression of pIKKα-Ser176 (images taken at 400x magnification; scale bar represents 100μm). Histograms show distribution of histoscores for (C) cytoplasmic and (D) for nuclear expression of pIKKα-Ser176.*

### 5.2.2.1 Association of pIKKα-Ser176 with clinical outcome in the Belfast cohort

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pIKKα-Ser176 were plotted as shown in Figure 5.5. The log rank test was used to compare low and high expression. High cytoplasmic expression of pIKKα-Ser176 (HR 2.14 95% CI 1.05-4.34, P=0.031, Figure 5.5) and nuclear pIKKα-Ser176 was associated with decreased overall survival (HR 2.73 95% CI 1.35-5.52, P=0.009, Figure 5.5). Similarly, high expression of cytoplasmic pIKKα-Ser176 (HR 2.22 95% CI 1.21-4.09 P=0.004, Figure 5.5) and nuclear pIKKα-Ser176 was associated with decreased recurrence free survival (HR 2.73 95% CI 1.35-5.52, P=0.011, Figure 5.5).

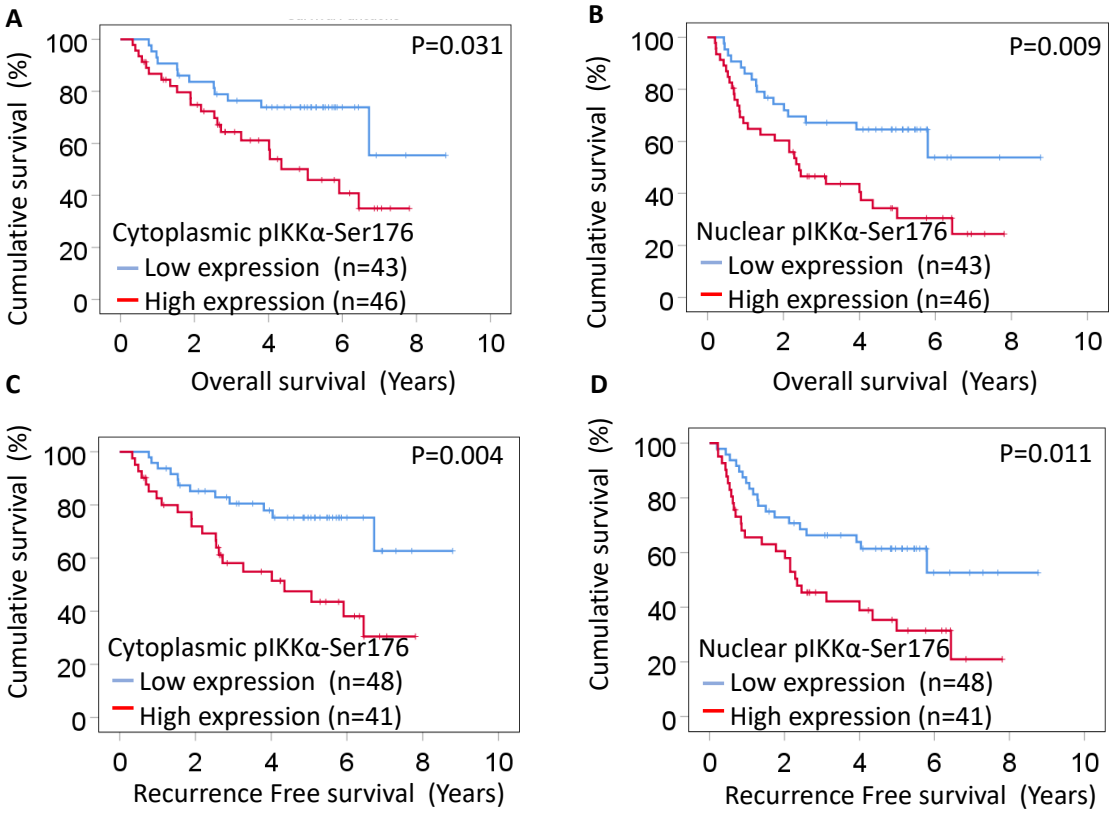


**Figure 5.5 Expression of pIKKα-Ser176 and clinical outcome in the Belfast cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pIKKα-Ser176 with overall survival (A and B) and recurrence free survival (C and D)*

Multivariate analysis suggested that nuclear expression of pIKKα-Ser176 was an independent prognostic marker for overall survival when combined with tumour size and lymph node status (HR 02.60 95% CI 1.27-5.31, P= 0.009) (Table 5.3).

| Patient characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 2.72 (0.36-20.87) | 0.336 | | |
| Size (mm)<br>20/ 21-50/>50 | 1.99 (1.27-3.10) | **0.003** | 2.07 (1.21-3.53) | **0.008** |
| Grade<br>I/II/III | 1.59 (0.56-4.53) | 0.383 | | |
| Lymph node status<br>Negative/ Positive | 4.10 (2.11-7.96) | **<0.001** | 3.82 (1.84-7.93) | **<0.001** |
| Cytoplasmic pIKKα-Ser176<br>Low/High | 2.14 (1.05-4.34) | **0.035** | 1.20 (0.41-3.54) | 0.743 |
| Nuclear pIKKα-Ser176<br>Low/High | 2.73 (1.35-5.52) | **0.005** | 2.60 (1.27-5.31) | **0.009** |

**Table 5.4 Univariate and multivariate analysis for overall survival of pIKKα-Ser176 and clinicopathological characteristic in the Belfast cohort**

Multivariate analysis suggested that expression of cytoplasmic pIKKα-Ser176 was an independent prognostic marker for recurrence free survival when combined with tumour size and lymph node status (HR 2.33 95% CI 1.26-4.32, P= 0.007) Table 5.5.

| Patient characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Age<br>50/>50 | 2.00 (0.27-15.00) | 0.501 | | |
| Size (mm)<br>20/ 21-50/>50 | 1.89 (1.28-2.79) | **0.001** | 1.85 (1.20-2.85) | **0.005** |
| Grade<br>I/II/III | 1.73 (0.68-4.37) | 0.250 | | |
| Lymph node status<br>Negative/ Positive | 3.39 (1.95-5.88) | **<0.001** | 2.93 (1.61-5.34) | **<0.001** |
| Cytoplasmic pIKKα-Ser176<br>Low/High | 2.22 (1.21-4.09) | **0.010** | 2.33 (1.26-4.32) | **0.007** |
| Nuclear pIKKα-Ser176<br>Low/High | 2.12 (1.18-3.82) | **0.012** | 1.30 (0.57-2.95) | 0.535 |

**Table 5.5 Univariate and multivariate analysis for recurrence free survival of pIKKα-Ser176 and clinicopathological characteristic in the Belfast cohort**

### 5.2.2.2 Associated of pIKKα-Ser176 and clinic-pathological characteristics

Chi-squared analysis was performed in order to determine whether expression of pIKKα-Ser176 was associated with any clinicopathological characteristic of the patients in the Belfast cohort as shown in Table 5.6. Expression of cytoplasmic pIKKα-Ser176 was associated only with patient age (P=0.008). Similarly, expression of nuclear pIKKα-Ser176 was associated with patient age (P<0.001).

| Clinicopathological characteristics | Cytoplasmic pIKKα-Ser176 | | | Nuclear pIKKα-Ser176 | | |
|---|---|---|---|---|---|---|
| | Low | High | P | Low | High | P |
| Age | | | | | | |
| ≤50 | 26 (60.5) | 15 (32.6) | **0.008** | 32 (66.7) | 9 (22.0) | **<0.001** |
| >50 years) | 17 (39.5) | 31 (57.4) | | 16 (33.3) | 32 (78.0) | |
| Size (mm) | | | | | | |
| ≤20 | 11 (25.6) | 14 (30.4) | 0.504 | 14 (29.2) | 11 (26.8) | 0.653 |
| 21-50 | 23 (53.5) | 19 (41.3) | | 24 (50.0) | 18 (43.9) | |
| >50 | 9 (20.9) | 13 (28.3) | | 10 (20.8) | 12 (29.3) | |
| Grade | | | | | | |
| II | 6 (14.0) | 4 (8.7) | 0.513 | 5 (10.4) | 5 (12.2) | 1.000 |
| III | 37 (86.0) | 42 (91.3) | | 43 (89.6) | 36 (87.8) | |
| Lymph node status | | | | | | |
| Negative | 26 (60.5) | 26 (57.8) | 0.798 | 29 (60.4) | 23 (57.5) | 0.782 |
| Positive | 17 (39.5) | 19 (42.2) | | 19 (39.6) | 17 (42.5) | |

**Table 5.6 Association of pIKKα-Ser176 with clinic-pathological characteristics of the Belfast cohort.** *Chi-squared analysis explored the relationship of pIKKα-Ser176 with various characteristics of the cohort.*

## 5.2.3 Expression of pIKKα-Thr23 in the Belfast cohort

Expression of pIKKα-Thr23 was assessed in 90 patients within the Belfast cohort. The histoscore for cytoplasmic expression of pIKKα-Thr23 ranged from 0 to 265 with a median histoscore of 100 (IQR=64.9-101.5). The histoscore for nuclear pIKKα-Thr23 ranged from 0 to 300 and a median histoscore of 100 (IQR=61.7-107.2). The median histoscore of 100 and 110 for cytoplasmic and nuclear pIKKα-Thr23 observed in this cohort was therefore used to subdivide patients into those with low and high expression. 67 patients were found to have low expression of cytoplasmic pIKKα-Thr23, and 23 patients were classified as having high expression. 48 patients were found to have low expression of nuclear pIKKα-Thr23, and 42 patients were classified as having high expression. An example of immunostaining of low and high expression of pIKKα-Thr23 is shown in Figure 5.6. Histograms showing the distribution of histoscores for cytoplasmic and nuclear expression of pIKKα-Thr23 are shown Figure 5.6. There was moderated and good correlation between

observers with ICCC score of 0.93 for cytoplasmic and 0.86 for nuclear expression, respectively.

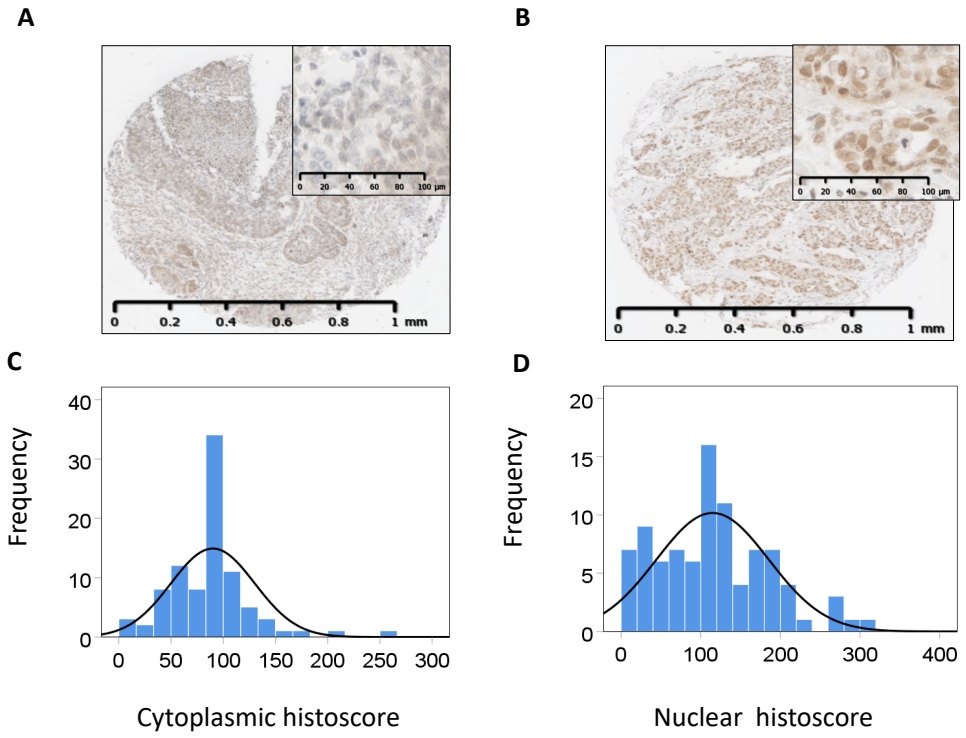


**Figure 5.6 Expression of pIKKα-Thr23** *(A) Example of low expression and (B) of high expression of pIKKα-Thr23 (images taken at 400x magnification; scale bar represents 100μm). Histograms show distribution of histoscores for (C) cytoplasmic (D) and nuclear expression of pIKKα-ThrT23.*

### 5.2.3.1 Association of pIKKα-Thr23 with clinical outcome in the Belfast cohort

Kaplan-Meier survival curves for cytoplasmic and nuclear expression of pIKKα-Thr23 were plotted as shown in Figure 5.7. The log rank test was used to compare low and high expression. Expression of cytoplasmic pIKKα-Thr23 (HR 0.98 95%CI 0.46-2.10, P=0.995) and nuclear pIKKα-Thr23 (HR 1.43 95% CI 0.72-2.81, P=0.301) was not associated with overall survival. Similarly, expression of cytoplasmic pIKKα-Thr23 1.18 (HR 1.18 95% CI 0.62-2.25, P=0.605) and nuclear pIKKα-Thr23 (HR 1.29 95% CI 0.73-2.31, P=0.382) was not associated with recurrence free survival.

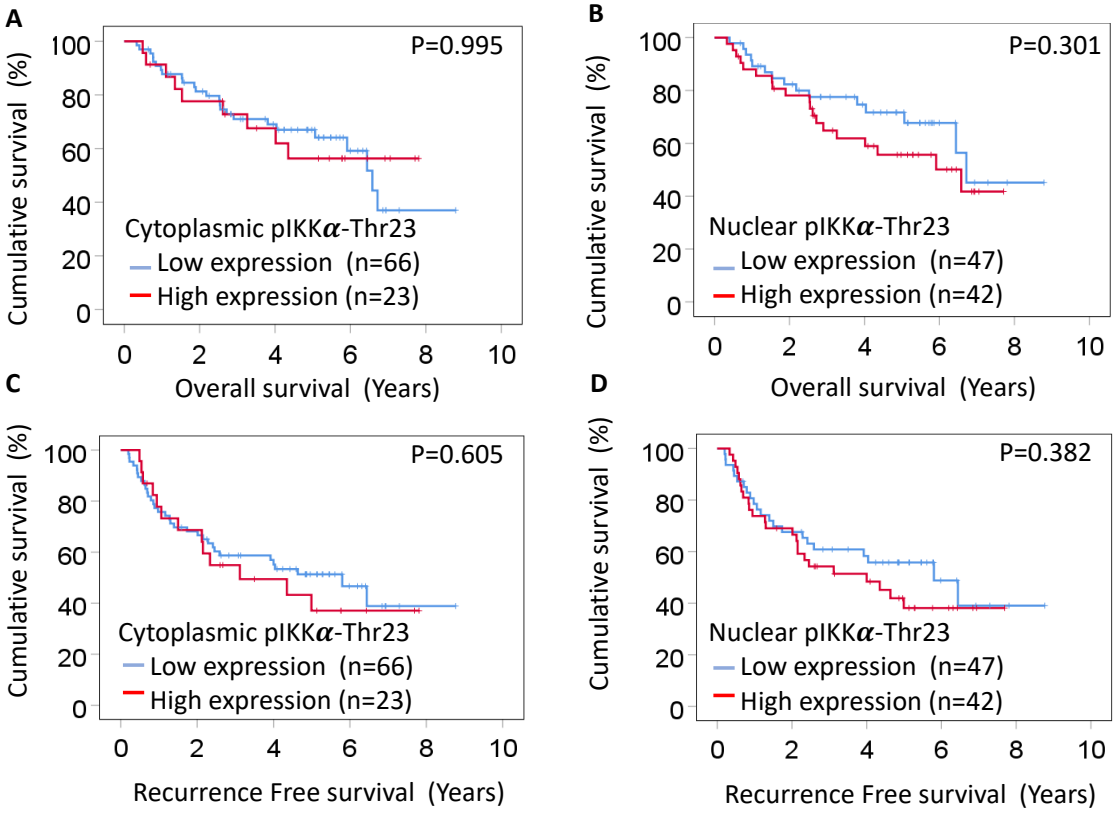


**Figure 5.7 Expression of pIKKα-Thr23 and clinical outcome in the Belfast-cohort.** *Kaplan Meier curves showing associations of cytoplasmic and nuclear pIKKα-SerThr23 with overall survival (A and B) and recurrence free survival (C and D)*

### 5.2.4 Correlation between expression of IKKα, pIKKα-Ser176 and pIKKα-Thr23

Correlation between the upstream regulators and IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (199). There was no statistically significant correlation between expression of cytoplasmic IKKα and pIKKα-Ser176 (Cytoplasmic; Spearman's rho=0.177, P=0.099; nuclear pIKKα-Ser176; Spearman's rho=0.038, P=0.722). There was a statistically significant, weak positive correlation between expression of cytoplasmic IKKα and expression of cytoplasmic pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.273, P=0.009). However, no association was observed between cytoplasmic IKKα and nuclear pIKKα-Thr23 (Spearman's rho=0.077, P=0.475).

There was no statistically significant correlation between expression of cytoplasmic IKKα and pIKKα-Ser176 (Cytoplasmic; Spearman's rho=0.106, P=0.328; nuclear pIKKα-Ser176;

Spearman's rho=0.197, P=0.065). There was a statistically significant, weak positive correlation between expression of cytoplasmic IKKα and expression of pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.385, P<0.001; nuclear pIKKα-Thr23 Spearman's rho=0.363, P=0.001).

#### 5.2.4.1 Cytoplasmic IKKα and pIKKα-Ser17 as a combined biological marker of clinical outcomes

Although no significant correlation was observed between IKKα, pIKKα-Ser176, these two proteins were previously demonstrated as a prognostic biomarker in the earlier part of this chapter. Therefore, the present study hypothesised that a combined low cytoplasmic IKKα and high cytoplasmic or nuclear IKKα-Ser176 may associated with poor clinical outcomes, and we classified this group of patients as a 'high risk'. A combination of at least one low cytoplasmic IKKα or one high cytoplasmic/nuclear IKKα-Ser176 was classified as 'intermediate risk' and a combination of high cytoplasmic IKKα and low cytoplasmic IKKα-Ser176 was classified as a 'low risk' group. In the high-risk group, a combined expression of cytoplasmic IKKα and cytoplasmic pIKKα-Ser176 was associated with decreased overall survival (HR 2.42 95% CI 1.40-4.20, P=0.006) and decreased recurrence free survival (HR 1.92 95% CI 1.20-3.07, P=0.023) (Figure 5.8).

Similarly, a combined expression of cytoplasmic IKKα and nuclear pIKKα-Ser176 was associated with decreased overall survival (HR 2.77 95% CI 1.60-4.81, P=0.001) and decreased recurrence free survival (HR 1.83 95% CI 1.16-2.88, P=0.032) (Figure 5.8).

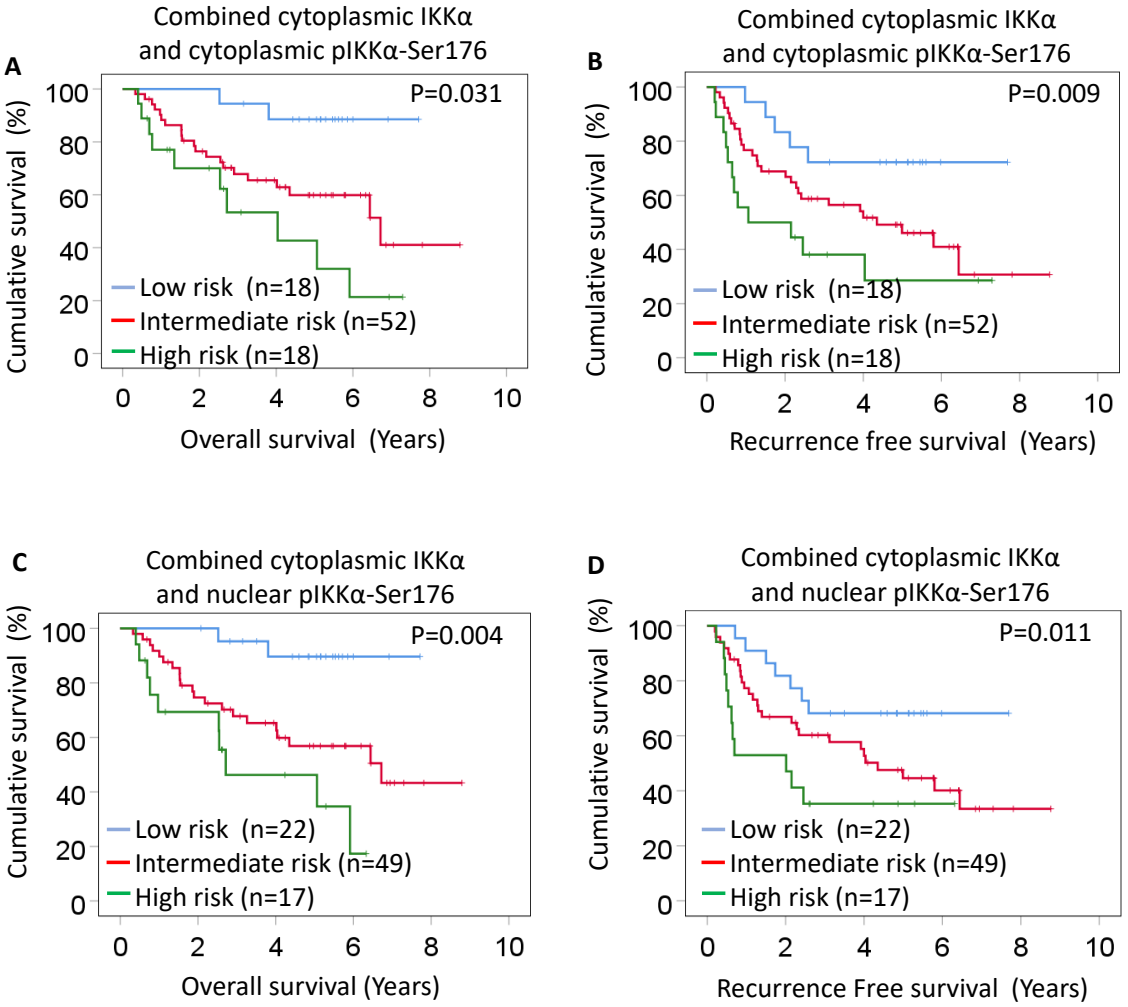


**Figure 5.8 A combined expression of cytoplasmic IKKα and pIKKα-Ser176.** *Kaplan Meier curves showing associations between combined cytoplasmic IKKα and cytoplasmic pIKKα-Ser176 and overall survival (A) and recurrence free survival (B). Kaplan Meier curves showing associations between combined cytoplasmic IKKα and nuclear pIKKα-Ser176 and overall survival (C) and recurrence free survival (B).*

## 5.3 Discussion

In the chapter of mixed breast cancer subtypes (the 1800-cohort), the expression of IKKα and pIKKα-Ser176 was not associated with clinical outcomes in TNBC tumours. However, decreased cancer specific survival was observed in patients with either high expression of cytoplasmic or nuclear pIKKα-Thr23.

Decreased recurrence free survival also was observed in patients with high expression of cytoplasmic pIKKα-Thr23. Conversely, it was observed in this chapter that the expression of IKKα was associated with improved overall survival and high expression of pIKKα-Ser176 was associated with worsen overall survival regardless of cellular distribution. The associated was not observed in patients with high expression of pIKKα-Thr23.

There are several possible explanations for this contradictory result. Since the primary outcome of present cohort was the overall survival looking at death from any cause and not just cancer death, which was different from the previous cohort. This might lead to an inconsistent result between the two cohort. Therefore, in order to confirm the prognostic association of these biomarkers, it need to be analysed using the same outcome determinants. Next, the variation on IHC result might affect the difference outcomes. The median histoscore for cytoplasmic and nuclear IKKα was 150 and 170 in the Belfast cohort whereas it was 120 and 100 in cohort of the 1800-cohort. The median histoscore for cytoplasmic and nuclear pIKKα-Ser176 was 170 and 140 in the Belfast cohort whereas it was 100 and 70 in the 1800-cohort. The median histoscore for cytoplasmic and nuclear pIKKα-Thr23 was 100 and 100 in the Belfast cohort whereas it was 80 and 90 in the 1800-cohort. This highlights the variability observed with levels of proteins and phosphoproteins, which may lead to discordance of the results. Several studies have demonstrated that variations in specimen fixation can influence immunoreactive of the proteins (219, 220). Lower expression of pAKT and pERK1/2 were observed in routine fixation compared to immediately core-cuts after tumour resection (219) . Another study showed that more than 20 percent decrease of pAKT-Ser473 whereas 20 percent increase of pSTAT1-Tyr701 within several hours of post excision (220). The 1800-cohort included the patients between 1980 and 1998 whereas the Belfast cohort included the patients between 2000 and 2013. As the tissue handling time point was different between the two cohort, it may lead to the variation of method for fixation, duration of fixation and fixative formula, which can affect an intensity of IHC staining (221-223).

In conclusion, these findings were inconsistent with the TNBC subgroup in the 1800 cohort. Several limitations were noted, as previously discussed. Further study with an adequate number of patients and using the optimal cut-off value across the different cohorts and using the same outcome measurement was needed to demonstrate their potential prognostic role.

# Chapter 6 : Expression of AR in breast cancer clinical specimens

## 6.1 Introduction

Androgen receptor (AR) is thought to have crucial role in breast cancer. High androgen level is associated with increased risk of breast cancer in post-menopausal women (224, 225). Although, the mechanism by which AR contributes to development of breast cancer is not well understood, it has been shown that androgen can promote proliferation in breast tissue and induce tumour formation in animal models (226). There was consistent agreement that AR expression is a favourable prognostic marker in ER-positive disease (227, 228). However, there was disagreement on AR expression being prognostically relevant in TNBC (45, 47, 229, 230). Currently, AR is a potential target for advance breast cancer and anti-androgen agents such as bicalutamide and enzalutamide have been investigated for tolerance and anti-tumour activity in preclinical and clinical trials (50).

Several studies have demonstrated crosstalk between the NF-κB pathway and AR to promote tumour progression in prostate cancer and induce tumour resistance to treatment (231, 232). Recent work from our lab demonstrated the prognostic role of cytoplasmic IKKα and its phosphorylation of threonine 23 in prostate cancer (52). High expression of IKKα was associated with low AR expression and higher cancer specific survival in hormone naïve prostate cancer. A high level of nuclear IKKα was associated with a lower time to death from recurrence in castration-resistant prostate cancer (52). It has been demonstrated a multifunction of IKKα either NF-κB-dependent and NF-κB-independent mechanism as previously described in chapter 1. Several NF-κB-independent functions of IKKα has been reported, including the potential crosstalk with AR pathway through the phosphorylation of SRC3 (233). The schematic represents the potential crosstalk between IKKα/NF-κB and AR pathway has been shown in Figure 6.1.

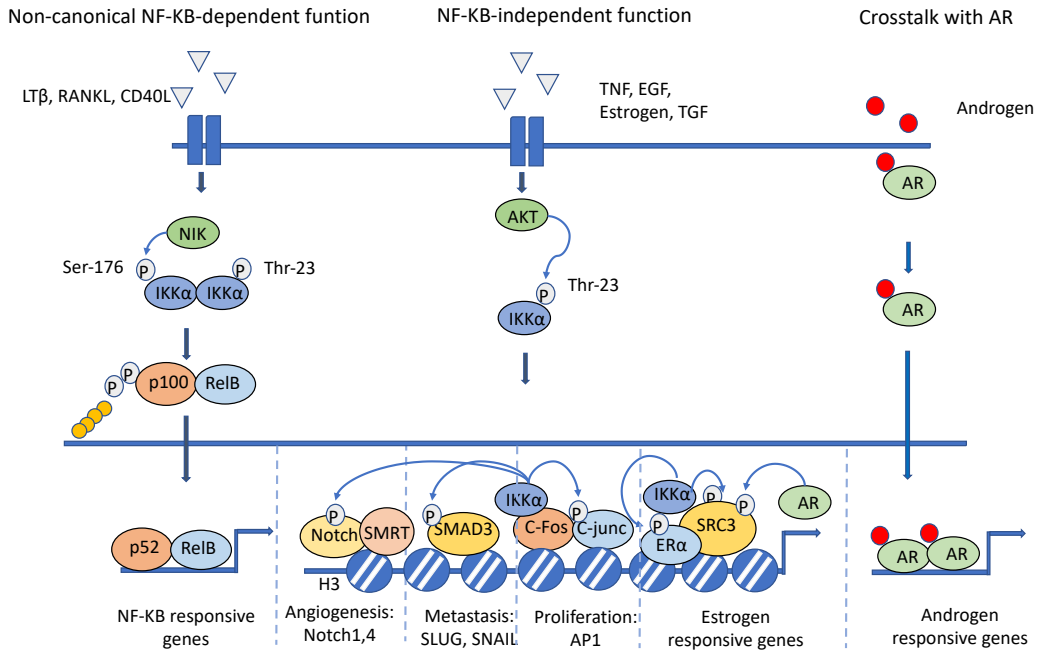


**Figure 6.1 The schematic represents the potential crosstalk between IKKα/NF-κB and AR pathway**

There is limited evidence on the crosstalk between NF-κB and AR in breast cancer. Therefore, this thesis will also investigate the role of AR in combination with IKKα and its phosphorylation to demonstrate if they have an enhancing effect in the TNBC subgroup.

Androgen receptor expression is vary based on the cut-off value that is used to define its positivity. Currently, it is still a discussion regarding the cut-off values of AR because they vary between studies. In a systemic review of 19 studies with a total of 7693 women with early-stage breast cancer, the cut-off values were 1%, 5% and 10% (42). Similarly, most studies use either 1% or 10% of AR-positive cells as a positive value (234).

In this chapter, AR expression was classified as positive when 1% of the tumour had nuclei stained. This threshold was concordance with a phase I/II clinical trial that assess the use of selective inhibitors of CDK4/6 in combination with bicalutamide for treatment of AR-positive metastatic TNBC (235). This cut-off value is also used in clinical trials to evaluate adjuvant enzalutamide for early-stage AR-positive TNBC (236). Since it was no consensus regarding the AR positivity cut-off, a value of 1% will be used in the chapter.

## 6.2 Comparison of clinicopathological characteristic between TNBC tumours in the 1800-cohort and the Belfast TNBC cohort

The patient characteristics of the two cohorts were compared as shown in Table 6.1. The majority of patients were over 50 years old, were tumour grade II and III, had lymph node negative disease, received chemotherapy and were still alive. No significant difference was noted for any clinical characteristic between the two TNBC cohorts.

| Clinicopathological characteristics | TNBC tumour in the 1800-cohort (n=176) (n, %) | The Belfast-cohort (n=109) (n, %) | P value |
|---|---|---|---|
| Age | | | |
| ≤50 | 68 (38.5) | 47 (43.1) | 0.195 |
| >50 | 108 (61.4) | 62 (56.9) | |
| Size (mm) | | | |
| ≤20 | 91 (51.7) | 31 (28.4) | 0.293 |
| 21-50 | 75 (42.6) | 53 (48.6) | |
| >50 | 9 (5.1) | 25(22.9) | |
| Grade | | | |
| I | 6 (3.4) | 0 (0%) | 0.153 |
| II | 42 (23.9) | 13 (11.9) | |
| III | 126(71.6) | 96 (88.1) | |
| Missing | 2(1.1) | 0 (0%) | |
| Lymph node status | | | |
| Negative | 101 (57.4) | 63 (57.8) | 0.192 |
| Positive | 75(42.6) | 45 (41.3) | |
| Missing | 0 (0%) | 1(0.9) | |
| AR status | | | |
| Negative | 94 (53.4) | 43 (39.4) | 0.825 |
| Positive | 66(37.5) | 45 (41.3) | |
| Missing | 16 (9.1) | 21(19.3) | |
| Chemotherapy | | | |
| No | 69 (39.2) | 16 (14.7) | 0.272 |
| Yes | 106 (60.2) | 75(68.8) | |
| Missing | 1(0.6) | 18(16.5) | |
| Patient status | | | |
| Alive | 95(54.0) | 67 (61.5) | 0.648 |
| Death | 50 (28.4) | 41(37.6) | |
| Missing | 25(14.2) | 1(0.9) | |
| Recurrence status | | | |
| No recurrence | 121 (68.8) | 53 (48.6) | 0.712 |
| Recurrence | 51(29.0) | 55(40.5) | |
| Missing | 1(0.6) | 1(0.9) | |

**Table 6.1 Clinicopathological characteristics of TNBC tumour from the 1800-cohort and the Belfast cohort**

## 6.3 Expression of AR and clinical outcome in the 1800 cohort.

AR expression levels were available in the database and were classified as positive when 1% of tumour had nuclei stained. Kaplan-Meier survival curves for AR expression were plotted for the entire cohort as shown in Figure 6.2. The log rank test was used to compare negative and positive expression. Expression of AR was not associated with overall survival (HR 0.92 95% CI 0.72-1.18, P=0.519), cancer specific survival (HR 0.92 95% CI 0.67-1.23, P=0.293) nor recurrence free survival (HR 0.85 95% CI 0.62-1.17, P=0.307).

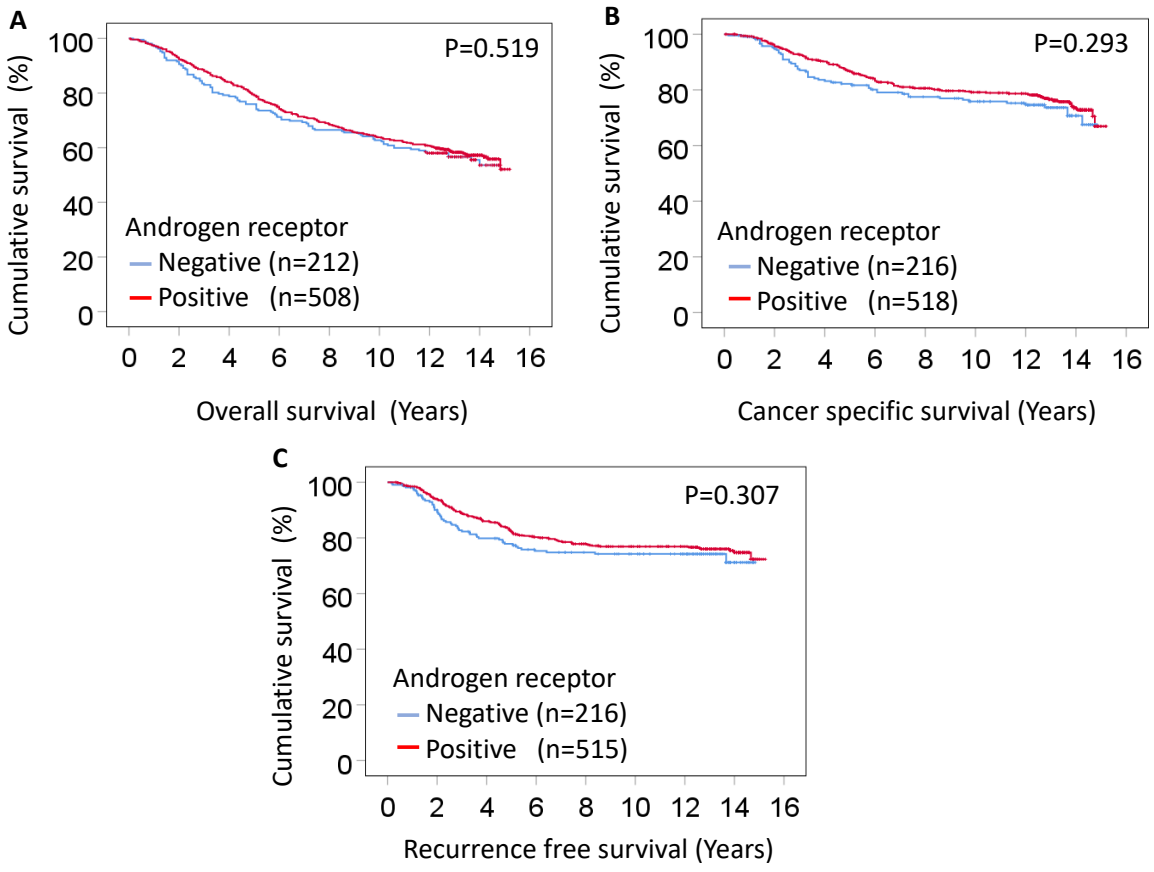


**Figure 6.2 Expression of AR and clinical outcome in the 1800-cohort.** *Kaplan Meier curves showing associations of AR with overall survival (A) cancer specific survival (B) and recurrence free survival (C).*

Kaplan-Meier survival curves for AR expression were plotted for the TNBC tumour within the 1800 cohort as shown in Figure 6.3. The log rank test was used to compare negative and positive expression. Expression of AR was not associated with overall survival (HR 1.49 95% CI 0.93-2.40, P=0.094) but was associated with decreased cancer specific survival (HR 1.86 95% CI 1.04-3.33, P=0.033 and decreased recurrence free survival (HR 1.82 95% CI 1.03-3.21, P=0.036).

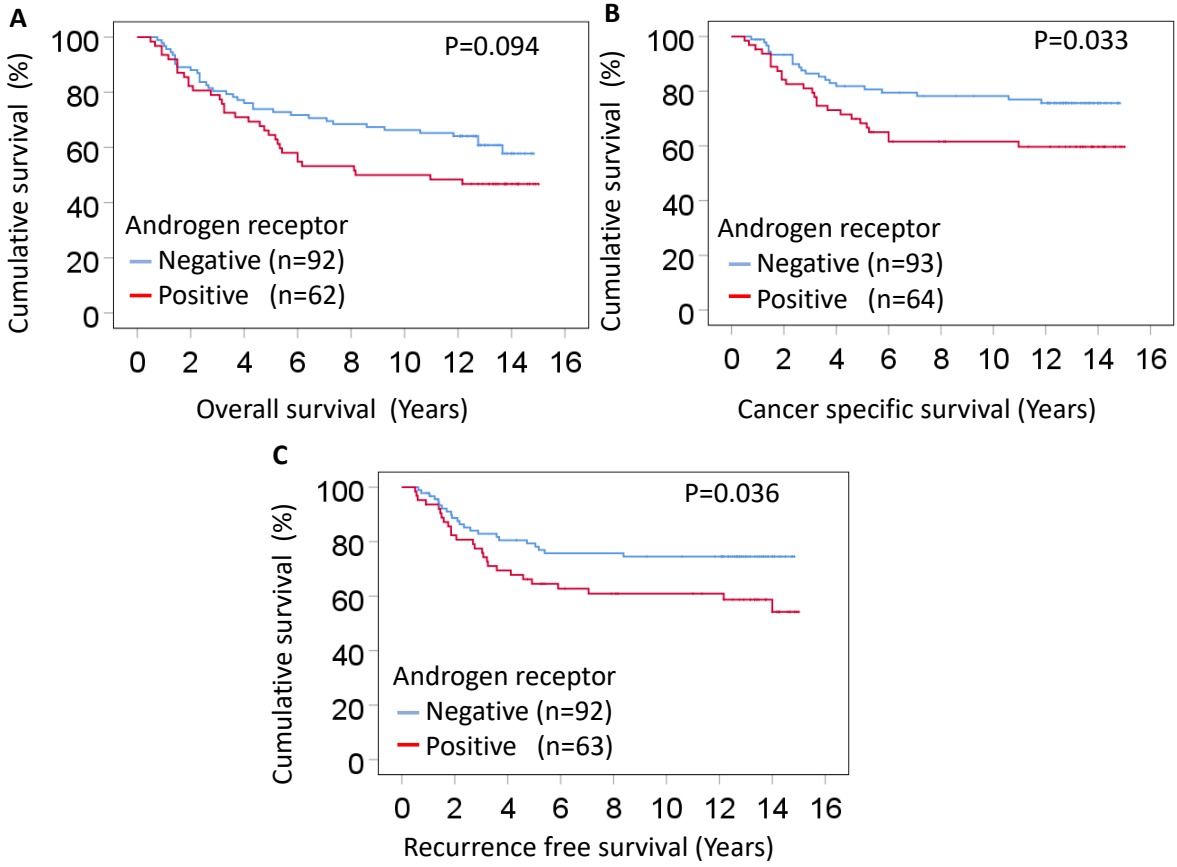


**Figure 6.3 Expression of AR and clinical outcome in TNBC tumour in the 1800-cohort.**
*Kaplan Meier curves showing associations of AR with overall survival (A) cancer specific survival (B) and recurrence free survival (C).*

## 6.4 Correlation between expression of AR, IKKα, pIKKα-S176 and pIKKα-T23 in TNBC tumours in the 1800 cohort.

As the present chapter aimed to investigate if the prognostic power of AR was enhanced when combined with IKKα, correlations between AR, IKKα, pIKKα-Ser176, and pIKKα-Thr23 were examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (2). There was a statistically significant correlation between expression of AR and IKKα (Cytoplasmic; Spearman's rho=0.144, P=0.023; nuclear Spearman's rho=-0.156, P=0.013). However, there was no statistically significant correlation between expression of AR and pIKKα-Ser176 (cytoplasmic; Spearman's rho=0.058, P=0.350; nuclear; Spearman's rho=0.051, P=0.415) or between expression of AR and pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.057, p=0.357; nuclear pIKKα-Thr23 Spearman's rho=-0.102, P=0.099).

## 6.5 Combined expression of AR, IKKα, pIKKα-Ser176, pIKKα-Thr23 and clinical outcome in the 1800 cohort.

### 6.5.1 Combined expression of AR, IKKα and clinical outcomes

In order to determine whether combined expression of AR and IKKα was associated with different clinical outcomes, the cohort was subdivided into four groups. These included tumours with low expression of both, tumours with at least one high and tumours with high expression of both AR and IKKα. In the entire cohort, combined expression of both AR and IKKα was not associated with overall survival (HR 1.04 95% CI 0.93-1.16, P=0.193), cancer specific survival (HR 1.06 95% CI 0.91-1.23, P=0.283), nor recurrence free survival (HR 1.04 95% CI 0.89-1.23, P=0.333) (Figure 6.4).

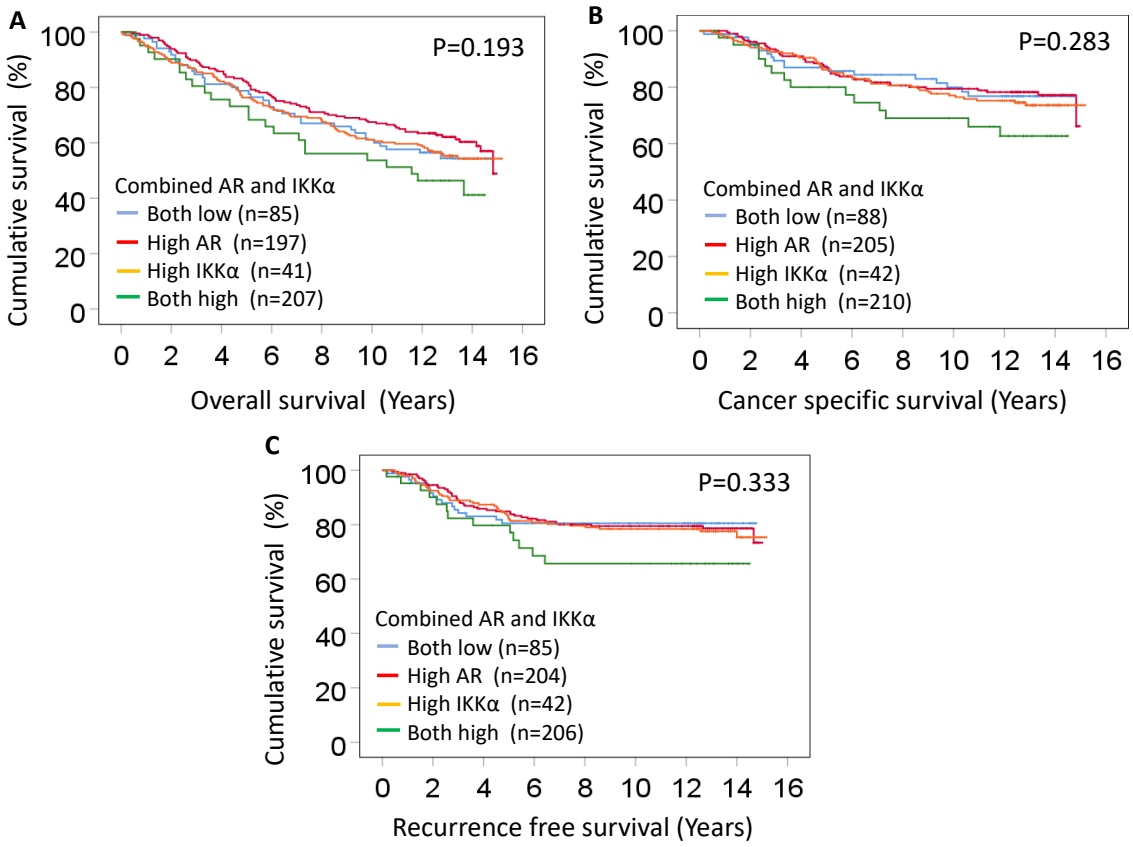


**Figure 6.4 Combined expression of cytoplasmic IKKα, AR and clinical outcome in the 1800 cohort.** *Kaplan Meier curves showing associations of combined expression of cytoplasmic IKKα and AR with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

In the TNBC subgroup, combined expression of AR and cytoplasmic IKKα had a trend towards a statistically significant association with decreased cancer specific survival (HR 1.26 95% CI 0.93-1.70, P=0.052). However, combined expression of both AR and cytoplasmic IKKα was not associated with overall survival (HR 1.23 95% CI 0.96-1.57, P=0.128), nor recurrence free survival (HR 1.18 95% CI 0.87-1.62, P=0.457) (Figure 6.5).

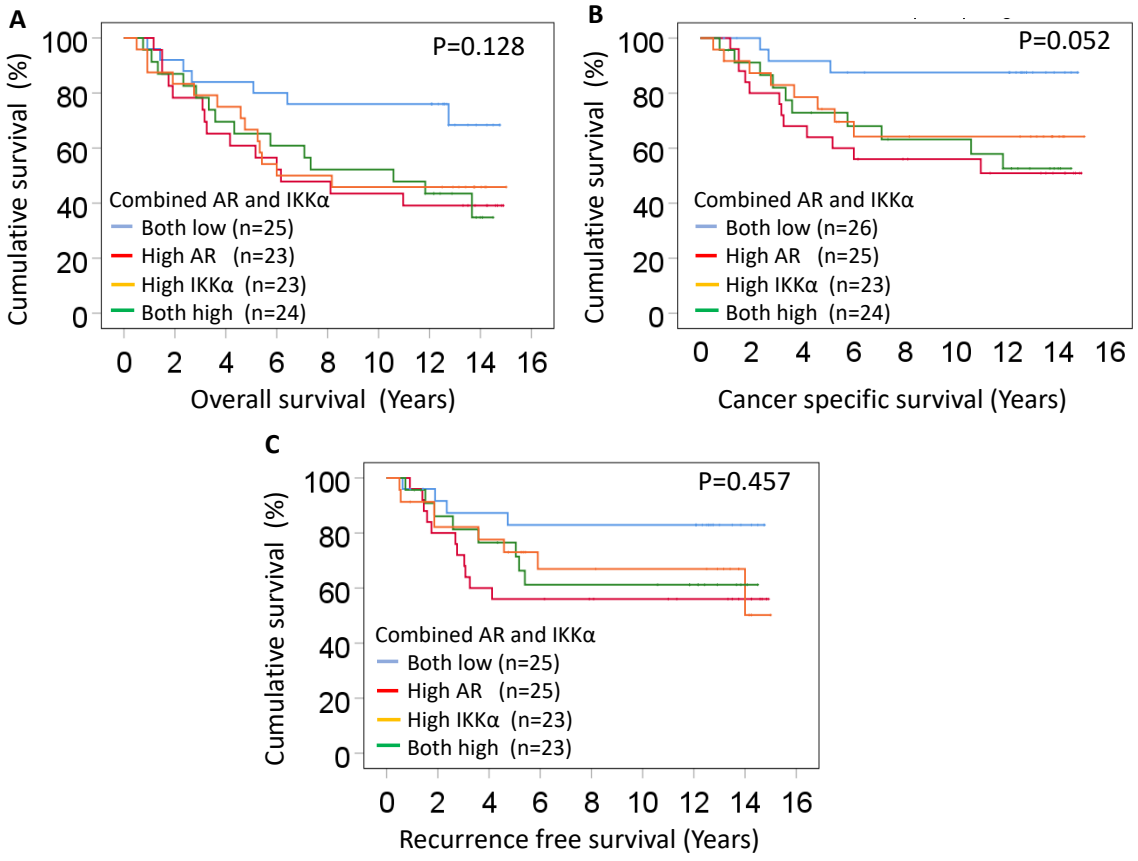


**Figure 6.5 Combined expression of AR, cytoplasmic IKKα and clinical outcome in the TNBC subgroup in the 1800 cohort.** *Kaplan Meier curves showing associations of combined expression of cytoplasmic IKKα and AR with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

## 6.5.2 Combined expression of AR, pIKKα-Ser176 and clinical outcomes

To determine whether combined expression of AR and pIKKα-Ser176 was associated with different clinical outcomes, the cohort was subdivided into four groups. These included tumours with low expression of both, tumours with at least one high and tumours with high expression of both AR and pIKKα-Ser176.

In the entire cohort, combined expression of AR and pIKKα-Ser176 was associated with overall survival (HR 0.84 95% CI 0.75-0.95, P=0.038) and had a trend towards a statistically significant association with cancer specific survival (HR 0.84 95% CI 0.71-0.98, P=0.051) with patients with high AR doing the worst and those with high IKKα-Ser176 doing the best. However, the combined expression was not associated with recurrence free survival (HR 0.85 95% CI 0.72-1.00, P=0.243) (Figure 6.6).

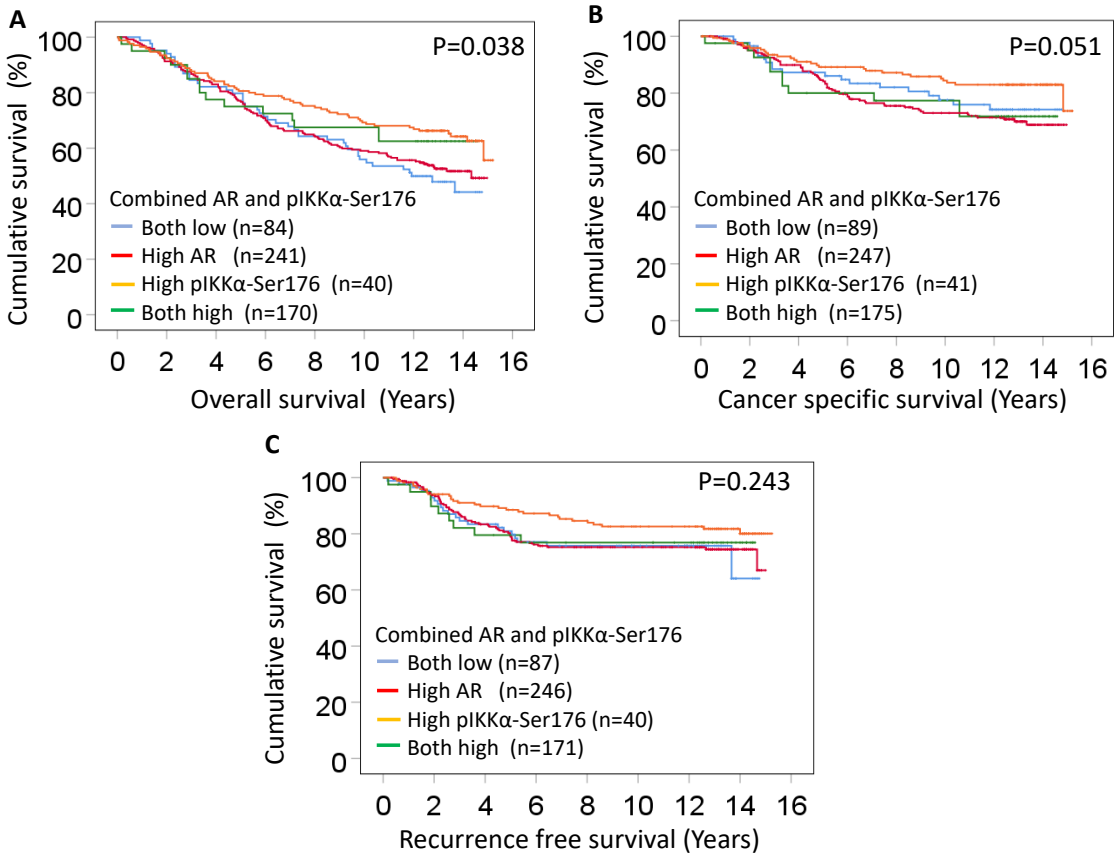


**Figure 6.6 Combined expression of AR, cytoplasmic pIKKα-Ser176 and clinical outcomes of the 1800-cohort.** *Kaplan Meier curves showing associations of combined AR, cytoplasmic pIKKα-Ser176 with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

In the TNBC subgroup, combined expression of AR and cytoplasmic pIKKα-Ser176 was associated with overall survival (HR 3.38 95% CI 1.16-9.84, P=0.026) with patients with high AR doing the worst and those with high IKKα-Ser176 doing the best. However, it was not associated with cancer specific survival (HR 1.39 95% CI 0.33-5.83, P=0.083) and recurrence free survival (HR 0.90 95% CI 0.63-1.28, P=0.347) (Figure 6.7).

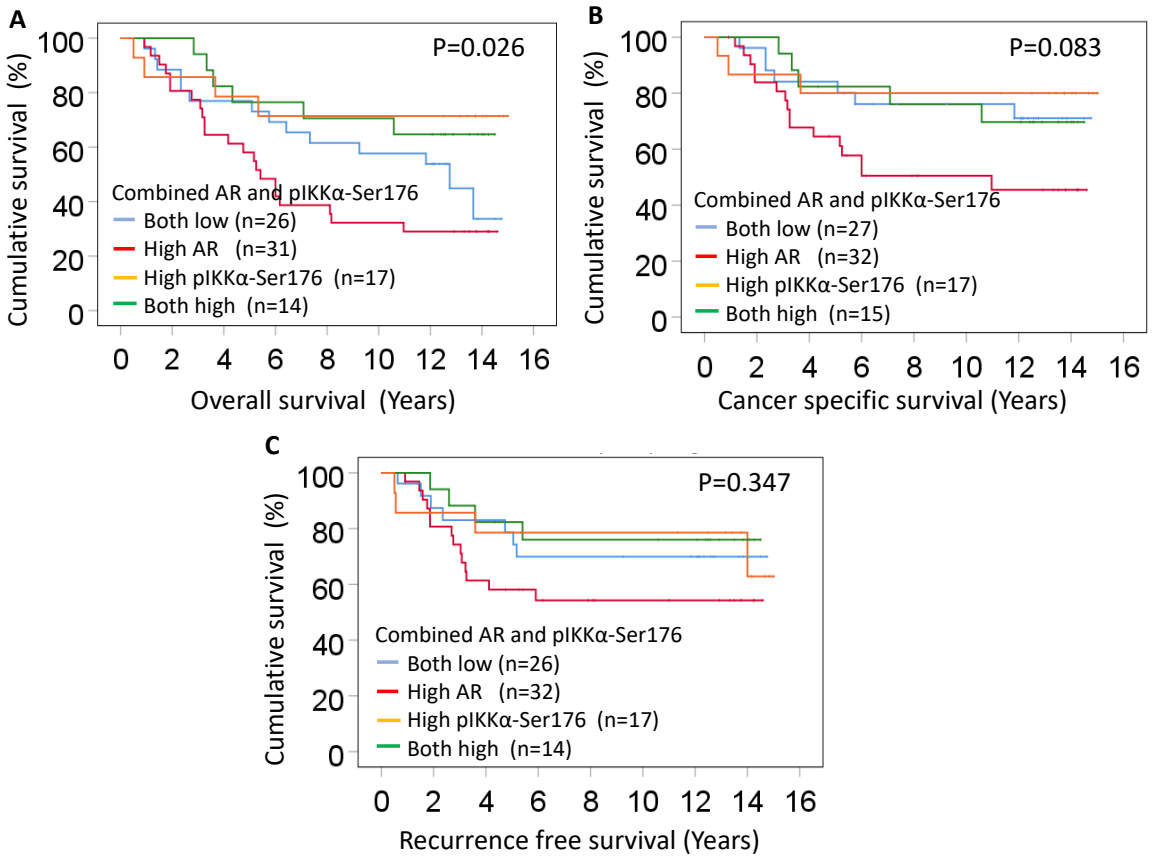


**Figure 6.7 Combined expression of AR, cytoplasmic pIKKα-Ser176 and clinical outcomes in TNBC in the 1800-cohort.** *Meier curves showing associations of combined AR, cytoplasmic pIKKα-Ser176 with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

### 6.5.3 Combined expression of AR and pIKKα-Thr23 and clinical outcomes

To determine whether combined expression of AR and pIKKα-Thr23 was associated with different clinical outcomes, the cohort was subdivided into four groups. These included tumours with low expression of both, tumours with at least one high and tumours with high expression of both AR and pIKKα-Thr23.

In the entire cohort, combined expression of AR and cytoplasmic pIKKα-Thr23 was not associated with overall survival (HR 0.96 95% CI 0.85-1.04, P=0.574), cancer specific survival (HR 1.03 95% CI 0.88-1.21, P=0.503) nor recurrence free survival (HR 1.05 95% CI 0.90-1.24, P=0.443) (Figure 6.8).

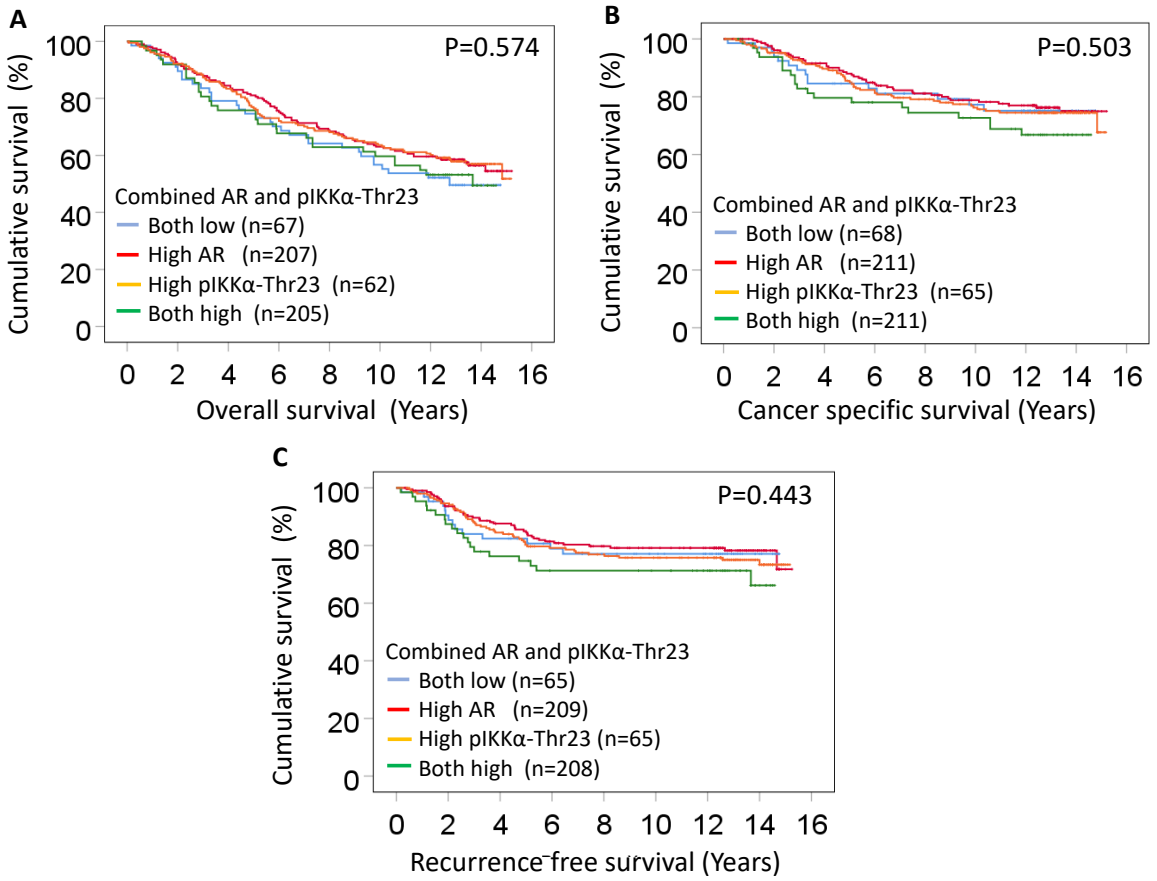


**Figure 6.8 Combined expression of AR, cytoplasmic pIKKα-Thr23 and clinical outcomes of the 1800-cohort.** *Kaplan Meier curves showing associations of combined AR, cytoplasmic pIKKα-Ser176 with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

In the entire cohort, combined expression of AR and nuclear pIKKα-Thr23 was not associated with overall survival (HR 0.89 95% CI 0.79-1.00, P=0.191), cancer specific

survival (HR 0.95 95% CI 0.81-1.11, P=0.635) nor recurrence free survival ((HR 0.96 95% CI 0.82-1.12, P=0.627) (Figure 6.9).

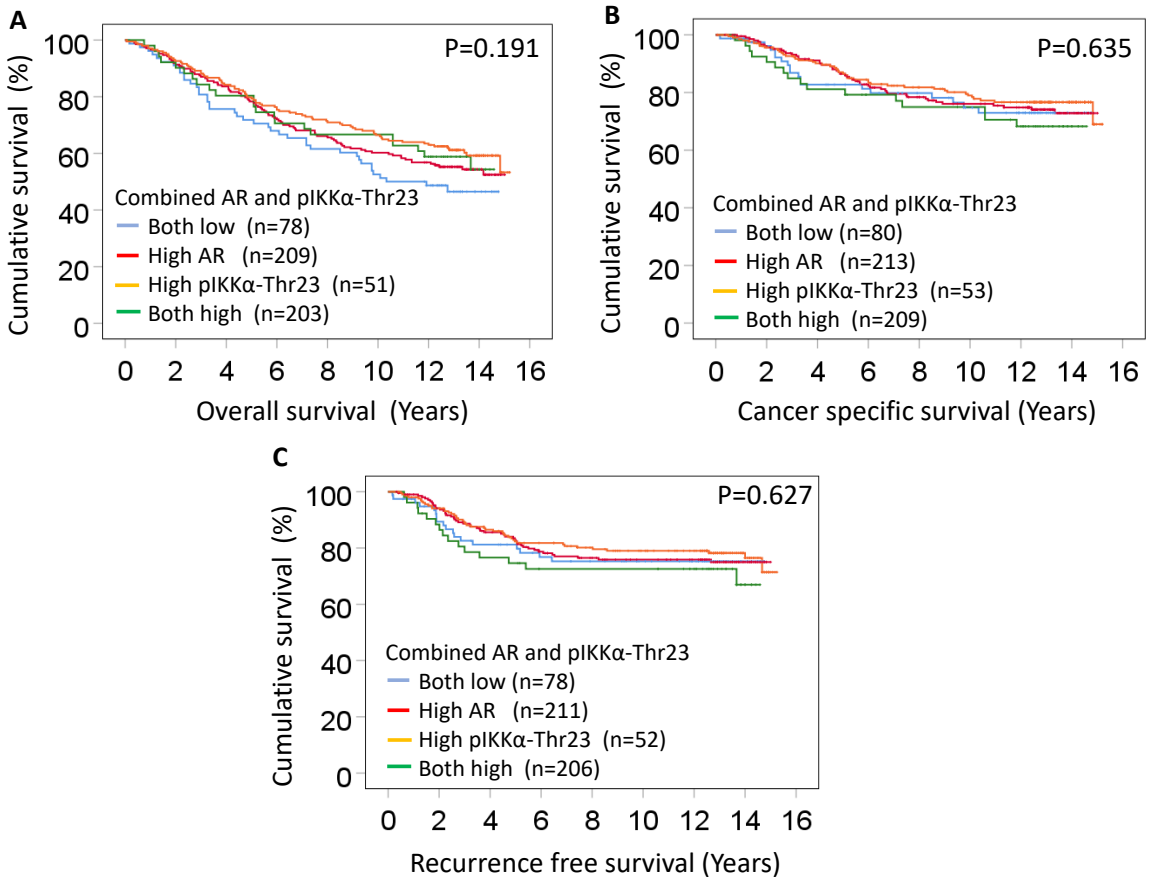


**Figure 6.9 Combined expression of AR, nuclear pIKKα-Thr23 and clinical outcomes of the 1800-cohort.** *Kaplan Meier curves showing associations of combined AR, cytoplasmic pIKKα-Thr23 with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

In the TNBC subgroup, combined expression of AR and cytoplasmic pIKKα-Thr23 was associated with cancer specific survival (HR 1.62 95% CI 1.15-2.30, P=0.040) with high IKKα-Thr23 doing the worst but was not associated with overall survival (HR 1.32 95% CI 1.00-1.73, P=0.173), nor recurrence free survival (HR 1.52 95% CI 1.07-2.15, P=0.095) (Figure 6.10).

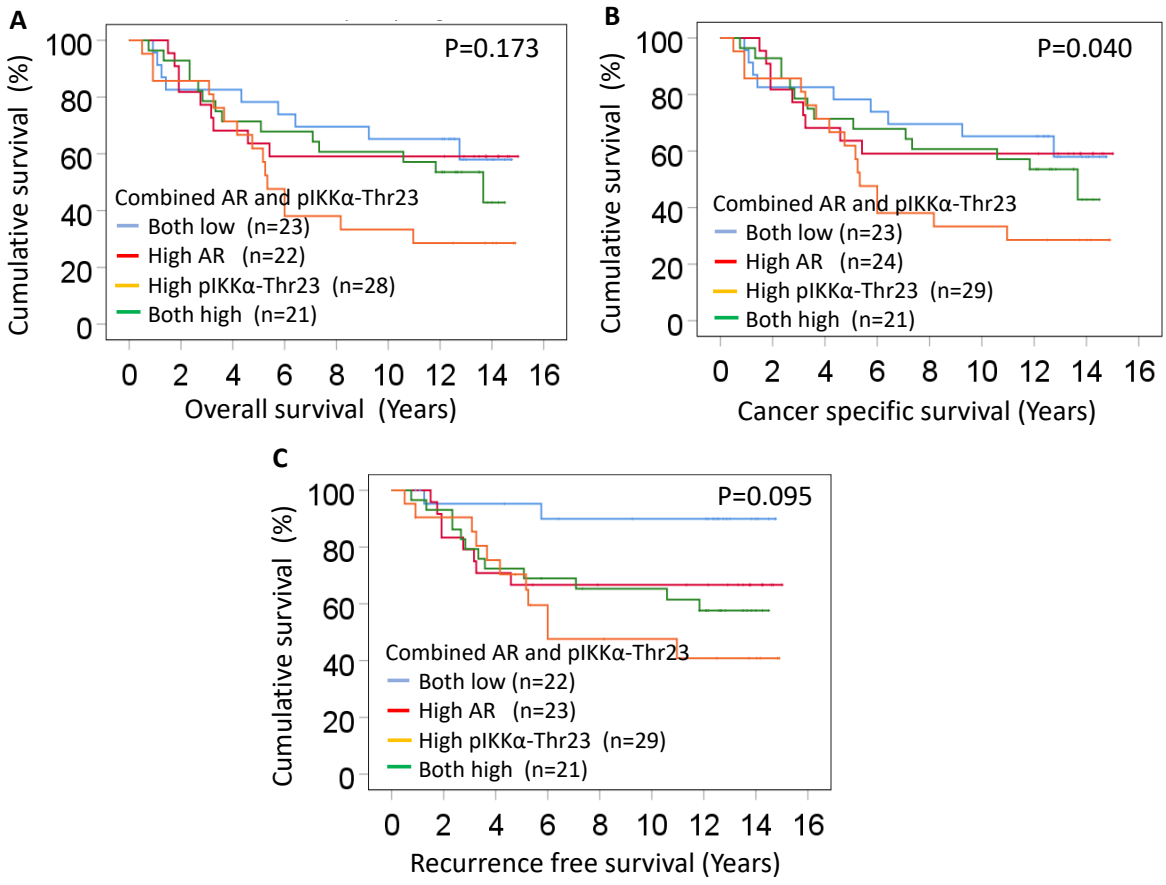


**Figure 6.10 Combined expression of AR, cytoplasmic pIKKα-Thr23 and clinical outcomes in TNBC in the 1800-cohort.** *Kaplan Meier curves showing associations of combined AR, cytoplasmic pIKKα-Ser176 with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

In the TNBC subgroup, combined expression of AR and nuclear pIKKα-Thr23 was associated with cancer specific survival (HR 3.19 95% CI 1.00-10.18, P=0.044) with high IKKα-Thr23 doing the worst but was not associated with overall survival (HR 1.24 95% CI 0.55-2.80, P=0.085) and recurrence free survival (HR 2.08 95% CI 0.68-6.35, P=0.100) (Figure 6.11).

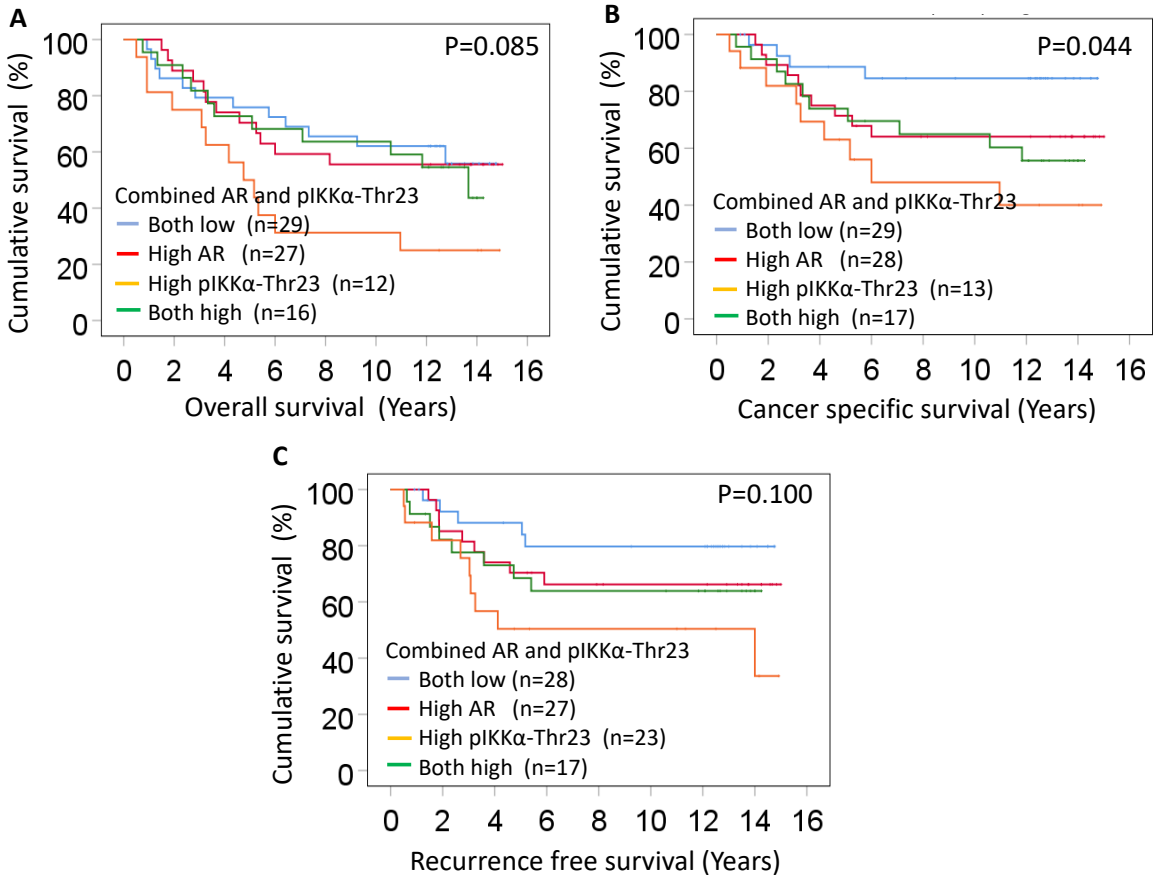


**Figure 6.11 Combined expression of AR, nuclear pIKKα-Thr23 and clinical outcomes in TNBC in the 1800-cohort.** *Kaplan Meier curves showing associations of combined AR, cytoplasmic pIKKα-Ser176 with overall survival (A), cancer specific survival (B) and recurrence free survival (C)*

## 6.6 Expression of AR and clinical outcome in the Belfast cohort

Kaplan-Meier survival curves for AR expression in the Belfast cohort were plotted as shown in Figure 6.12. The log rank test was used to compare negative and positive expression. Expression of AR was not associated with overall survival (HR 0.60 95% CI 0.25-1.45, P=0.249) nor recurrence free survival (HR 1.27 95% CI 0.66-2.43, P=0.473).

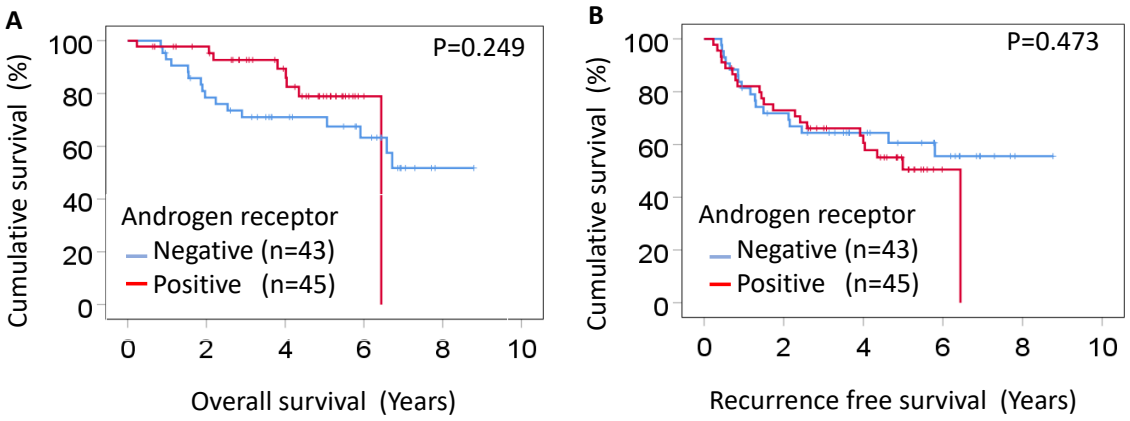


**Figure 6.12 Expression of AR and clinical outcome in the Belfast cohort.** *Kaplan Meier curves showing associations of AR with overall survival (A) and recurrence free survival (B).*

## 6.7 Correlation between expression of AR, IKKα, pIKKα-Ser176 and pIKKα-Thr23 in TNBC tumours in the Belfast cohort

The correlation between AR, IKKα, pIKKα-Ser176, and pIKKα-Thr23 was examined by the Spearman rank correlation coefficient. Preliminary analysis showed the relationship to be monotonic, as assessed by visual inspection of a scatterplot (199). There was no statistically significant correlation between expression of AR and IKKα (Cytoplasmic; Spearman's rho=0.150, P=0.234; nuclear Spearman's rho=-0.026, P=0.837). There was a statistically significant, moderate correlation between expression of AR and cytoplasmic pIKKα-Ser176 (Spearman's rho=0.444, P<0.001) and a statistically significant, weak correlation between AR and nuclear pIKKα-Ser176 (Spearman's rho=-0.309, P=0.014). There was no statistically significant correlation between expression of AR and expression of pIKKα-Thr23 (Cytoplasmic; Spearman's rho=0.167, p=0.183; nuclear pIKKα-Thr23 Spearman's rho=-0.001, P=0.994).

## 6.8 Combined expression of AR, IKKα, pIKKα-S176, pIKKα-Thr23 and clinical outcome

### 6.8.1 Combined expression of AR and IKKα and clinical outcomes

In order to determine whether combined expression of AR and IKKα was associated with different clinical outcomes, the cohort was subdivided into four groups. These included tumours with low expression of both, tumours with at least one high and tumours with high expression of both AR and IKKα. Combined expression of AR and IKKα was not associated with overall survival (HR 0.70 95% CI 0.48-1.02, P=0.093) and recurrence free survival (HR 0.93 95% CI 0.70-1.25, P=0.908) (

Figure 6.13).

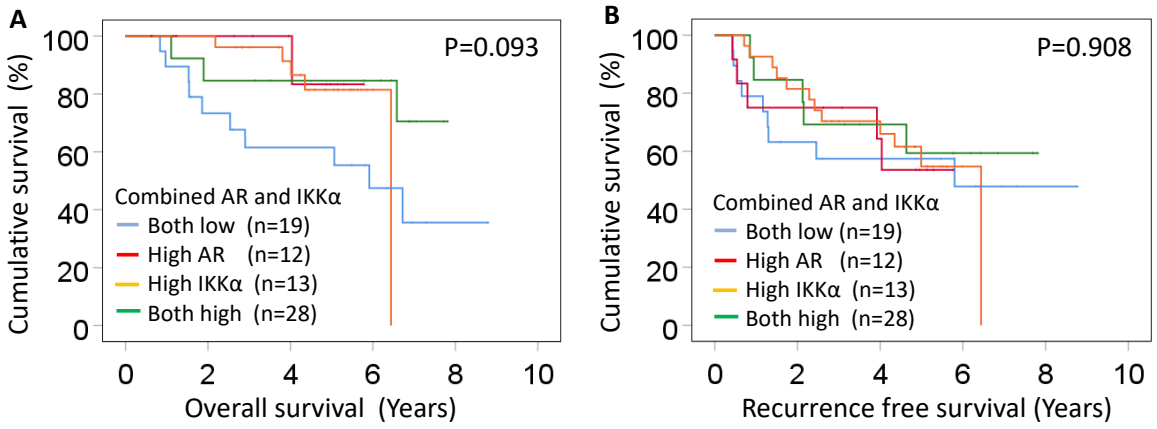


**Figure 6.13 Combined expression of AR, cytoplasmic IKKα and clinical outcome in the Belfast cohort.** *Kaplan Meier curves showing associations of combined expression of cytoplasmic IKKα and AR with overall survival (A) and recurrence free survival (B)*

## 6.8.2 Expression of AR, pIKKα-Ser176 and clinical outcomes

Kaplan-Meier survival curves were plotted and the log rank test for overall trend was reported for overall survival and recurrence free survival (Figure 6.14). Combined expression of AR and pIKKα-Ser176 was not associated with overall survival (HR 1.05 95% CI 0.67-1.65, P=0.081). However, it was associated with recurrence free survival (HR 1.35 95% CI 0.96-1.91, P=0.009).

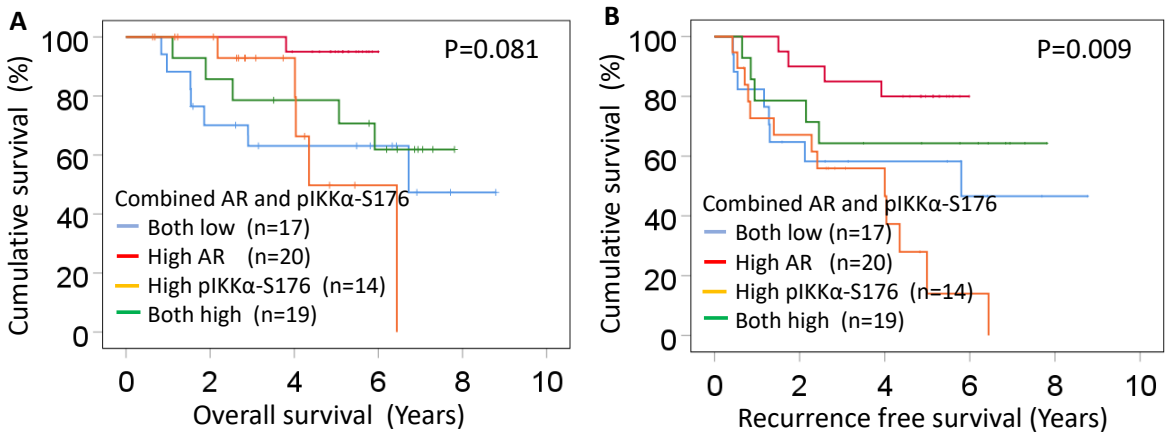


**Figure 6.14 Combined expression of AR, cytoplasmic pIKKα-Ser176 and clinical outcome in the Belfast cohort.** *Kaplan Meier curves showing associations of combined expression of AR and cytoplasmic pIKKα-Ser176 with overall survival (A) and recurrence free survival (B)*

### 6.8.3 Expression of AR, pIKKα-Thr23 and clinical outcomes

Kaplan-Meier survival curves were plotted and the log rank test for overall trend was reported for overall survival and recurrence free survival (Figure 6.15). Combined expression of both AR and cytoplasmic pIKKα-Thr23 was not associated with overall survival (HR 0.74 95% CI 0.44-1.25, P=0.294) nor recurrence free survival (HR 1.08 95% CI 0.74-1.55, P=0.953).

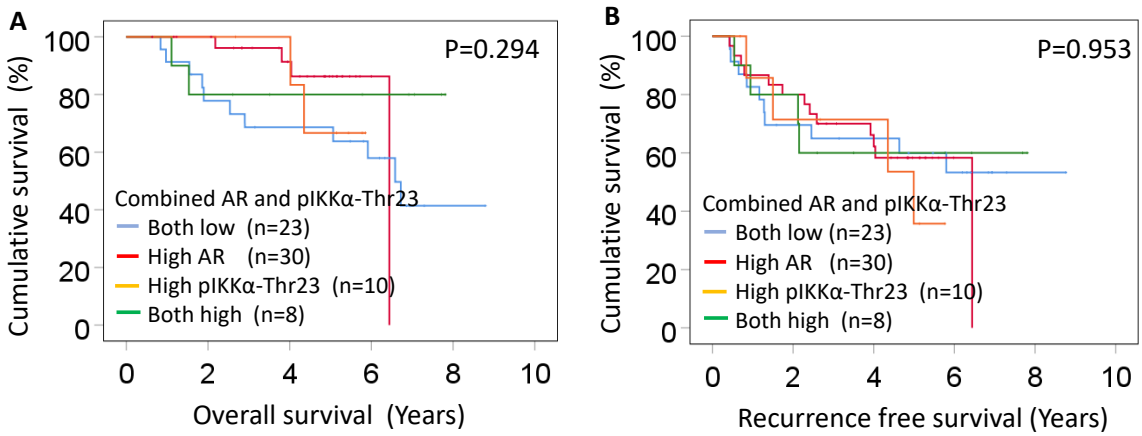


**Figure 6.15 Combined expression of AR, cytoplasmic pIKKα-Thr23 and clinical outcome in the Belfast cohort.** *Kaplan Meier curves showing associations of combined expression of AR and cytoplasmic pIKKα-Thr23 with overall survival (A) and recurrence free survival (B)*

Kaplan-Meier survival curves were plot and the log rank test for overall trend was reported for overall survival and recurrence free survival (Figure 6.16). Combined expression of both AR and nuclear pIKKα-Thr23 was not associated with overall survival (HR 1.00 95% CI 0.64-1.56, P=0.679) nor recurrence free survival (HR 1.13 95% CI 0.80-1.58, P=0.454).

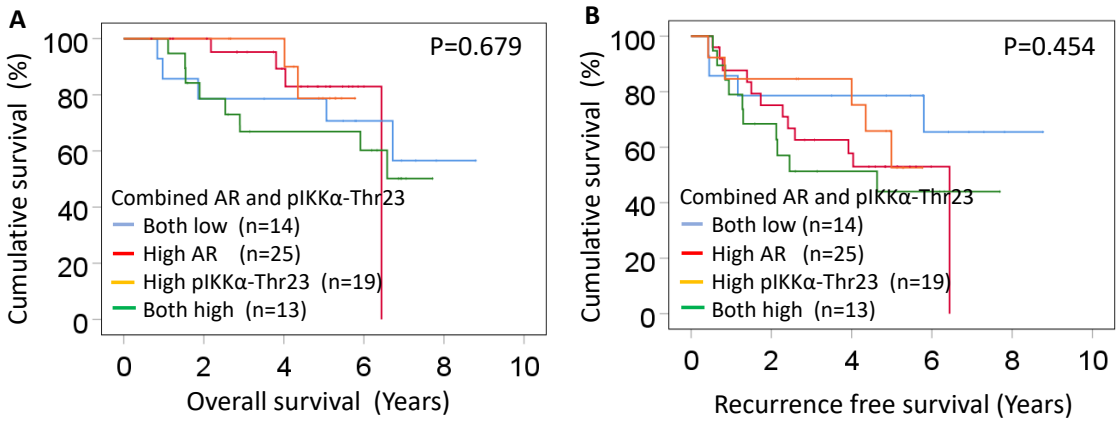


**Figure 6.16 Combined expression of AR, nuclear pIKKα-Thr23 and clinical outcome in the Belfast cohort.** *Kaplan Meier curves showing associations of combined expression of AR and nuclear pIKKα-Thr23 with overall survival (A) and recurrence free survival (B)*

## 6.9 Summary the whole thesis aims and finding from patient's tissue studies

The current thesis hypothesis is that IKKα and its phosphorylation are associated with breast cancer progression, resistance to treatment, and potentially a novel therapeutic target for breast cancer treatment. To address this hypothesis, the first objective was to establish if the expression of IKKα, pIKKα-Ser176, or pIKKα-Thr23 could be employed as prognostic markers in breast cancer. NIK and pAKT were also assessed as they are the upstream kinases of IKKα. The schematic represents the whole thesis aims showed in Figure 6.17.

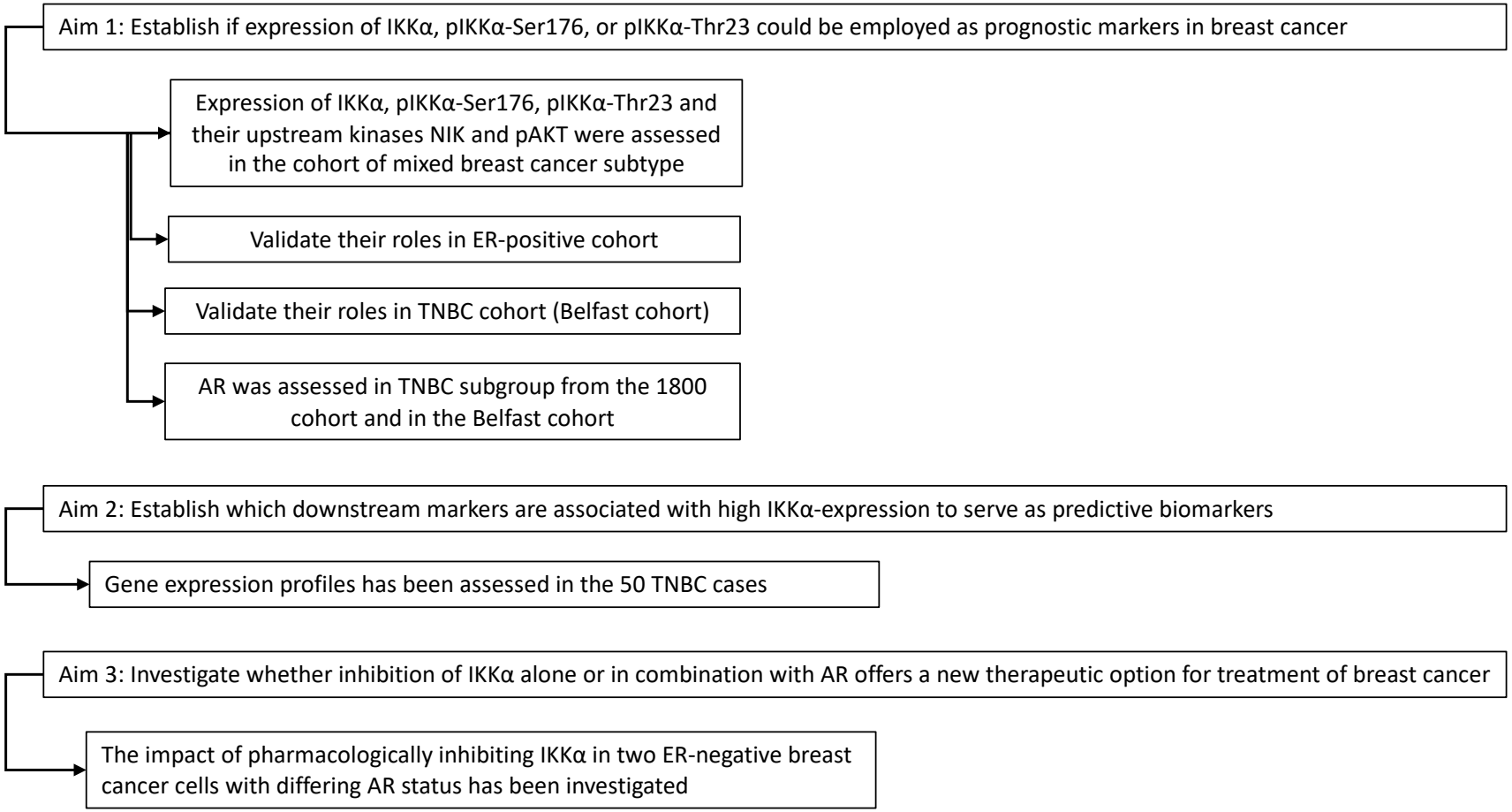


**Figure 6.17 Schematic represents the whole thesis aims.**

Firstly, the expression of these proteins was assessed in the cohort of mixed breast cancer subtypes. IKKα was not associated with survival outcomes. pIKKα-Ser176 was associated with longer cancer specific survival in luminal B. pIKKα-Thr23 was associated with decreased cancer specific survival and recurrence free survival in TNBC, decreased recurrence free survival in luminal A but was associated with increased cancer specific survival and recurrence free survival in HER2-positive. NIK was associated with longer cancer specific survival and recurrence free survival. pAKT was associated with decreased recurrence free survival. Although IKKα was not associated with the outcome measures in this cohort, expression of IKKα was also assessed in the cohort of ER-positive patients. This finding may be inconclusive since the 95% CI for IKKα was broad (HR 1.14 95% CI 0.81-1.60). Therefore, we decided to confirm this finding in the ER-positive cohort.

The next step was validating the findings in the ER-positive tamoxifen-treated cohort. The result showed that cytoplasmic IKKα was associated with decreased cancer specific survival, recurrence free survival and recurrence on tamoxifen. Cytoplasmic pIKKα-Ser176 was associated with increased cancer specific survival. Nuclear pIKKα-Thr23 was associated with decreased cancer specific survival, recurrence free survival and recurrence on tamoxifen. No associations were observed between NIK, pAKT and clinical outcomes.

The findings were also validated in the TNBC Belfast cohort. The findings demonstrated that cytoplasmic IKKα was associated with longer overall survival. pIKKα-Ser176 was associated with decrease overall survival and recurrence free survival. pIKKα-Thr23 was not associated with outcomes. NIK and pAKT were not assessed in the Belfast cohort because it the limitations of tissue samples. Up to this step, the prognostic role of IKKα, pIKKα-Ser176 and pIKKα-Thr23 differed among breast cancer subtypes and their cellular locations. However, several limitations might affect these findings and will be summarised and discussed in chapter 9.

The next step of the thesis was assessing the expression of AR and clinical outcomes in the TNBC subgroup from the 1800 cohort and the TNBC Belfast cohort. The rationale for performing this step was that AR expression had been extensively studied in TNBC and used for subtyping TNBC into the luminal AR subtypes (42). The evidence of crosstalk between AR and NF-κB has been demonstrated in prostate cancer but is still unknown in breast cancer (237). Recent work from our lab demonstrated the prognostic role of cytoplasmic IKKα and its phosphorylation of threonine 23 in prostate cancer (52). A high level of nuclear IKKα

was associated with a lower time to death from recurrence in castration-resistant prostate cancer (52). Therefore, this thesis will also investigate the role of AR in combination with IKKα and its phosphorylation to demonstrate if they have an enhancing effect in the TNBC subgroup. The results for this step have been previously demonstrated.

Another aim of the thesis was to establish which downstream markers are associated with high IKKα-expression to serve as predictive biomarkers. Profiles identified will be matched with suitable antibody panels for IHC and IHC performed on our validation cohort to establish that expression of the novel biomarkers correlates with high IKKα expression and clinical outcome measures. This experiment is designed to establish if markers associated with high levels of IKKα in clinical specimens can be employed as predictive markers of response to IKKα inhibitors clinically. The results have been demonstrated and discussed in chapter 7.

The last step was to investigate whether inhibition of IKKα alone or in combination with AR offers a new therapeutic option for the treatment of breast cancer. Our proprietary preclinical selective IKKα inhibitors, androgen blockade and a combination of both will be investigated in breast cancer cell lines to establish if IKKα represents a clinical target for breast cancer treatment alone or in combination therapy. The results have been demonstrated and discussed in chapter 8.

## 6.10 Discussion

As there is limited evidence on crosstalk between AR and NF-κB in breast cancer, the present chapter investigated if the prognostic power of AR is enhanced when combined with IKKα and its phosphorylation site.

In the 1800-cohort, expression of AR was not associated with survival outcome, but was in the TNBC subgroup. In the TNBC subgroup AR expression was associated with cancer specific survival and recurrence free survival but not overall survival. Unfortunately for the Belfast cohort, cancer specific survival was not available, and AR expression was not associated with recurrence free survival or overall survival. The prognostic role of AR was similar when looking at overall survival. The lack of association with recurrence in the cohort could be due to a lack of power in the 1800 cohort there were 176 TNBC patients with 12.5 years median follow-up, however in the Belfast cohort there were 109 TNBC patients with only 3.70 years median follow-up. This may account of the discrepancies observed.

Such disparity is evident also in the literature, with some studies reporting that AR is a poor prognostic marker for early stage TNBC tumours correlating with older age and lower histologic grade (45, 238), with other studies reporting that AR is a good prognostic marker (47, 48). Although the prognostic role of AR in early stage TNBC being inconclusive, it has still been suggested that targeting the AR-positive tumour may be beneficial (50, 239, 240).

Reports suggest crosstalk between AR and NF-κB in prostate cancer (232, 237). Prostate cancer cell lines that expressed p52 exhibited better survival ability after being exposed to enzalutamide (232). Expression of p52 enhanced the level of AR splice variants that are responsible for resistance to enzalutamide (232). Silencing AR splice variants down regulated the expression of p52 leading to increased sensitivity to enzalutamide (232). However, evidence demonstrating a link between AR and NF-κB in breast cancer is limited. Therefore, the present chapter also investigated the prognostic role of the combined expression of AR and IKKα to investigate if combining these markers provided additive prognostic value.

In the 1800 cohort, combined expression of AR and cytoplasmic IKKα was not associated with any survival outcome, but those patients expressing both AR and IKKα, did have the shortest median survival. This observation was not upheld in the TNBC subgroup, as similar

survival was observed for those with high AR or IKKα or both high, but those patients with low AR and IKKα did have the best prognosis, suggesting that dual targeting might be of benefit for those expressing high AR or high IKKα. In the Belfast TNBC cohort, no associations with survival were noted, but those patients with low expression of both had the worst prognosis. This result be explained by the finding that, in the Belfast cohort, patient with either low AR or IKKα had shorter overall survival. Therefore, the combination of low AR with low IKKα suggest a shorter overall survival, however, this was not statistically significant. As patient numbers were limited in the Belfast cohort, the findings require to be validated in a larger independent study.

In contrast, in both cohorts no additive association was observed for AR when combine with cytoplasmic pIKKα-Ser176, suggesting that classical activation of the IKKα non canonical pathway does not synergise with AR signalling. Those patients with high pIKKα-Ser176 expression were associated with good prognosis, in the 1800 cohort, suggesting that targeting IKKα might not be a favourable option, however it is unknow if it is IKKα expression or phosphorylation status that is associated with response to inhibition, this would need to be explored in in vitro and in vivo studies.

In the 1800 cohort, combined expression of AR and pIKKα-Thr23 was not associated with survival outcomes when analysed for the entire cohort regardless of its cellular distribution. However, a significant association was observed in the TNBC subgroup for both cytoplasmic and nuclear expression. Combined expression did not provide additive prognostic power with high pIKKα-Thr23 both in the cytoplasm and nucleus having the worst prognosis compared to the other groups. Again, this effect was not validated in the Belfast TNBC cohort and could be due to an insufficiently powered cohort as discussed above.

In conclusion, the current study has not been able to state any firm conclusions when analysing combined expression of AR and IKKα. The weight of evidence suggests that total expression or phosphorylation at the two sites did not provide any additional prognostic power over the two factors alone for any survival measure. Overall, high AR and high pIKKα-Thr23 associated with poor cancer specific survival in TNBC when expressed separately with pIKKα-Thr23 having the more detrimental effect. These results were could not be validated in the Belfast TNBC cohort as cancer specific survival was not available. However, it does suggest that targeting these two factors in patients with high expression

may be beneficial. However, as the same trend was not noted with Ser176, in contrast low expression of this combined with high AR was the worse prognostic group. Therefore, it is clear, that co-targeting AR and IKKα could be beneficial or detrimental therefore further studies that include a greater number of TNBC patients are required to fully explore the prognostic value of AR and IKKα and to determine their therapeutic potential.

# Chapter 7 : Differential gene expression profiles associated with high IKKα, pIKKα-Ser176 and pIKK-Thr23 expression

## 7.1 Introduction

In chapter 3, which contained both ER-positive and ER-negative breast cancers, the expression of cytoplasmic pIKKα-Ser176 was associated with a good prognosis. However, in the ER-positive cohort (The STB cohort), the expression of cytoplasmic IKKα and nuclear pIKKα-Thr23 were associated with poor prognosis. In chapter 5, the TNBC cohort (The Belfast cohort), cytoplasmic IKKα was associated with a good prognosis, but pIKKα-Ser176 with worse prognosis. Therefore, as IKKα and pIKKα-Ser176 had opposing associations with prognosis in ER-negative cancer, we were interested in investigating if this resulted in differing gene profiles.

The gene expression and molecular pathways associated with the expression of IKKα pIKKα-Ser176 and pIKKα-Thr23 are incompletely known. Therefore, this chapter aimed to evaluate differential gene expression associated with high expression of IKKα, pIKKα-Ser176 and pIKKα-Thr23. Such expression can further characterise a functional group by performing the Gene Ontology (GO) term and pathway analysis. The GO term and pathway analysis can further identify such DEGs associated with distinct biological functions, representing a common mechanism of tumours with high expression of IKKa, pIKKα-Ser176 and pIKKα-Thr23.

In this chapter, the Templated Oligo assay with Sequencing readout (TempO-Seq) was utilized on FFPE cancer specimens to identify differentially expressed gene profiles for tumours with low versus high cytoplasmic IKKα, for tumours with low versus high cytoplasmic pIKKα-Ser176 and for tumours with low versus high nuclear pIKKα-Thr23 in ER-negative tumours. The gene profiles of these three groups will then be compared.

The only samples available to use were FFPE, however tissue fixation results in formaldehyde modification of RNA leading to degradation and fragmentation during extraction. Therefore, to overcome this, the Templated Oligo-Sequencing (TempO-Seq) (Bioclavis) assay was employed which does not require RNA extraction and is therefore more reliable and less prone to error than traditional methods. TempO-Seq is a highly efficient multiplexed molecular profiling platform, which uses sample barcoding and pooling into libraries for quick high throughput profiling of the whole protein-coding transcriptome of ~20,000 genes. The extremely high analytical sensitivity (down to single-cell level) and specificity (99.8% for a single base mismatch) of TempO-Seq is derived (in part) due to proprietary ligation and enzymatic discrimination.

Therefore, in the present study, as FFPE tissue was the only tissue available for analysis, gene expression profiling was performed on ER negative cancer specimens who's cytoplasmic IKKα, cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23 expression levels were known to allow comparison between both sample groups.

## 7.2 Material and method

### 7.2.1 Cohort selection

Gene expression profiles of the archival FFPE tissue samples from 50 TNBC of the 1800 cohort were available for analysis. Of the 50 samples, 48 had IHC profiles for IKKα, 47 had IHC profiles for pIKKα-Ser176, and 44 had IHC profiles for pIKKα-Thr23.

Among the 48 cases with the IHC profiles for IKKα, 33 had high, and 15 had low expression of cytoplasmic IKKα. Of the 47 cases with the IHC profiles for pIKKα-Ser176, 28 had high, and 19 had low expression of cytoplasmic pIKKα-Ser176. Of those 44 cased with the IHC profiles for pIKKα-Thr23, 23 had high, and 21 had low expression of nuclear pIKKα-Thr23. The clinicopathological characteristics of these samples were available.

Kaplan-Meier survival curves for expression of cytoplasmic pIKK, cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23 were plotted as shown in Figure 7.1. The log rank test was used to compare low and high expression. There was no association found between cancer specific survival and cytoplasmic IKKα (HR = 0.98 95% CI 0.34-2.81, P=0.962), cytoplasmic pIKKα-Ser176 (HR = 0.92 95% CI 0.34-2.49, P=0.879) and nuclear pIKKα-Thr23 (HR = 1.90 95% CI 0.69-5.26, P=0.203) (Figure 7.1). Since there was a small sample size in each group, the study was underpowered to detect the difference in survival outcomes.

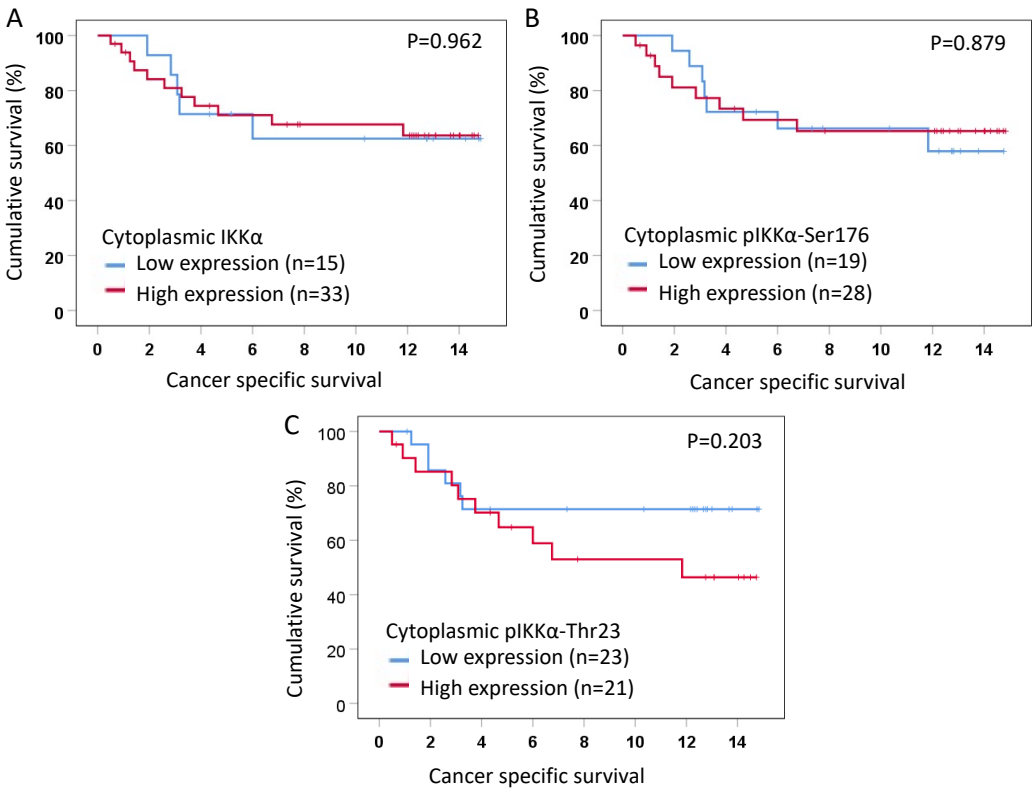


**Figure 7.1 Expression of IKKα, pIKKα-Ser176, pIKKα-Thr23 and cancer specific survival and.** *Kaplan Meier curves showing associations of cancer specific survival and expression of cytoplasmic IKKα (A), cytoplasmic pIKKα-Ser176 (B) and nuclear pIKKα-Thr23 (C)*

Similarly, no association was observed between recurrence free survival and cytoplasmic IKKα (HR = 1.20 95% CI 0.43-3.37, P=0.732), cytoplasmic pIKKα-Ser176 (HR = 1.08 95% CI 0.42-2.82, P=0.973) and nuclear pIKKα-Thr23 (HR = 1.79 95% CI 0.69-4.63, P=0.225) (Figure 7.2). Again, as there was a small sample size in each group, the study was underpowered to detect the difference in survival outcomes.

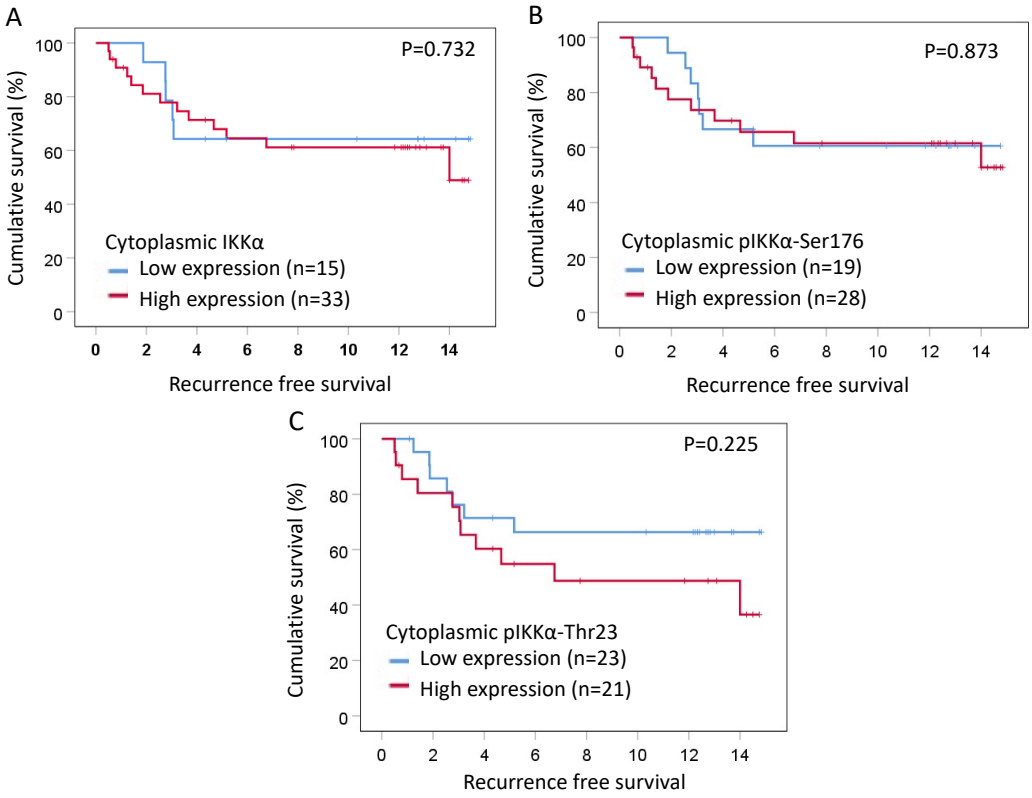


**Figure 7.2 Expression of IKKα, pIKKα-Ser176, pIKKα-Thr23 and recurrence free survival and.** *Kaplan Meier curves showing associations of recurrence free survival and expression of cytoplasmic IKKα (A), cytoplasmic pIKKα-Ser176 (B) and nuclear pIKKα-Thr23 (C)*

For cytoplasmic IKKα expression there was a significant difference in tumour size between specimens with high and low expression (P=0.031). However, no significant difference between expression groups in terms of patients age (P=0.153), tumour grade (P=0.198) and lymph node status (P=0.613) (Table 7.1).

For cytoplasmic pIKKα-Ser176, no significant difference in baseline clinicopathological characteristics in terms of patient age (P=0.433), tumour size (P=0.168), tumour grade (P=0.181) and lymph node status (P=0.859) was observed for patients grouped by low or high expression of cytoplasmic pIKKα-Ser176 (Table 7.1).

For nuclear pIKKα-Thr23, no significant difference in baseline clinicopathological characteristics in terms of patient age (P=0.138), tumour size (P=0.339), tumour grade (P=0.128) and lymph node status (P=0.231) was observed for patients grouped by low or high expression of cytoplasmic pIKKα-Ser176 (Table 7.1).

| Clinicopathological characteristics | Cytoplasmic IKKα (n=48) | | | Cytoplasmic pIKKα-Ser176 (n=47) | | | Nuclear pIKKα-Thr23 (n=44) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low (n=15) | High (n=33) | P value | Low (n=19) | High (n=28) | P value | Low (n=23) | High (n=21) | P value |
| Age | | | | | | | | | |
| ≤50 | 7 (46.7) | 11 (33.3) | 0.153 | 6 (31.6) | 12 (42.9) | 0.433 | 7 7 (30.4) | 11 (52.4) | 0.138 |
| >50 | 8 (53.3) | 22 (66.7) | | 13 (68.4) | 16 (57.1) | | 16 (69.6) | 10 (47.6) | |
| Size(mm) | | | | | | | | | |
| ≤20 | 6 (40.0) | 12 (36.4) | **0.031** | 8 (42.1) | 9 (32.1) | 0.168 | 10 (43.5) | 5 (23.8) | 0.339 |
| 21-50 | 9 (60.0) | 13 (39.4) | | 10 (52.6) | 12 (42.9) | | 10 (43.5) | 11 (52.4) | |
| >50 | 0 (0) | 8 (24.2) | | 1 (5.3) | 7 (25.0) | | 3 (13.0) | 5 (23.8) | |
| Grade | | | | | | | | | |
| I | 0 (0) | 3(9.1) | 0.198 | 0 (0) | 3 (10.7) | 0.181 | 3 (13.0) | 0 (0.0) | 0.128 |
| II | 3 (20.0) | 10(30.3) | | 5 (26.3) | 8 (28.6) | | 6 (26.1) | 5 (23.8) | |
| III | 12 (80.0) | 20 (60.6) | | 14 (73.7) | 17 (60.7) | | 14 (60.9) | 16 (76.2) | |
| LN status | | | | | | | | | |
| Negative | 7 (46.7) | 18 (54.5) | 0.613 | 10 (52.6) | 14 (50.0) | 0.859 | 14 (60.9) | 9 (42.9) | 0.231 |
| Positive | 8 (53.3) | 15 (45.5) | | 9 (47.4) | 14 (50.0) | | 9 (39.1) | 12 (57.1) | |

**Table 7.1 Baseline clinicopathological characteristics of tumours with TempO-Seq data**

### 7.2.2 RNA sequencing using TempO-Seq®

RNA sequencing was performed at Bioclavis as described in chapter 2.

### 7.2.3 Data pre-processing

Raw sequencing data in form of FASTQ files were initially analysed and aligned with human whole transcriptome by Bioclavis using TempO-SeqR software package (BioSpyder Technologies, Inc., Carlsbad, CA, United States).

### 7.2.4 Differential expression analysis and clustering

Differentially expressed genes (DEGs) were identified using RStudio Team (2020) (RStudio: Integrated Development for R. RStudio, PBC, Boston, MA). The targeted RNA sequencing methods focus on assessment of differentially expressed genes (DEGs) between tumours with high and low expression of cytoplasmic IKKα, pIKKα-Ser176 and nuclear pIKK-Thr23.

A total of 22,133 genes were identified between tumours with low and high expression of cytoplasmic IKKα. A total of 22,538 and 22,235 genes were identified between tumours with low and high expression of cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23, respectively.

The genes with a statistically significant fold change were identified. A log2 fold change of 0.5 in both directions and an adjusted P value (padj) of <0.1 were considered to determine the final DEGs. “Top” genes were determined by ranking the padj. Cluster analysis was then used to group the top 20 DEGs into clusters using RStudio. The cluster analysis was able to group the top 20 DEGs based on the similarity in their expression profiles.

### 7.2.5 Gene Ontology and pathway enrichment analyses

The Gene Ontology (GO) is a system for unification of biology, including cellular component, biological process and molecular function (190). The Reactome pathway and The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases were used for the identification of correlating gene sets into their specific pathway (241, 242). The STRING database 10.0 was utilised to identify the DEGs regarding their GO and relating pathways (191-193).

### 7.2.6 Protein-Protein Interaction (PPI) network construction

The DEGs with a significant adjusted P value were analysed for their function interaction. These interaction networks were constructed and visualised using STRING online database with a required interaction score of > 0.4 (medium confidence), a false discovery rate (FDR) < 0.05 and A PPI enrichment P-value <0.05 (191). Maximum number of interactors to show the first and the second shell were limited to no more than 10 interactions.

## 7.3 Differential gene expression profiles associated with high IKKα

Prior to performing differential expression analysis, the read counts were normalised for the differential library size and for the composition bias. The box plot for normalised data was created. The median value of log2 counts per million (cpm) were almost on a straight line, indicating all data were normalised and had adequate quality for further analysis (Figure 7.3).

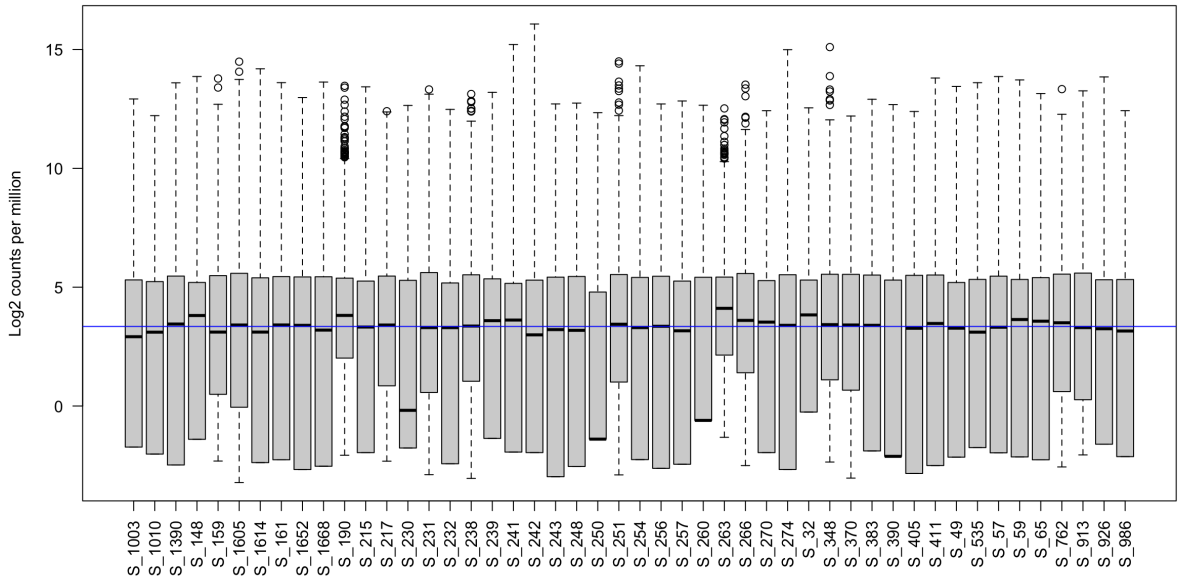


**Figure 7.3 Box plot of sample data.** *The horizontal axis represents the names of the samples, and the vertical axis represents the log2 counts per million of the genes. The blue horizontal line indicated the median value of log2 counts per million (cpm) of the genes.*

### 7.3.1 Identifying differentially expressed genes (DEGs) in tumours with high versus low expression of cytoplasmic IKKα

DEGs that differ between high and low expression of cytoplasmic IKKα were identified using the DESeq 2 package. DEGs were considered significant with an adjusted P value of 0.1 and the logarithm of fold change (logFC) > ± 0.5. A total of 22,133 and the top 20 DEGs between low and high expression of cytoplasmic IKKα were identified. Of those 20 DEGs, 14 DEGs had a significant adjusted P value of 0.1 Table 7.2. Among these 20 DEGs, 13 DEGs were identified as up regulated, including ACTA2, ASH2L, MERTK, PDCD6IP, ACE, DCAF11, EFHD2, MVP, FAM214B, HIST2H3D, MYL6, CNEP1R1, CNEP1R1 and PGM5. 7 DEGs were identified as downregulated, including SERHL2, ALCAM, KCNMA1, CD9, PRSS8 and RAPGEFL1.

| Name | Description | log2FoldChange | padj |
|---|---|---|---|
| ACTA2 | Actin, aortic smooth muscle | 2.549218156 | **2.75E-05** |
| SERHL2 | Serine hydrolase-like protein 2 | -3.295756276 | **0.014721301** |
| ALCAM | CD166 antigen | -2.124043417 | **0.020334861** |
| ASH2L | Set1/Ash2 histone methyltransferase complex subunit ASH2 | 0.704621508 | **0.025602136** |
| MERTK | Tyrosine-protein kinase Mer | 1.788762279 | **0.025839315** |
| KCNMA1 | Potassium calcium-activated channel subfamily M alpha 1 | -1.616715558 | **0.051434731** |
| PDCD6IP | Programmed cell death 6-interacting protein | 1.232439482 | **0.051434731** |
| ACE | Angiotensin-converting enzyme | 1.604501373 | **0.099223665** |
| CD9 | CD9 antigen | -1.004228594 | **0.099223665** |
| DCAF11 | DDB1- and CUL4-associated factor 11 | 1.139722667 | **0.099223665** |
| EFHD2 | EF-hand domain-containing protein D2 | 1.098916206 | **0.099223665** |
| MVP | Major vault protein | 0.954344648 | **0.099223665** |
| PRSS8 | Prostasin | -1.352448615 | **0.099223665** |
| RAPGEFL1 | Rap guanine nucleotide exchange factor-like 1 | -1.858646317 | **0.099223665** |
| GPR160 | Probable G-protein coupled receptor 160 | -1.765572344 | 0.119759882 |
| FAM214B | Protein FAM214B | 1.20298226 | 0.12626319 |
| HIST2H3D | Histone H3.2 | 1.477823014 | 0.12626319 |
| MYL6 | Myosin light polypeptide 6 | 1.394644752 | 0.12626319 |
| CNEP1R1 | Nuclear envelope phosphatase-regulatory subunit 1 | 1.835609973 | 0.202117825 |
| PGM5 | Phosphoglucomutase-like protein 5 | 1.352590717 | 0.202117825 |

**Table 7.2 The top 20 DEGs comparing high and low cytoplasmic expression of IKKα.**

### 7.3.2 Cluster analysis of tumour with high versus low expression of cytoplasmic IKKα

The heatmap of relative rlog-transformed values across samples was performed to cluster the top 20 DEGs. Expressional status of cytoplasmic IKKα is shown with colour bars at the top of the heatmap. Each row represents a single gene and each column represent a tumour sample. As shown in the colour bar, red indicates up-regulation and blue represents down regulation. There were notable differences between high and low expression according to their gene expression profiles. The signature of those tumours with low expression differed from those tumours with high expression, however, there was more variation between tumours with low expression than those tumours with high expression.

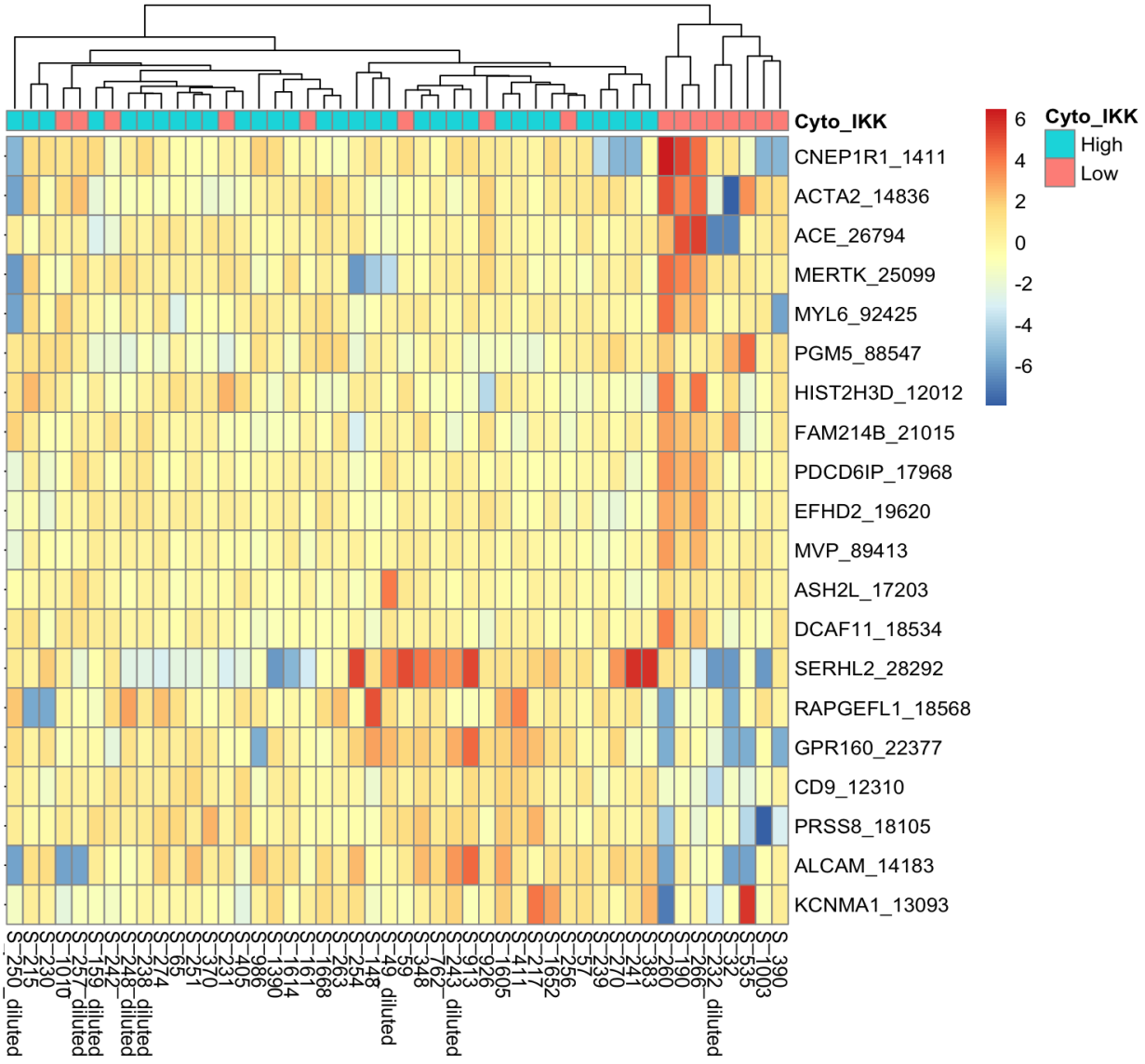


**Figure 7.4 Heatmap of log2 fold changes for the top 20 DEGs with smallest adjust P value.** *Hierarchical clustering analysis and heatmap of the top 20 DEGs identified between tumour with low and high cytoplasmic IKKα. Each row represents a single gene and each column represent a*

*tumour sample. As shown in the colour bar, red indicates up-regulation and blue represent down regulation.*

Principal component analysis (PCA) plots were generated using DESeq2 to determine whether samples in each group (high vs low cytoplasmic expression of IKKα) clustered with each other or other groups. Principal component 1 (PC1) and principal component 2 (PC2) were identified by variance stabilizing transformation in DESeq2. The percentage of variance indicates how much variance was explained by PC1 and PC2. The PCA results demonstrated that the two groups of samples form weak clusters with some overlap based on expression of the top 20 DE genes. PC1 and PC2 components accounted for 43% of the total variations around the PCs for the top 20 DEGs (Figure 7.5).

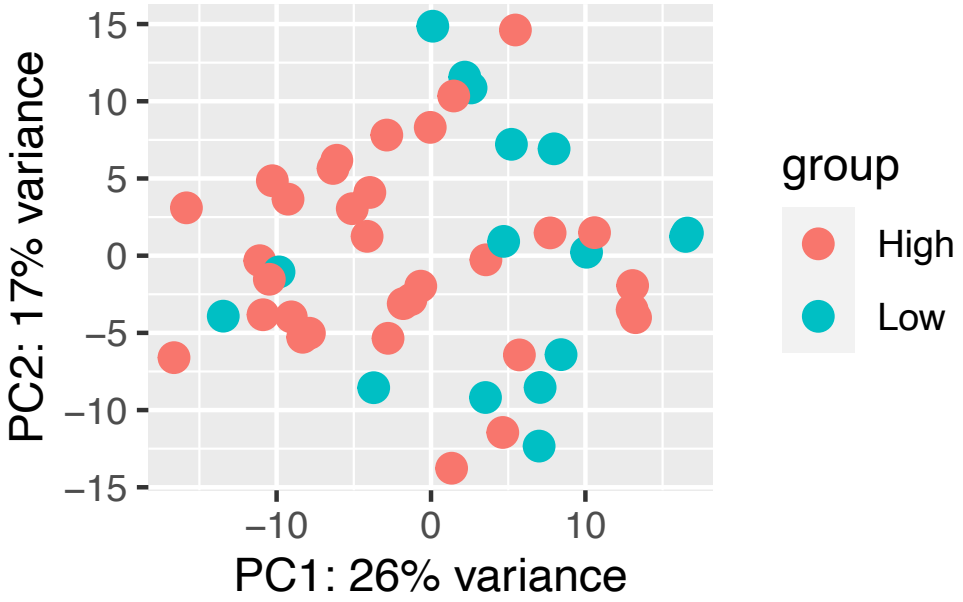


**Figure 7.5 Principal component analysis (PCA) scatter plots.** *PCA plots of top 20 (A) differential expression (DE) genes identified between tumour with high (Rede) and low (Green) expression of cytoplasmic IKKα.*

The reads count for a single DEG that had a significant adjusted P value was plotted. The specific gene which had the smallest adjusted P value from the DEGs tables was plotted first. Among the DEGs, reads counts for CHUK (IKKα) gene were also identified, however the level of CHUK did not significantly differ between the two group, suggesting that mRNA levels do not always reflect protein levels (Figure 7.6).

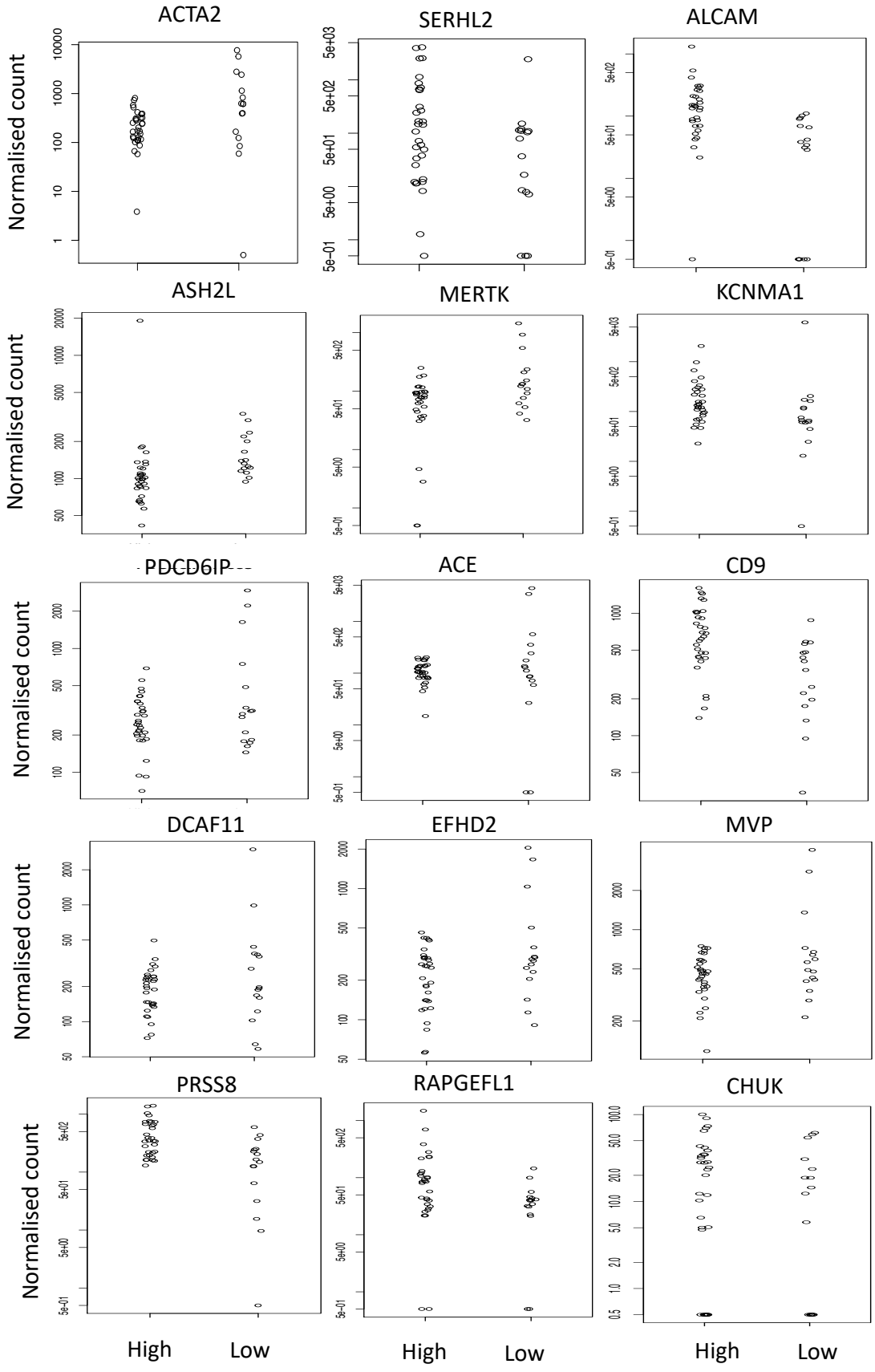


**Figure 7.6 Plot counts.** *The counts represent reads for a single gene GO enrichment analyses*

### 7.3.3 GO enrichment analyses

The DEGs were analysed and categorised into the three categories in GO, including cellular component, biological process and molecular function (190). The enriched terms regarding cellular component are shown in Table 7.3. These included Set1C/COMPASS complex, transferase complex, extracellular exosome, immunological synapse, external side of plasma membrane and extracellular space.

| **Cellular components** | **Matching proteins** | **FDR** |
|---|---|---|
| Set1C/COMPASS complex (A conserved protein complex that catalyses methylation of histone H3) | ASH2L | 7.84E-13 |
| Transferase complex | ASH2L, DCAF11 | 0.00013 |
| Extracellular exosome | ACE, CD9, PDCD6IP | 0.00160 |
| Immunological synapse | ALCAM, PDCD6IP | 0.00650 |
| External side of plasma membrane | ACE, ALCAM, CD9 | 0.01680 |
| Extracellular space | ACE, CD9, MERTK, PDCD6IP, PRSS8 | 0.04740 |

**Table 7.3 Enriched GO terms: Cellular components**

The enriched terms regarding biological processes are shown in Table 7.4. These terms included histone H3-K4 methylation, regulation of megakaryocyte differentiation, regulation of hemopoiesis, peptidyl-amino acid modification, regulation of immune system process, beta-catenin-TCF complex assembly, protein metabolic process, regulation of protein autophosphorylation and smooth muscle contraction.

| **Biological process** | **Matching proteins** | **FDR** |
|---|---|---|
| Histone H3-K4 methylation | ASH2L | 7.01E-10 |
| Regulation of megakaryocyte differentiation | ASH2L | 1.43E-06 |
| Regulation of hemopoiesis | ACE, ASH2L | 0.00011 |
| Peptidyl-amino acid modification | ASH2L, MERTK | 0.00045 |
| Regulation of immune system process | ACE, ASH2L, MERTK | 0.00960 |
| Beta-catenin-TCF complex assembly | ASH2L | 0.01600 |
| Protein metabolic process | ACE, ASH2L, DCAF11, MERTK, PDCD6IP, PRSS8 | 0.02200 |

| | | |
|---|---|---|
| Regulation of protein autophosphorylation | ACE, MVP | 0.03060 |
| Smooth muscle contraction | ACTA2, KCNMA1 | 0.0405 |

**Table 7.4 Enriched GO terms for biological process**

The enriched terms regarding molecular function are shown in Table 7.5. These terms included histone-lysine N-methyltransferase activity, catalytic activity, acting on a protein, methylated histone binding, protein binding, beta-catenin binding and transferase activity.

| **Molecular function** | **Matching proteins** | **FDR** |
|---|---|---|
| Histone-lysine N-methyltransferase activity | ASH2L | 3.90E-10 |
| Catalytic activity, acting on a protein | ACE, ASH2L, MERTK, PRSS8 | 0.0011 |
| Methylated histone binding | RBBP5 | 0.0176 |
| Protein binding | ACE, ACTA2, ALCAM, ASH2L, CD9, KCNMA1, MVP, PDCD6IP, RAPGEFL1 | 0.0203 |
| Beta-catenin binding | ASH2L | 0.0260 |
| Transferase activity | ASH2L, MERTK | 0.0314 |

**Table 7.5 Enriched GO terms for molecular function**

### 7.3.4 Pathway enrichment analyses

To determine biological pathways associated with DEGs in the high cytoplasmic IKKα expression group, the STRING online database was also used. The up-regulated genes, including ASH2L, ACTA2, and ACE were linked with three KEGG pathways, including Cushing's syndrome, renin secretion, and vascular smooth muscle contraction. However, the down-regulated gene, KCNMA1 was linked with vascular smooth muscle contraction (Table 7.6).

| **KEGG pathway** | **Matching proteins** | **FDR** |
|---|---|---|
| Cushing's syndrome | ASH2L | 0.0070 |
| Renin secretion | ACE, KCNMA1 | 0.0126 |
| Vascular smooth muscle contraction | ACTA2, KCNMA1 | 0.0242 |

**Table 7.6 KEGG pathway enrichment analysis.**

### 7.3.5 PPI network construction

PPI analyses were examined using the STRING online database and showed 19 nodes and 23 edges (Figure 7.7). Each node represents a gene, and the edges indicate the interaction between nodes. Each colour represents their response molecular function, however there were two nodes with white colour that shown as unidentified function based on the STRING online database. The PPI enrichment P value was 2.34e-3. Within a PPI network, 2 group of proteins were found that had interaction with each other. However, there were 8 proteins that did not have interactions with other proteins.

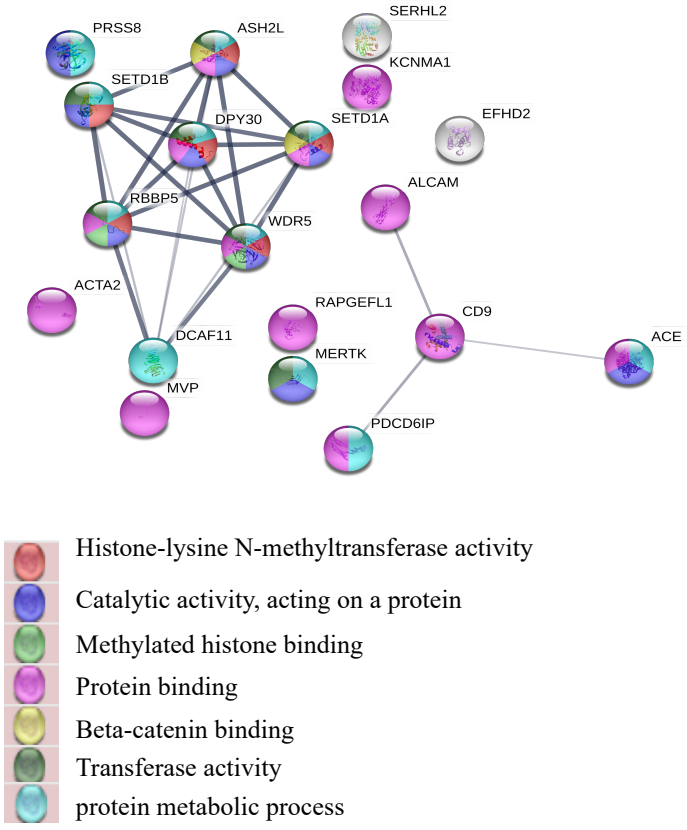


**Figure 7.7 Protein-Protein Interaction of DEGs.** *The thickness of the edge indicates the strength of data that support an interaction between the two proteins*

## 7.4 Differential gene expression profiles associated with high pIKKα-Ser176

### 7.4.1 Identifying differentially expressed genes (DEGs) of tumours with high versus low expression of cytoplasmic pIKKαSer176

DEGs that differ between high and low expression of cytoplasmic pIKKα-Ser176 were identified. DEGs were considered significant with an adjusted P value of 0.1 and the logarithm of fold change (logFC) > ± 0.5. The top 20 DEGs between low and high expression of cytoplasmic pIKKα-Ser176 were identified. However, none of the 20 DEGs had a significant adjusted P value of ≤0.1 (Table 7.7). Among these 20 DEGs, all but one was identified as downregulated.

| Name | Description | log2FoldChange | padj |
|---|---|---|---|
| FAM180A | Protein FAM180A | -1.927033531 | 0.24498439 |
| MEPE | Matrix extracellular phosphoglycoprotein | -4.004526847 | 0.24498439 |
| PCP4 | Calmodulin regulator protein PCP4 | -6.159782628 | 0.24498439 |
| ARSD | Arylsulfatase D | -1.379771273 | 0.249755024 |
| DDIT3 | DDIT3 upstream open reading frame protein | -0.925749578 | 0.249755024 |
| ECI1 | Enoyl-CoA delta isomerase 1 | -0.788665418 | 0.249755024 |
| KLK12 | Kallikrein-12 | -5.039632445 | 0.249755024 |
| SERHL2 | Serine hydrolase-like protein 2 | -2.76750705 | 0.249755024 |
| KRTAP8 | Keratin-associated protein 8-1 | -4.474780627 | 0.300884633 |
| ASB15 | Ankyrin repeat and SOCS box protein 15 | -4.4779224 | 0.455678557 |
| RPL19 | Ribosomal protein L19 | -1.295317005 | 0.495821951 |
| HIST1H2AL | Histone H2A type 1 | 1.549271182 | 0.62562286 |
| OR13C5 | Olfactory receptor 13C5 | -5.533858162 | 0.62562286 |
| GOLGA8H | Golgin A8 family member H; Belongs to the GOLGA8 family | -2.369531007 | 0.625626107 |
| ATPM | ATP synthase subunit, mitochondrial | -0.74415746 | 0.645263066 |
| OR11H1 | Olfactory receptor 11H12 | -3.746269853 | 0.645263066 |
| OR51A7 | Olfactory receptor 51A7 | -5.460423419 | 0.678214829 |
| SNAP91 | Clathrin coat assembly protein AP180 | -4.544733924 | 0.678214829 |
| RAB33A | Ras-related protein Rab-33A | -4.855783484 | 0.681574111 |
| SLC36A3 | Proton-coupled amino acid transporter 3 | -5.781506513 | 0.681574111 |

**Table 7.7 The top 20 DEGs comparing high and low cytoplasmic expression of pIKKα-Ser176.**

### 7.4.2 Cluster analysis of tumours with high versus low expression of cytoplasmic pIKKα-Ser176

The heatmap of relative rlog-transformed values across samples was performed to cluster the top 20 DEGs (Figure 7.8). Expressional status of cytoplasmic pIKKα-Ser176 is shown with colour bars at the top of the heatmap. Each row represents a single gene and each column represent a tumour sample. As shown in the colour bar, red indicates up-regulation and blue represents down regulation. There were no notable overall differences between high and low expression according to their gene expression profiles. However, 5 patients with low expression were all noted to have low SRHL2 expression and cluster together, suggesting this may be an interesting target to investigate further.

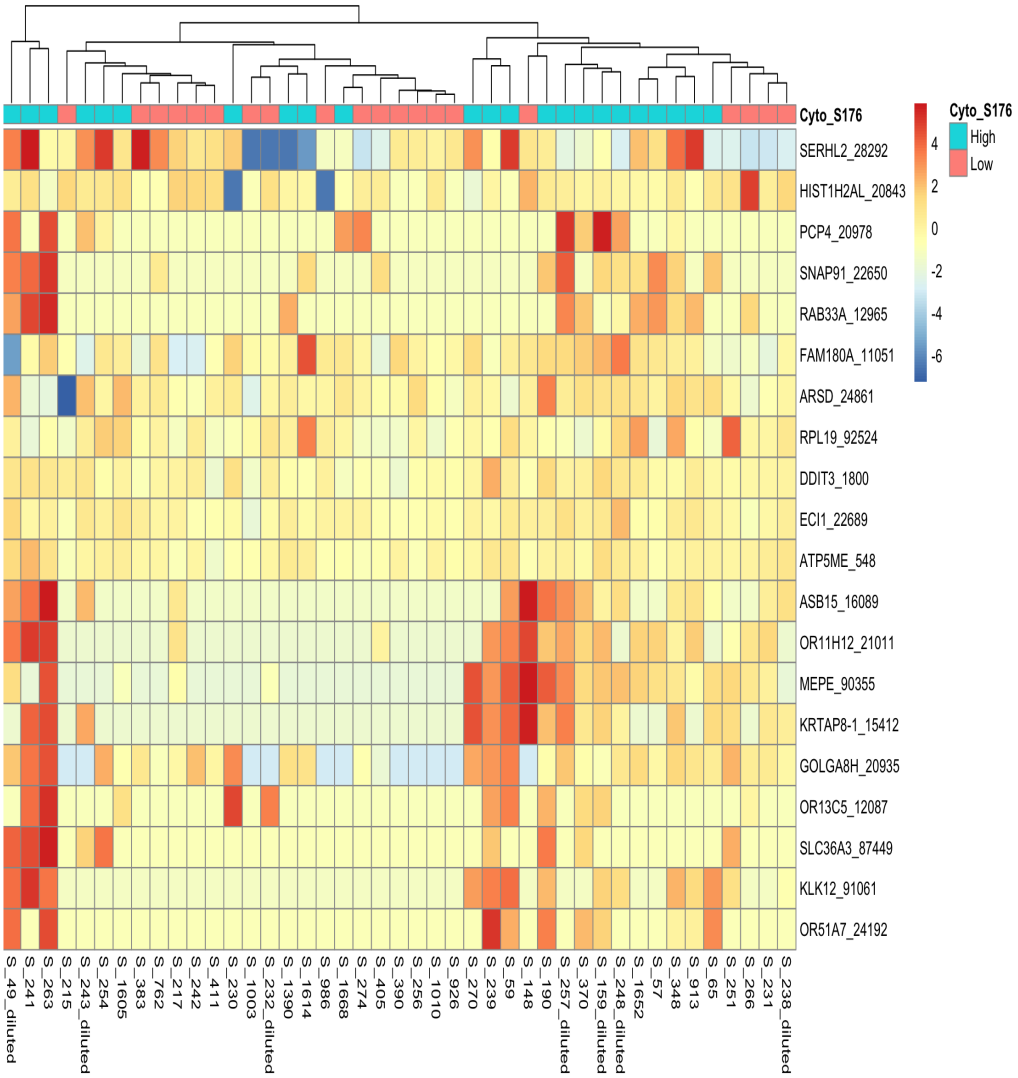


**Figure 7.8 Heatmap of log2 fold changes for the top 20 DEGs with smallest adjust P value.** *Hierarchical clustering analysis and heatmap of the top 20 DEGs identified between tumour with low and high cytoplasmic pIKKα-Ser176 expression. Each row represents a single gene and each column represent a tumour sample. As shown in the colour bar, red indicates up-regulation and blue represent down regulation.*

PCA plots were generated using DESeq2 to determine whether samples in each group (high vs low cytoplasmic expression of pIKKα-Ser176) clustered with each other or other groups (Figure 7.9). The PCA results demonstrated that no obvious clusters were observed.

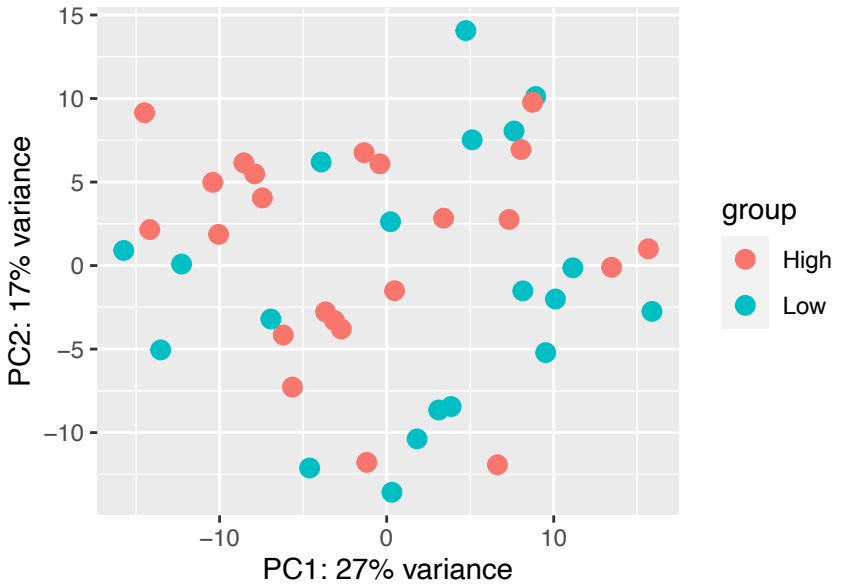


**Figure 7.9 Principal component analysis (PCA) scatter plots.** *PCA plots of top 20 DEGs identified between tumour with high (Rede) and low (Green) expression of cytoplasmic pIKKα-Ser176.*

The read counts for CHUK mRNA expression, which is the encoded gene for IKKα were plotted (Figure 7.10). However, there was no significant difference in CHUK expression between tumours with low and high expression of cytoplasmic pIKKα-Ser176, again suggesting that mRNA levels do not always reflect protein levels.

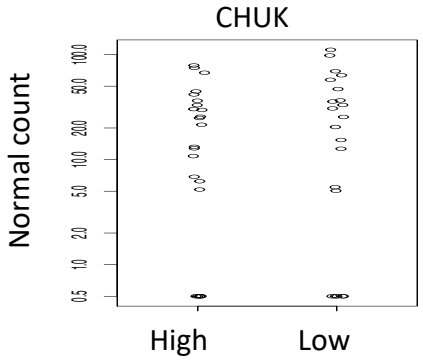


**Figure 7.10 Plot counts.** *The counts represent reads for a single gene.*

## 7.5 Differential gene expression profiles associated with high pIKKα-Thr23

### 7.5.1 Identifying differentially expressed genes (DEGs) of tumours with high versus low expression of nuclear pIKKα-Thr23

The DEGs that differ between high and low expression of cytoplasmic pIKKα-Thr23 were identified. The DEGs were considered significant with an adjusted P value of 0.1 and the logarithm of fold change (logFC) > ± 0.5. The top 20 DEGs between low and high expression of nuclear pIKKα-Thr23 were identified. Of those 20 DEGs, only 3 DEGs had a significant adjusted P value of 0.1, including IGLV3-1, GPR82 and LHX4 (Table 7.8). Among these 20 DEGs, 13 DEGs were identified as up-regulated, including GPR82, LHX4, SLC10A1, LRP2, OR6Y1, C12orf77, CCDC7, KCNK9, TRBV5-3, MAP3K, GLRX, MEFV, and STXBP1. 7 DEGs were identified as down-regulated, including IGLV3-1, KRTAP3-1, IGHV3-15, IGHV3-21, RPL19, FEV and CHAC1.

| Name | Description | log2FoldChange | padj |
|---|---|---|---|
| IGLV3-1 | Immunoglobulin lambda variable 3-1 | -4.1186419 | **0.02876632** |
| GPR82 | Probable G-protein coupled receptor 82 | 6.31249609 | **0.04140756** |
| LHX4 | LIM/homeobox protein Lhx4 | 5.64545783 | **0.08499775** |
| SLC10A1 | Sodium/bile acid cotransporter | 5.82577118 | 0.21357753 |
| KRTAP3-1 | Keratin-associated protein 3-1 | -5.5335639 | 0.67208111 |
| LRP2 | Low-density lipoprotein receptor-related protein 2 | 2.39214553 | 0.67208111 |
| OR6Y1 | Olfactory receptor 6Y1 | 6.39042721 | 0.67208111 |
| C12orf77 | Putative uncharacterized protein C12orf77 | 6.22613007 | 0.68844784 |
| CCDC7 | Coiled-coil domain-containing protein 7 | 1.30700911 | 0.68844784 |
| IGHV3-15 | Immunoglobulin heavy variable 3-15 | -3.0455192 | 0.68844784 |
| KCNK9 | Potassium channel subfamily K member 9 | 3.87904779 | 0.70576317 |
| TRBV5-3 | T cell receptor beta variable 5-3 | 3.83931295 | 0.70576317 |
| IGHV3-21 | Immunoglobulin heavy variable 3-21 | -2.7096511 | 0.84218453 |
| RPL19 | 60S ribosomal protein L19 | -1.3246582 | 0.85952987 |
| MAP3K1 | Mitogen-activated protein kinase kinase kinase 1 | 0.9163187 | 0.86680894 |
| FEV | Protein FEV | -4.1901857 | 0.89381189 |
| CHAC1 | Glutathione-specific gamma-glutamylcyclotransferase 1 | -0.3767762 | 0.999193 |
| GLRX | Glutaredoxin-1 | 0.16988235 | 0.999193 |
| MEFV | Pyrin | 0.389033 | 0.999193 |
| STXBP1 | Syntaxin-binding protein 1 | 0.22151055 | 0.999193 |

**Table 7.8 The top 20 DEGs comparing high and low nuclear expression of pIKKα-Thr23.**

### 7.5.2 Cluster analysis of tumours with high versus low expression of nuclear pIKKα-Thr23

The heatmap of relative rlog-transformed values across samples was performed to cluster the top 20 DEGs (Figure 7.11). Expressional status of nuclear pIKKα-Thr23 was shown with colour bars at the top of the heatmap. Each row represents a single gene and each column represent a tumour sample. As shown in the colour bar, red indicates up-regulation and blue represents down regulation. There was no clear clustering of gene expression profile between tumours with high and low expression. Interestingly, tumours with low expression seem to be clustered into two groups with different signatures.

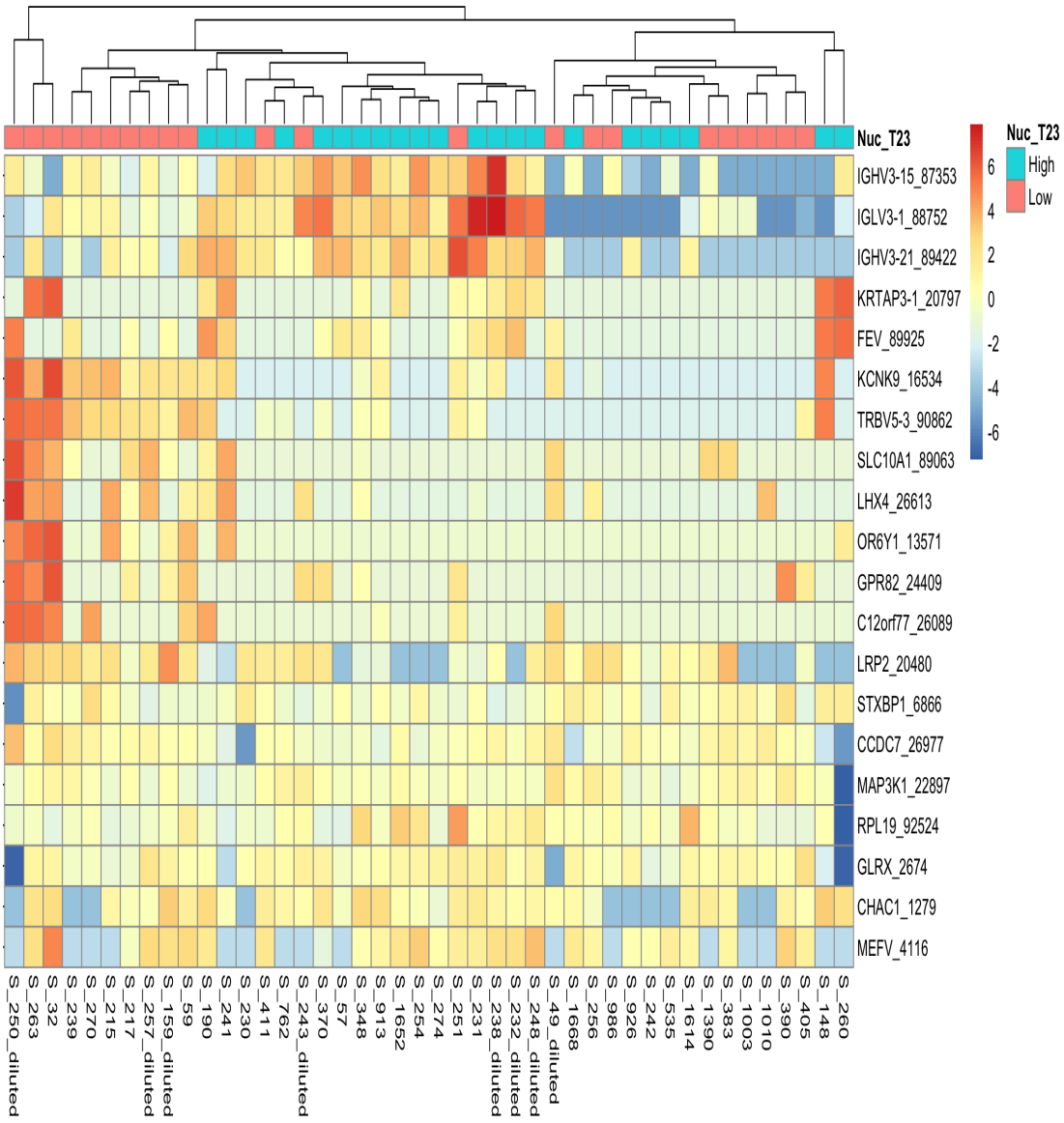


**Figure 7.11 Heatmap of log2 fold changes for the top 20 DEGs with smallest adjust P value.** *Hierarchical clustering analysis and heatmap of the top 20 DEGs identified between tumour with low and high nuclear pIKKα-Thr23 expression. Each row represents a single gene and each*

*column represent a tumour sample. As shown in the colour bar, red indicates up-regulation and blue represent down regulation.*

PCA plots were generated using DESeq2 to determine whether samples in each group (high vs low nuclear expression of pIKKα-Thr23) clustered with each other or other groups (Figure 7.12). The PCA results demonstrated that no obvious clusters were observed.

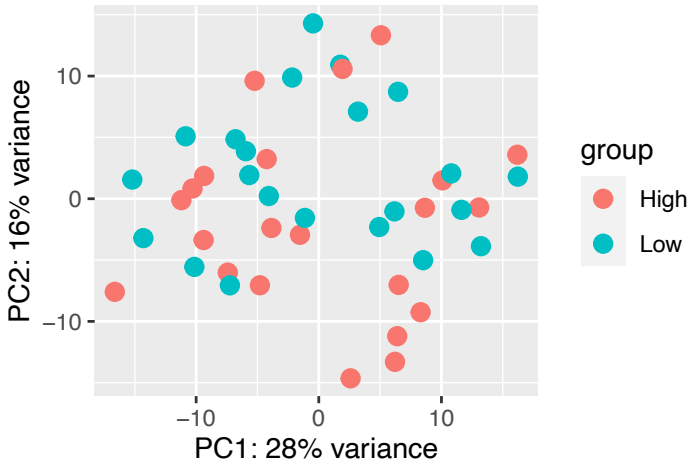


**Figure 7.12 Principal component analysis (PCA) scatter plots.** *PCA plots of top 20 DEGs identified between tumour with high (Red) and low (Green) expression of nuclear pIKKα-Thr23.*

Three DEGs that had a significant adjusted P value in high nuclear expression group, and the read counts for these DEGs were plotted (Figure 7.13). The read counts for CHUK mRNA expression, which is the encoded gene for IKKα was also plotted (Figure 7.13). Again, no significantly different expression of CHUK was observed in tumours with low and high expression of nuclear IKKα-Thr23

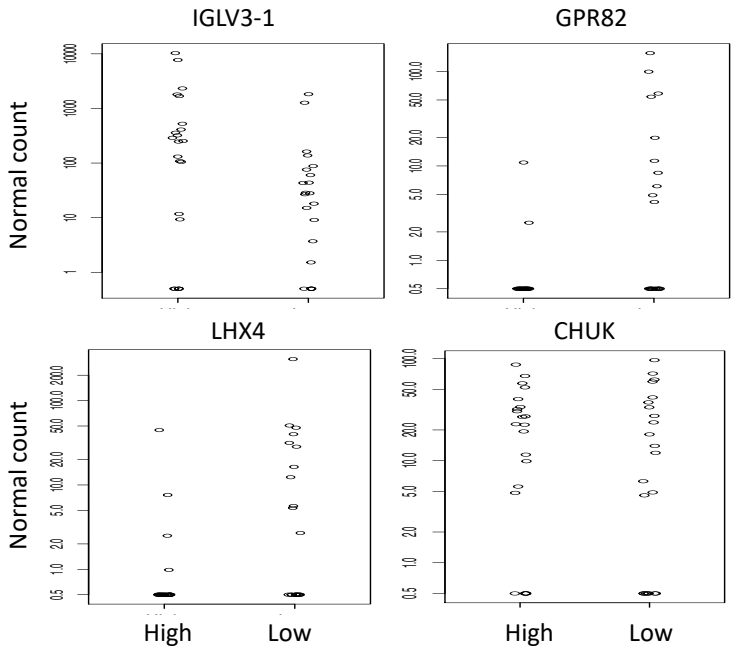


**Figure 7.13 Plot counts.** *The counts represent reads for a single gene.*

## 7.5.3 GO enrichment analyses

The DEGs were analysed using both STRING online tool and DAVID online to provided information for these three DEGS. The DEGs were analysed and categorised into the three categories in GO, including cellular component, biological process and molecular function (190). The enriched terms regarding cellular component are shown in Table 7.9. These include extracellular region, plasma membrane, immunoglobulin complex, plasma membrane, integral component of membrane and nuclear.

| **Cellular component** | **Matched proteins** | FDR |
|---|---|---|
| Extracellular region, plasma membrane, immunoglobulin complex | IGLV3-1 | - |
| Plasma membrane, integral component of membrane | GPR82 | 0.0031 |
| Nuclear | LHX4 | - |

**Table 7.9 Enriched GO terms for cellular components**

The enriched terms regarding biological process are shown in Table 7.10. The down-regulated gene, IGLV3-1 is associated with adaptive immunity. The up-regulated genes, GPR82 and LHX4 are associated with G protein-coupled receptor signalling pathway, transcription by RNA polymerase II, positive regulation of gene expression and negative regulation of apoptotic process.

| Biological process | Matching proteins | FDR |
|---|---|---|
| Adaptive immunity | IGLV3-1 | - |
| G protein-coupled receptor signaling pathway | GPR82 | 1.74E-07 |
| Transcription by RNA polymerase II | LHX4 | 0.00072 |
| Positive regulation of gene expression | LHX4 | 0.001 |
| Negative regulation of apoptotic process | LHX4 | 0.0115 |

**Table 7.10 Enriched GO terms for biological process**

The enriched terms regarding molecular function are shown in Table 7.11. The down-regulated gene, IGLV3-1 is associated with antigen binding. The up-regulated genes, GPR82 and LHX4 are inked with G protein-coupled receptor activity, axon guidance, positive regulation of transcription by RNA polymerase II, negative regulation of apoptotic process and regulation of cellular metabolic process.

| Molecular function | Matching proteins | FDR |
|---|---|---|
| Antigen binding | IGLV3-1 | - |
| G protein-coupled receptor activity | GPR82 | 1E-09 |
| Axon guidance | LHX4 | 4.48E-07 |
| Positive regulation of transcription by RNA polymerase II | LHX4 | 0.0012 |
| Negative regulation of apoptotic process | LHX4 | 0.0075 |
| Regulation of cellular metabolic process | LHX4 | 0.0439 |

**Table 7.11 Enriched GO terms for molecular function**

## 7.5.4 Pathway enrichment analyses

To determine the biological pathways associated with the DEGs in the high nuclear pIKKα-Thr23 expression group, the STRING online database was also used (Table 7.12). Up-regulated genes, GPR82 and LHX4 were linked with Reactome pathways, including G alpha (s) signalling events and KEGG pathway, including Axon guidance.

| Pathway enrichment | Matching proteins | FDR |
|---|---|---|
| G alpha (s) signalling events (Reactome) | GPR82 | 0.0424 |

| | | |
|---|---|---|
| Axon guidance (KEGG) | LHX4 | 0.0035 |

**Table 7.12 Pathway enrichment analysis**

## 7.5.5 PPI network construction

Three proteins, including IGLV3-1, LHX4 and GPR82 were entered into STRING online database for PPI network analysis. There was no available PPI network database for IGLV3-1 in STRING. The PPI network for LHX4 and GPR82 has been demonstrated. However, there was no significant interaction between these proteins. For LHX4, a total of 5 proteins, including ISL1, LDB1, LHX3, ROBO2 and SLIT2 were added to show a network around the input protein with the PPI enrichment P value of 2.02e-4. There were 15 edges among these 6 nodes. For the GPR82, a total of 10 proteins, including EFHC2, GPR142, GPR15, GPR150, GPR162, GPR26, GPR27, GPR52, GPR61, GPR82 and CASK were added to demonstrate a network around the input protein. There were 35 edges among these 11 proteins with the PPI enrichment P value of $9.30e^{-10}$. Each colour represents their response molecular function based on the STRING online database (Figure 7.14).

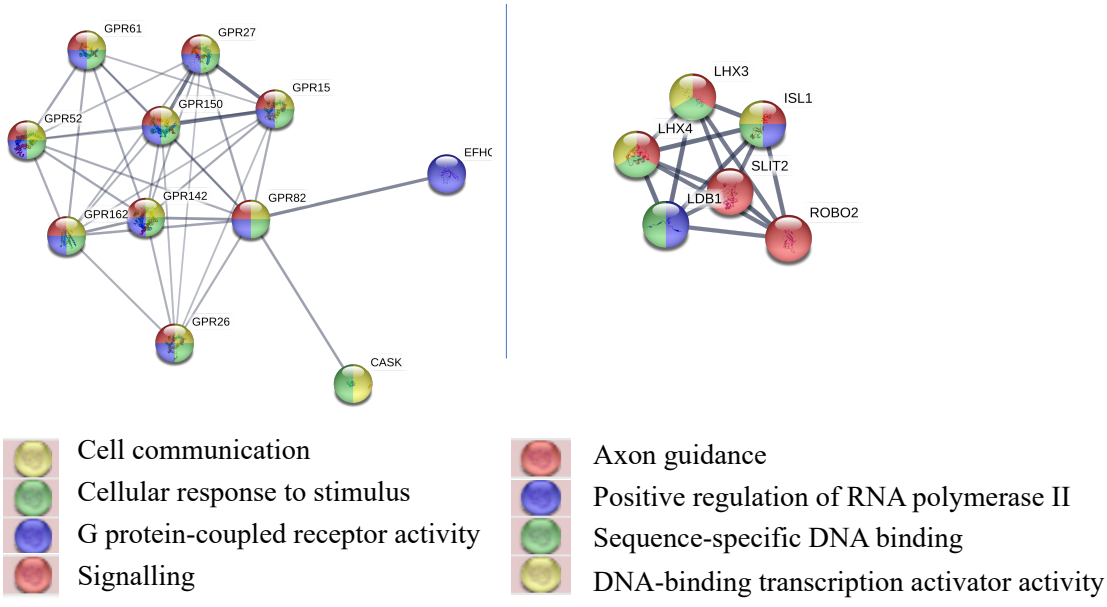


**Figure 7.14 Protein-Protein Interaction of DEGs.** *The thickness of the edge indicates the strength of data that support an interaction between the two proteins*

## 7.6 Comparison of DEGs profiles associated with high IKKα, pIKKα-Ser176 and pIKKα-Thr23

A bar plot of log2 fold change of top 20 DEGs identified between tumours with low and high expression of cytoplasmic IKKα, cytoplasmic pIKKα-Ser176 and nuclear pIKKα -Thr23, was constructed. Most tumours with high cytoplasmic IKKα had up-regulated genes, including ACTA2, ASH2L, MERTK, PDCD6IP, ACE, DCAF11, EFHD2, MVP, FAM214B, HIST2H3D, MYL6, CNEP1R1, CNEP1R1 and PGM5. Seven DEGs were identified as downregulated, including SERHL2, ALCAM, KCNMA1, CD9, PRSS8 and RAPGEFL1. Among these 20 DEGs, all but one was identified as downregulated, which was HIST1H2AL. Most tumours with high nuclear pIKKα-Thr23 had up-regulated genes, including GPR82, LHX4, SLC10A1, LRP2, OR6Y1, C12orf77, CCDC7, KCNK9, TRBV5-3, MAP3K, GLRX, MEFV, and STXBP1. 7 DEGs were identified as down-regulated, including IGLV3-1, KRTAP3-1, IGHV3-15, IGHV3-21, RPL19, FEV and CHAC1.

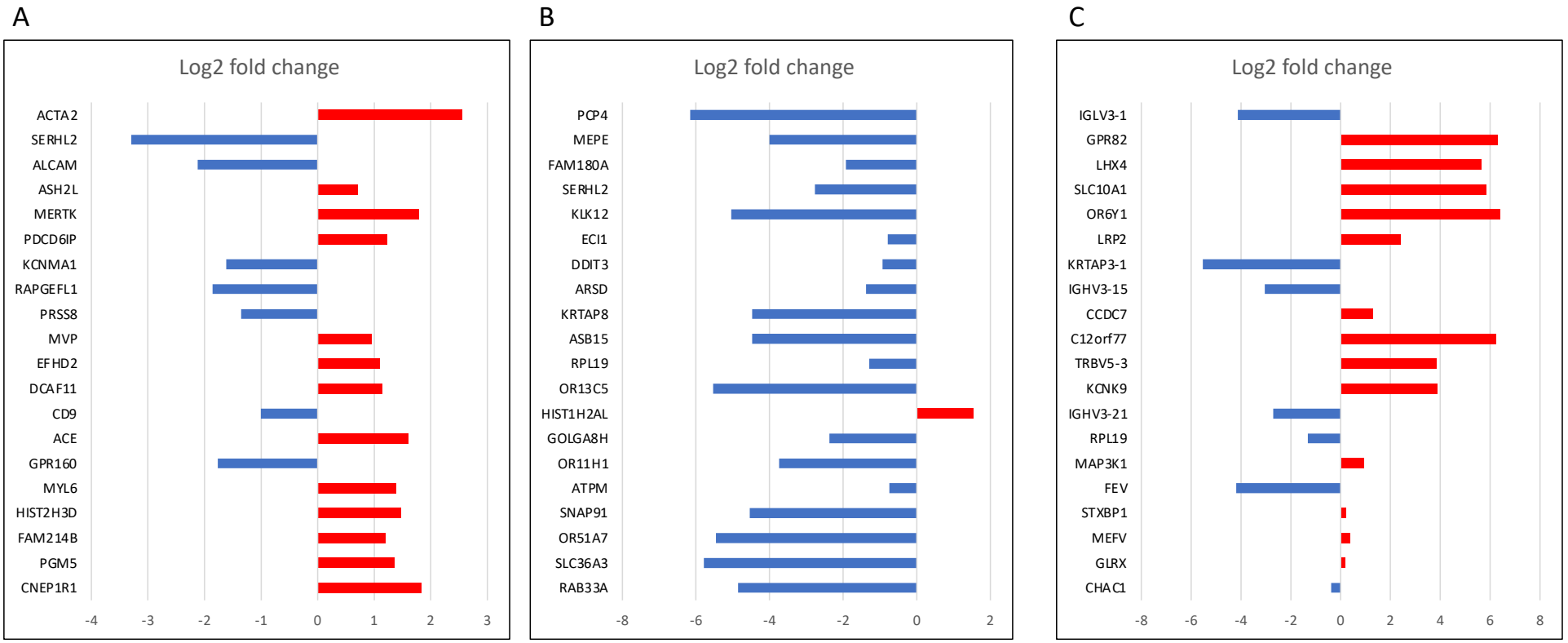


**Figure 7.15 Comparison log2 Fold Change of 20 DEGs.** *Log2 fold change of top 20 DEGs identified between tumour with low and high cytoplasmic IKKα (A), cytoplasmic pIKKα-Ser176 (B) and nuclear pIKKα-Thr23 (C), As shown in the colour bar, red indicates up-regulation and blue represent down regulation.*

Venn diagram of the top 20 DEGs identified between tumours with low and high expression of cytoplasmic IKKα, pIKKα-Ser176 and nuclear pIKKα-Thr23, was plotted to demonstrated up and downregulated genes in each group. There was no overlap of upregulated genes among the groups. Thirteen upregulated genes were observed in IKKα and pIKKα-Thr23 group. Only one upregulated gene was observed in the pIKKα-Ser176 group.

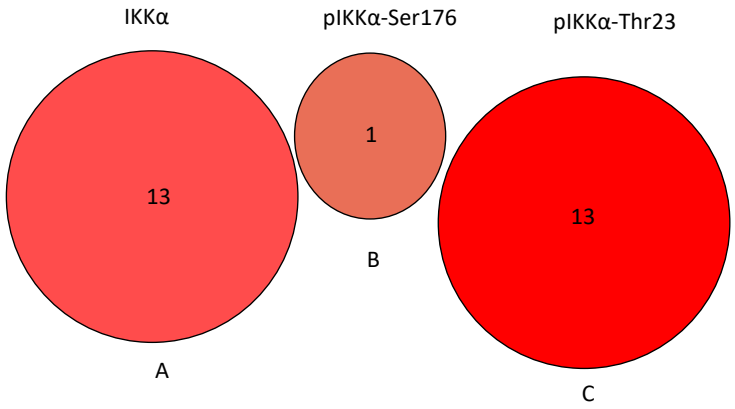


**Figure 7.16 Venn diagram of the upregulated genes in the top 20 DEGs.** *The number in each circle represents the amount of differentially expressed genes identified between tumours with low and high expression of cytoplasmic IKKα (A), cytoplasmic pIKKα-Ser276 (B) and nuclear pIKKα-Thr23 (C). The non-overlapping numbers specify the genes unique to each group.*

Six downregulated genes were observed in the IKKα and the pIKKα-Thr23 group. Nineteen genes were downregulated in the pIKKα-Ser176 group. Of the19 genes, one gene was overlapping with the IKKα group, and another one was overlapping with the pIKKα-Thr23 group. No overlapping gene was observed among the IKKα and the pIKKα-Thr23 groups.

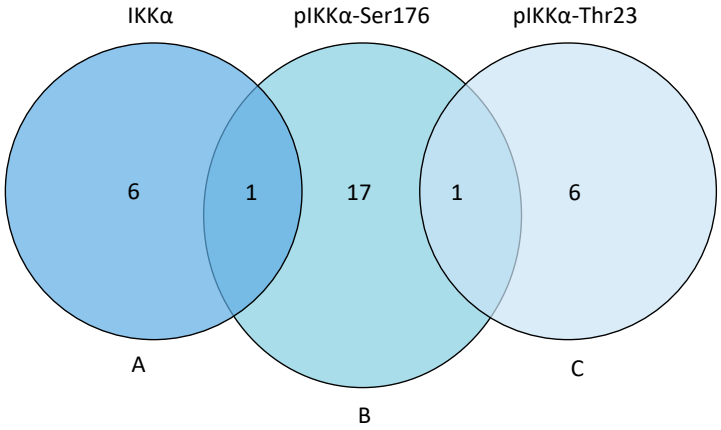


**Figure 7.17 Venn diagram of the downregulated genes in the top 20 DEGs.** The number in each circle represents the amount of differentially expressed identified between tumours with low and high expression of cytoplasmic IKKα (A), cytoplasmic pIKKα-Ser276 (B) and nuclear pIKKα-Thr23 (C). The overlapping number stands for the mutual differentially expressed genes between the group, and the non-overlapping numbers specify the genes unique to each group.

## 7.7 Discussion

In this chapter, the TempO-Seq platform was utilised to identify differentially expressed gene profiles for tumours with low versus high cytoplasmic IKKα, cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23 in ER-negative tumours. The gene profiles for three of these were analysed and compared.

The majority of DEGs in high expression of cytoplasmic IKKα and nuclear pIKKα-Thr23 groups were up-regulated. Conversely, the DEGs in high expression of cytoplasmic pIKKα-Ser176 were down-regulated, however few of these genes reached significance, suggesting that phosphorylation at Ser176 is not associated with any obvious gene signatures. This finding might explain the result from the previous chapter that demonstrated an opposite prognostic role between cytoplasmic IKKα and cytoplasmic pIKKα-Ser176 but identical prognostic role between cytoplasmic IKKα and nuclear pIKKα-Thr23.

The results of the cluster analysis demonstrated that different gene profiles were notable between tumours with low versus high expression of cytoplasmic IKKα. However, the samples did not perfectly cluster together. Conversely, signatures for tumours with low versus high expression of cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23 had no clear clustering.

The functional analysis from GO allowed the translation of DEGs into the underlying biological and molecular mechanisms. The enriched GO terms from the tumours with high cytoplasmic IKKα were associated with histone modification, catalytic activity, protein binding and beta-catenin binding, which are essential for gene transcription, and protein activity. The result from pathway enrichment analysis demonstrated three pathways, including Cushing's syndrome, renin secretion, and vascular smooth muscle contraction, which are not known to be directly involved in cancer progression. As pathway analysis identifies differential expression patterns based on the group of genes rather than individual genes, this might explain the distinct biological functions observed. Therefore, each of the significant DEGs were subsequently investigated regarding their role in breast cancer. Among the 14 DEGs, that showed significant difference in tumour with high expression of IKKα, there were a number of DEGs in this group that have been reported to be involved in cancer. These included, ACTA2, ALCAM, PDCD6IP, CD9, MVP, and PRSS8.

ACTA2 expression was increased in HER2-positive breast cancer patients and was associated with decreased recurrence free survival. ACTA2 silencing significantly inhibited cell migration and invasion in HER2-overexpressed MDA-MB231 cells (243). Although evidence that demonstrates a direct interaction between ACTA2 and NF-κB is limited, there was a study that demonstrated that cellular inhibitor of apoptosis protein 2 (cIAP2) enhanced expression of EMT-associated markers, including E-cadherin, ACTA2, SNAI1, and SNAI2, resulting in enhanced EMT transition through activation of the AKT/NF-KB signalling pathway in triple-negative breast cancer cells. In line with this finding, as ACTA2 was found to be up-regulated in tumours with high expression of IKKα, it can be concluded that in response to cIAP2 activation, NF-κB associates with ACTA2 to promote EMT transition that might promote tumour progression in breast cancer.

Studies have demonstrated that expression of ALCAM was high in normal breast but was lost or reduced in breast cancer (244). Reduced ALCAM expression was associated with large tumour size, high tumour grade, high Ki67 and negative ER and PR status (244). Lost ALCM expression was associated with poorer overall survival and cancer-specific survival (244). This is consistent with the present study, where it was found to have down-regulated expression by a log fold change of 2.12 in tumours with high expression of cytoplasmic IKKα.

PDCD6IP encodes a protein called ALIX, which is a protein that controls exosome biogenesis. This protein has been reported to have a negative association with PD-L1 expression in basal-like breast cancer (1). ALIX controls PD-L1 surface presentation by enhancing PD-L1 packaging into exosomes. Depletion of ALIX results in decreased exosome packing and enhanced surface PD-L1 expression, leading to induced immunosuppressive properties in breast cancer cells (1). In line with this chapter, PDCD6IP had down-regulated expression by a log fold change of 1.23 in tumours with high expression of cytoplasmic IKKα.

CD9 encodes a member of the tetraspanins cell surface glycoproteins. In breast cancer, it has been reported that decreased expression of CD9 protein was associated with poor prognosis (2). It was also demonstrated that patients with negative CD9 had higher 5 years relapse-free survival (245). In addition, down-regulation of CD9 was associated with lymph node involvement (3). Concordance with the result from this chapter, CD9 was down-regulated in tumours with high expression of cytoplasmic IKKα.

The human major vault protein (MVP) is involved in cellular transport molecules, including anti-cancer agents (246). A study reported that MVP is upregulated in human breast cancer cell at the invasive front, where the tumour cell closely contacts to adipocytes (4). MVP induced doxorubicin (DOX) efflux and enhanced tumour cell resistance to DOX in cocultures with human mammary adipocytes (4). Consistent with the present chapter, MVP was found to be up-regulated in tumours with high cytoplasmic IKKα.

Protease serine 8 (PRSS8) mRNA and protein level were decreased in hepatocellular carcinoma when compared with normal adjacent tissue and was associated with poor overall survival (6). Restoring expression of PRSS8 inhibited cell proliferation and invasion in HCC cell lines (6). It has been reported that mRNA and protein level of PRSS8 were absent in MDA-MB-231 breast cancer cell line when compared with normal human mammary epithelial cells (5). This result was consistent with the present chapter that demonstrates down-regulation of PFSS8 in tumours with high expression of cytoplasmic IKKα.

Identification of PPI networks is a crucial step in finding the signal transduction pathways that interact. In this chapter, the result from the PPI networks of the tumours with high expression of cytoplasmic IKKα demonstrated that several of the proteins had interactions with other proteins. Among 14 DEGs input proteins, ALCAM, CD9, PDCD6IP and ACE were demonstrated to interact with each other. ASH2L and DCAF11 were demonstrated to interact with their partner proteins, which were added from the STRING database. These results suggest a variety of interactions and association among these proteins that influence breast cancer progression.

However, three DEGs were identified to be significantly different in tumours with high nuclear pIKKα-Thr23 expression. Furthermore, only data for GPR82 and LHX4 were available in the STRING database and were shown to have various molecular functions. These functions were involved in transcriptional process, regulation of apoptosis and cellular metabolic process. The PPI networks for the two proteins were also constructed. A number of connecting protein were also added by the STRING database. The interaction network of these proteins has a crucial role in various biological process such as cellular communication and response to stimulus, signalling, axon guidance, and regulation of transcriptional processes. It has been reported that LHX4 was significantly upregulated in tissue samples from colorectal cancer (CRC) and was associated with enhanced tumour cell proliferation. However, no evidence has demonstrated a correlation between LHX4 and breast cancer.

In conclusion, significantly different gene profiles were observed in tumours with high cytoplasmic IKKα and high nuclear pIKKα-Thr23 but was not observed in tumours with high cytoplasmic pIKKα-Ser176. Although DEGs in tumours with high cytoplasmic pIKKα-Ser176 were not shown to be significantly different from those with low expression at a gene level, the majority of them appeared to have down-regulated DEGs whereas up-regulated DEGs were observed in tumours with high cytoplasmic IKKα and high nuclear pIKKα-Thr23. This finding might explain the result of the previous chapter that showed a contradictory survival role between cytoplasmic pIKKα-Ser176 and cytoplasmic IKKα. However, due to limitation of samples size, further study is needed to confirm this result. Any further investigation would also require patients with different breast cancer subtypes to identify the particular signature for each subtype in relation to expression of cytoplasmic IKKα, cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23.

# Chapter 8 Impact of inhibiting IKKα in breast cancer cell lines

## 8.1 Introduction

Activation of the non-canonical NF-κB pathways has been reported to be elevated in breast cancer cell lines. In the current study we observed that expression of IKKα, pIKKα-Ser176 and pIKKα-Thr23 were associated with clinical outcomes in breast cancer patients. Expression of cytoplasmic IKKα was not associated with survival in our TNBC patients from our mixed cohort (1800 cohort) but in the independent Belfast TNBC cohort, did associate with good prognosis, suggesting cohort differences. Expression of cytoplasmic pIKKα-Ser176 trended towards good prognosis in TNBC patients from the mixed cohort (1800 cohort). However, it was associated with worse prognosis in the Belfast TNBC cohort, conflicting with the previous results. However, expression of cytoplasmic and nuclear pIKKα-Thr23 was associated with worse prognosis in TNBC tumour from the mixed cohort (1800 cohort) but this was not validated in the full Belfast TNBC cohort, but does show similar results in AR-positive patients in this cohort. Overall, this data demonstrates a complex relationship between IKKα and survival in TNBC patient dependent on phosphorylation site and potentially AR status.

Therefore, further investigation was performed to establish the impact of pharmacologically inhibiting IKKα in two ER-negative breast cancer cells with differing AR status. The cell lines were ER-negative/AR-negative MDA-MB-231 and ER-negative/AR-positive MDA-MB-453 cells. Proprietary selective preclinical IKKα inhibitors, SU1433 and SU1644 were used to investigate the sensitivity of the two cell lines to IKKα inhibitors. Impact of IKKα inhibition on p52 processing (non-canonical NF-κB pathway) was assessed by western blotting and phenotypic response was assessed by changes in cell viability. These inhibitors were generated through CRUK and MRC-DPFS programme awards, by Professor Simon Mackay at the University of Strathclyde.

## 8.2 Methods for treatment and western blotting

Lymphotoxin α1β2 has been reported to drive activation of the non-canonical NF-κB pathway and previously used in our lab. This ligand was selected to determine pathway activation in breast cancer cell lines.

In order to investigate an impact of IKKα inhibitor SU1433 and SU1644 on the non-canonical NF-κB pathway, ER-negative/AR-negative MDA-MB-231 and ER-negative/AR-positive MDA-MB-453 breast cancer cell lines were treated with the concentration of 0.1, 0.3, 1, 3 and 10mM for 30 minutes followed by stimulation with Lymphotoxin α1β2 (20mg/ml) for 24 hours. Proteins were then extracted, and the expression of p100/p52 was assessed by western blotting. ImageJ was used to quantify expression of p52 and tubulin. Expression of proteins level in a treated cell were then compared to untreated cells or controls (DMEM) and displayed as fold change to control in the bar graphs. Each treatment has been planned to repeat three time in triplicate. However, due to time restrictions and the impact of COVID-19, I was unable to complete all the experiments as planned. Therefore, statistical analysis was not performed.

## 8.3 IKKα inhibitor (SU1433) on non-canonical NF-kB signalling in ER-negative/ AR-negative MDA-MB-231 cells

Activation of the non-canonical NF-κB pathway was not observed in MDA-MB-231 breast cancer cell lines after treatment with lymphotoxin. MDA-MB-231 breast cancer cell lines were treated with IKKα inhibitor SU1433 to assess of target effect of the inhibitor. No decrease in p52 was observed at any concentration (Figure 8.1).

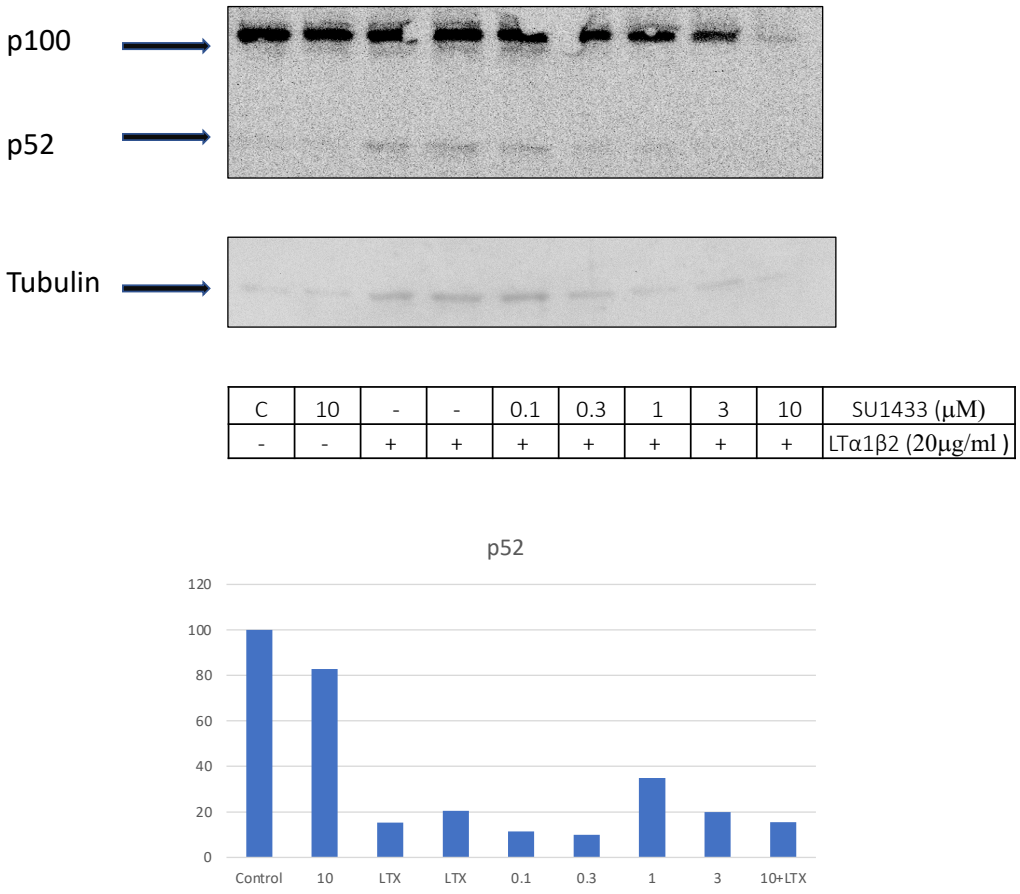


**Figure 8.1 Expression of the non-canonical NF-κB pathway in MDA-MB-231 cell lines following treatment with IKKα inhibitor (SU1433).** *MDA-MB231 cells were treated with SU1433. Protein was extracted, separated by SDS-PAGE and assessed for expression of p100/52. β-tubulin was used as a loading control. This is representative of 1 experiment. ImageJ was used to quantified expression of the proteins. Expression levels of each protein was compared to untreated cells and displayed as fold change to control in the bar graphs.*

## 8.4 IKKα inhibitor (SU1433) on non-canonical NF-kB signalling in ER-negative/AR-positive MDA-MB-453 cells

Activation of the non-canonical NF-κB pathway was observed in MDA-MB-453 breast cancer cell lines after treatment with lymphotoxin. Inhibition was also noted with 3uM of

SU1433 as seen in Figure 8.2. Again, it was unable to conclude the statistically significant result as this was only one experiment.

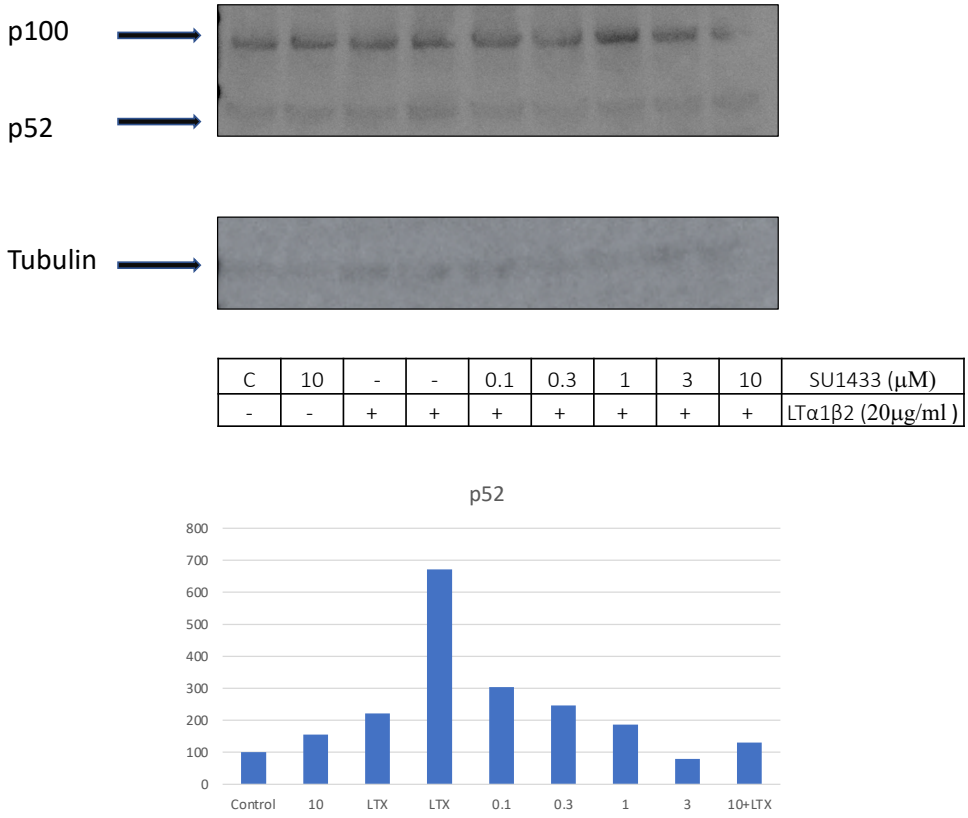


**Figure 8.2 Expression of the non-canonical NF-κB pathway in MDA-MB-453 cell lines following IKKα inhibitor (SU1433).** *MDA-MB-453 cells were treated with SU1433. Protein was extracted, separated by SDS-PAGE and assessed for expression of p100/52.. β-tubulin was used as a loading control. This is representative of 1 experiment.*

## 8.5 IKKα inhibitor (SU1644) on non-canonical NF-kB signalling in ER-negative/AR-negative MDA-MB-231 cells

SU1644 is a more recent IKKα inhibitor that was shown to be more potent in prostate cancer cell lines. Therefore, it was used to further investigate impact on the non-canonical NF-κB

pathway in our breast cancer cell line. Again, only a small stimulation was seen with lymphotoxin in these cells, but no effect of the inhibitor was seen as shown in Figure 8.3.

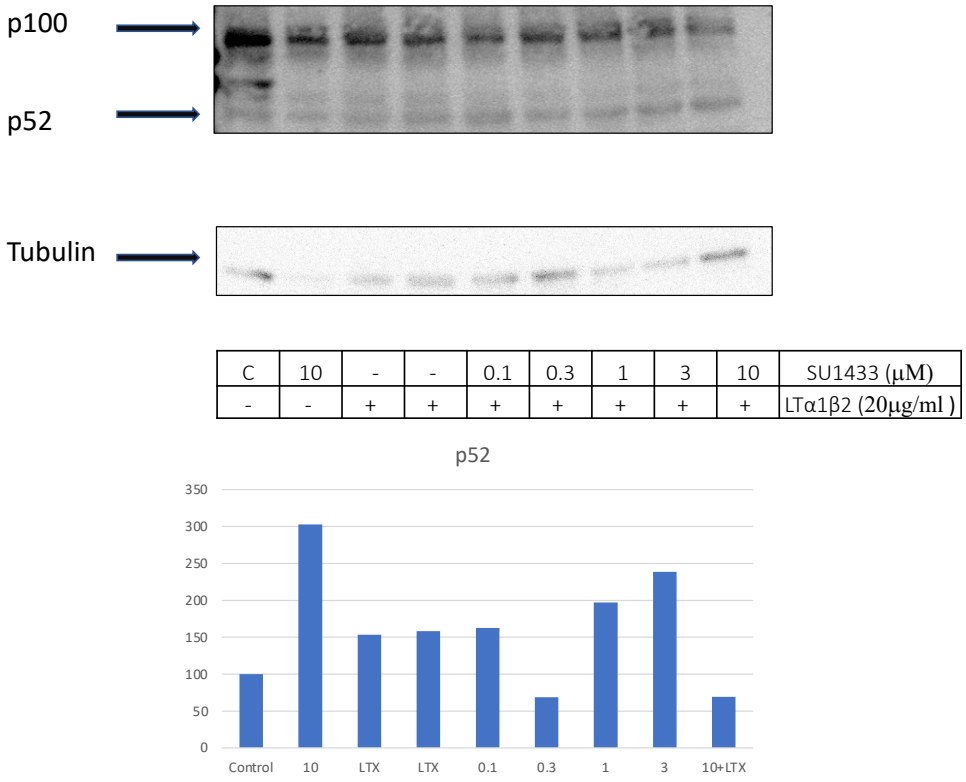


**Figure 8.3 Expression of the non-canonical NF-κB pathway in MDA-MB-231 cell lines following IKKα inhibitor (SU1644).** *MDA-MB231 cells were treated with SU1644. Protein was extracted, separated by SDS-PAGE and assessed for expression of p100/52. β-tubulin was used as a loading control. This is representative of 1 experiment. ImageJ was used to quantified expression of the proteins. Expression levels of each protein was compared to untreated cells and displayed as fold change to control in the bar graphs.*

Since the aim of the current chapter planned to investigate an impact of IKKα inhibition on p52 processing (non-canonical NF-κB pathway) was assessed by western blotting, the effect on pIKKα-Ser176 and pIKKα-Thr23 has not been assessed. Future work is needed to demonstrate the effect on pIKKα-Ser176 and pIKKα-Thr23 after treatment with IKKα inhibitor.

## 8.6 IKKα inhibitor (SU1644) on non-canonical NF-kB signalling in ER-negative/AR-positive MDA-MB-453 cells

Similarly, in the AR-positive cell line, little stimulation was noted with lymphotoxin and no effect was seen with the inhibitor in these cells as seen in Figure 8.4.

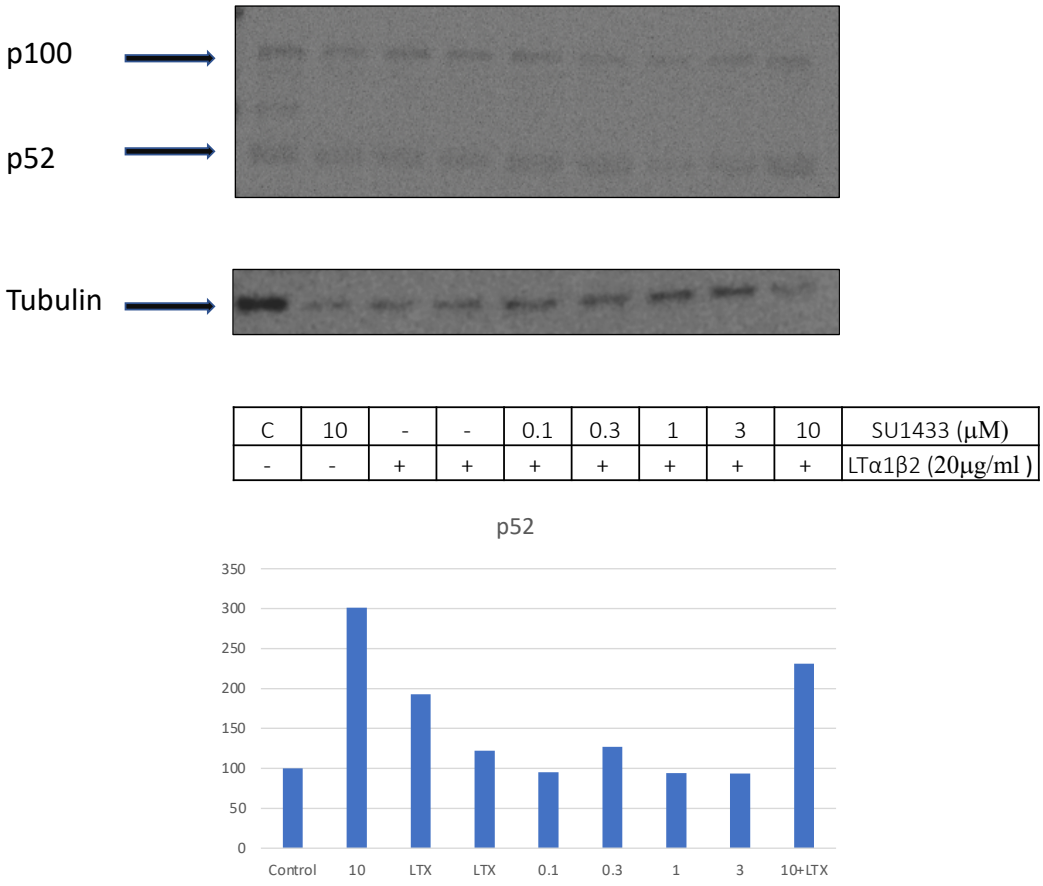


**Figure 8.4 Expression of the non-canonical NF-κB pathway in MDA-MB-453 cell lines following IKKα inhibitor (SU1644).** *MDA-MB-453 cells were treated with SU1644. Protein was extracted, separated by SDS-PAGE and assessed for expression of p100/52 (n=3). β-tubulin was used as a loading control. This is representative of 1 experiment. ImageJ was used to quantified expression of the proteins. Expression levels of each protein was compared to untreated cells and displayed as fold change to control in the bar graphs.*

## 8.7 Phenotypic impact of inhibiting IKKα in breast cancer cell line

Phenotypic assays using *in vitro* cell cultures that exhibit a phenotype of rapid unrestrained growth to predict effects of anti-cancer drugs has been used for many decades (247, 248). The 'hallmarks of cancer" as described by Hanahan and Weinberg, describes one way to define potential druggable phenotypes by investigating changes in sustained proliferation,

invasion, resistance to cell death, which can be measured in cancer cell lines (247) . Cell viability assays is one of the most common types of cell-based phenotypic assays that is used in anti-cancer drug discovery and there are many different assay formats. The main concept of these different assays involves mitochondrial activity, cellular metabolism, and the activity of enzymes that associated with viable or dead cells (249). Among different types of cell viability assays, the ATP content assay is the most novel and robust (249).

Adenosine triphosphate (ATP) is the "primary energy unit" that serves as a metabolite and signalling molecule in the living cells (250). Therefore, the measurement of ATP seems to be an excellent method for measuring cell viability and growth. In addition, the availability of a bioluminescence assay delivers a simple, rapid and more sensitive measure of ATP levels (251). In the presence of luciferin and luciferase, which is the enzyme that is extracted from the firefly, ATP produces light emission that can be quantified by a photometer (252). It has been confirmed that intracellular ATP levels that are measured by bioluminescence assays correlate with the viability of cancer cell lines. Therefore, the present study determined to use this cell viability assay to investigate if IKKα inhibitors could reduce the viability of breast cancer cell lines.

As demonstrated previously, we found that high expression of IKKα is associated with poor clinical outcome depending on phosphorylation site, ER and AR status. We hypothesised that inhibition of IKKα would reduce cell viability of some breast cancer cells. In order to address this hypothesis and explore the potential roles of IKKα as a novel target in breast cancer, IKKα inhibitors SU1644 and SU1433 were employed and the effect on cell viability were assessed. In addition, combination treatment of IKKα inhibitors SU1644 and SU1433 and an AR antagonist were employed to inhibit function of both IKKα and AR and assess effects on cell viability. Due to differences in clinical outcome measures observed between ER positive and ER negative breast cancer, and the differences in TNBC patients with different AR status, difference in results between ER negative/AR-negative MBA-MB 231 cells and ER negative/ AR-positive MBA-MB 453 cells were compared.

### 8.7.1 Methods for cell viability

Cell viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay. This method determines the number of viable cells based on quantitation of the ATP. After loss of membrane integrity, cells unable to synthesise ATP and endogenous ATPase rapidly reduce the remaining ATP from cytoplasm. This assay contains a mono-oxygenation of

beetle luciferin, which is catalysed by a recombinantly produced Luciferase with give rise to oxyluciferan + light. This reaction requires Mg2+ and ATP that release from the lysed cell. The extent of light generation is correlated to the number of live cells. An advantage design of this assay is only a single reagent is added to make luminescent signal that glows with a half-life of more than 5 hours. This assay is also the most sensitivity and proceeds linear reaction within the range of 15-50,000 cells, therefore this assay was used in the present study.

Each of breast cancer cell lines (MCF7, MDA-MB231, and MDA-MB 453) were treated with either SU1433 or SU1644 with the concentration of 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30μM at 24, 48 and 72 hours. 1μM Staurosporine was used as a positive control and 0.01% DMSO was used as a negative control. CellTiter-Glo reagent was added to each plate as described in chapter 2, before reading of luminescent signal.

Luminescent signal was recorded with Glomax multi detection system machine. The raw data was firstly analysed using Microsoft® Office Excel 16 (Microsoft, Redmond, WA, USA). Each treatment was repeat in triplicate on the plate and repeated to an N=3. The amount of luminescence from each treatment group was firstly average and then was normalized as a percentage of the mean intensity of untreated controls (DMSO). The results were then plotted, and statistical analysis of data was performed using GraphPad Prism (Version 8 San Diego, CA, USA). Cell viability assay in MCF-7 and MDA-MB-231cells were performed by Dr Daniel R. Croft **(**Drug Discovery Unit, Cancer Research UK Beatson Institute, Glasgow, United Kingdom).

## 8.7.2 Cell viability following IKKα inhibitor treatments

### 8.7.2.1 ER negative/AR negative MDA-MB-231

In MDA-MB-231 cells, treatment with SU1433 or SU1644 for 24 hours did not affect cell viability. However, a dose-dependent decrease in cell viability was observed following 48 and 72 hours of the treatment. At 72 hours, the effective concentration of a drug that gives half-maximal response (EC50) for SU1433 and SU1644 were 1.60μM and 0.33μM respectively (Figure 8.5).

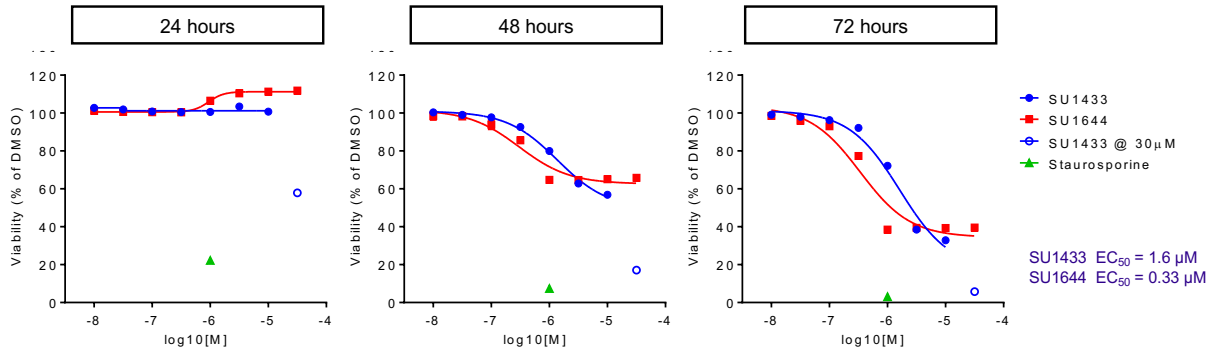


**Figure 8.5 Dose-response curves for cell viability as a percentage of controls in MDA-MB-231 cells.** *A cell viability relative to DMSO control was determined by CellTiter-Glo after treatment with IKKα inhibitor (SU1433 blue, SU1644 red). Results shown are means ± the standard error of mean (SEM).*

### 8.7.2.2 ER negative/AR positive MDA-MB-453 cells

In MDA-MB-453 cells, treatment with SU1433 at all time points showed a small dose-dependent decrease in cell viability. At 72 hours, the EC50 value for SU1433 was 0.097µM. However, no effect on cell viability was observed following treatment with SU1644 (Figure 8.6).

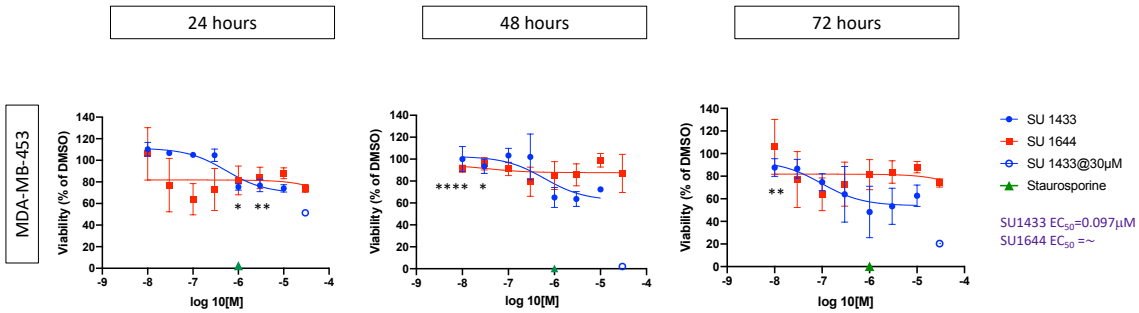


**Figure 8.6 Dose-response curves for cell viability as a percentage of controls in MDA-MB-453 cells.** *A cell viability relative to DMSO control was determined by CellTiter-Glo after treatment with IKKα inhibitor (SU1433 blue, SU1644 red). Results shown are means ± the standard error of mean (SEM). The symbols *, **, ***, and **** indicated P<0.05, <0.005, <0.0005 and <0.0001.*

## 8.8 Impact of AR antagonist on cell viability

In the 1800 cohort, combined expression was not associated with any survival outcome but those patients expressing both AR and IKKα did have the shortest median survival. However, Belfast TNBC cohort, significant results were seen for pIKKα-Thr23 in AR positive patients. Therefore, dual target might be benefit for those expressing high AR and

high IKKα or pIKKα-Thr23, especially in TNBC patients. Single agent of AR antagonist was assessed first, followed by combination of IKK inhibitor and AR antagonist for cell viability on each breast cancer cell line.

## 8.8.1 Cell viability following AR antagonist treatment

MCF-7, MDA-MB-231 and MDA-MB-453 cell lines were treated with enzalutamide at a concentration of 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30μM for 24, 48 or 72 hours. 1μM Staurosporine was used as a positive control and 0.01% DMSO was used as a negative control. CellTiter-Glo reagent was added to each plate before reading of luminescent signal.

### 8.8.1.1 ER-negative/AR-positive MDA-MB-453 cells

In MDA-MB-453 cell lines, no obvious dose-dependent decrease in cell viability was observed after 24 hrs treatment with enzalutamide (Figure 8.7). However, at 48 and 72 hrs, >1μM Enzalutamide showed a similar decrease in cell viability with an EC50 value of 0.61μM

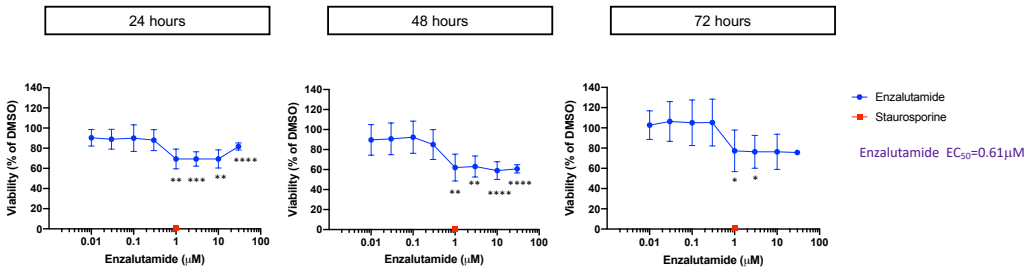


**Figure 8.7 Cell viability assay Enzalutamide single agent in MDA-MB-453.** *A cell viability relative to DMSO control was determined by CellTiter-Glo after treatment with Enzalutamide. Results shown are means ± the standard error of mean (SEM). The symbols *, **, ***, and **** indicated P<0.05, <0.005, <0.0005 and <0.0001.*

### 8.8.1.2 ER-positive/AR-positive MCF7 cells, ER-negative/AR-negative MDA-MB-231 cells

The effect of enzalutamide was also assessed in ER-positive/AR-positive MCF7 cells as a control as we planned to confirm the effective inhibition of enzalutamide on ER-negative/AR-positive cell lines. MCF-7 cell was also treated with the same treatment protocol as in MDA-MB-231 and MDA-MB-453 cell lines.

In MCF-7 cell lines no obvious dose-dependent decrease in cell viability was observed after treatment with enzalutamide. In MDA-MB 231 cell lines, no obvious dose-dependent decrease in cell viability was observed after treatment with enzalutamide. However, in MDA-MB-231 cell, 30µM enzalutamide reveal approximately 30% decrease in cell viability after 72 hours (Figure 8.8). As MDA-MB-231 cells are AR-negative this may just be due to off-target toxicity.

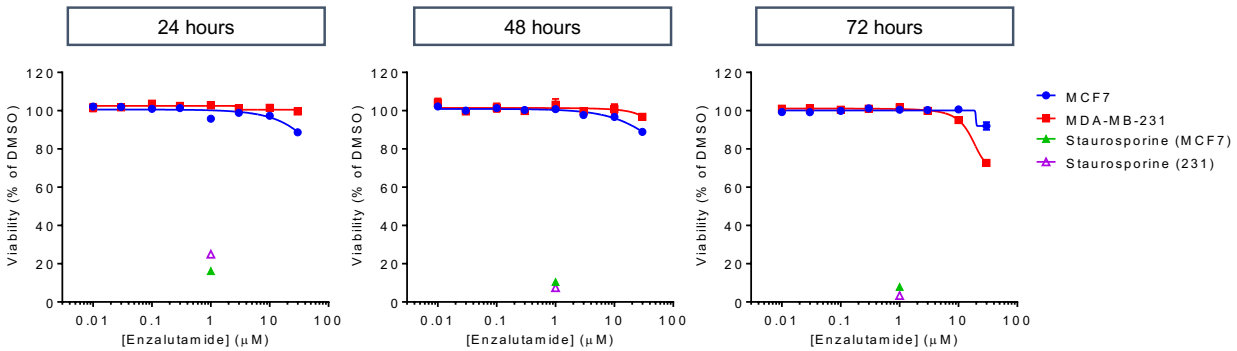


**Figure 8.8 Dose-response curves for cell viability as a percentage of controls in MCF-7 and MDA-MB-231 cells.** *A cell viability relative to DMSO control was determined by CellTiter-Glo after treatment with Enzalutamide (MCF-7 blue, MDA-MB-231 red). Results shown are means ± the standard error of mean (SEM).*

## 8.9 Cell viability following combined inhibition with IKKα inhibitor and AR antagonist

### 8.9.1 ER-negative/AR-negative MDA-MB-231 cells

MDA-MB-231 cell lines were treated with the combination of 3µM Enzalutamide and SU1433 or SU1644 with the concentration of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30µM. 1µM Staurosporine was used as a positive control and 0.01% DMSO was used as a negative control. CellTiter-Glo reagent was added to each plate before reading of luminescent signal.

In MDA-MB-231 cell lines, treatment with either SU1433 or SU1644 showed a dose-dependent decrease in cell viability (Figure 8.9). The dose-dependent decrease in cell viability was greater following after 48 and 72 hours of treatment. At 72 hours, the $EC_{50}$ for SU1433 and SU1644 were 1.97µM and 0.37µM, respectively. The combination of 3µM Enzalutamide with either SU1433 or SU1644 had no apparent enhanced effect on the viability of the MDA-MB-231 cell lines as expected in an AR-negative cell line.

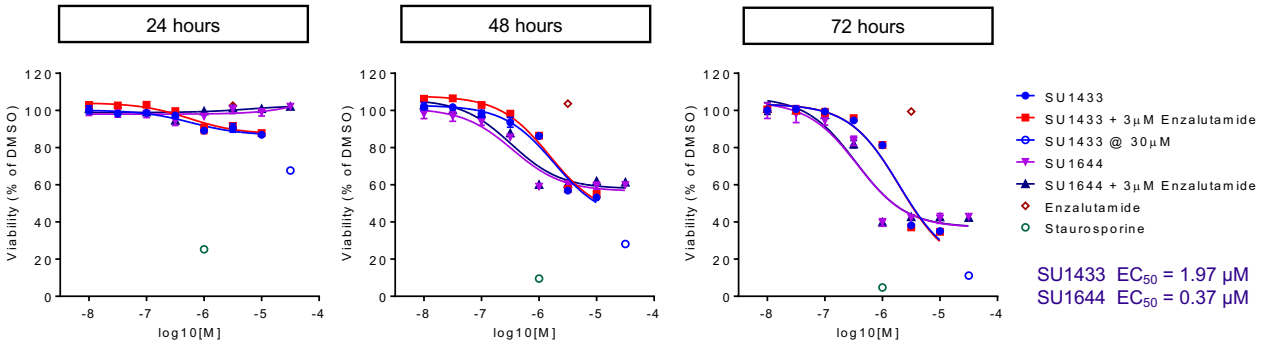


**Figure 8.9 Cell viability assay a combination of IKKα inhibitor and Enzalutamide in MDA-MB-231.** *A cell viability relative to DMSO control was determined by CellTiter-Glo after treatment with (SU1433 blue, SU1644 purple, SU1433+Enzalutamide red, SU1644+Enzalutamide dark blue). Results shown are means ± the standard error of mean (SEM).*

## 8.9.2 ER-negative/AR-positive MDA-MB-453 cells

MDA-MB-453 cell lines were treated with the combination of 3μM Enzalutamide and SU1433 or SU1644 with the concentration of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30μM. 1μM Staurosporine was used as a positive control and 0.01% DMSO was used as a negative control. CellTiter-Glo reagent was added to each plate before reading of luminescent signal.

In MDA-MB-453 cell lines, treatment with SU1433 showed a dose-dependent decrease in cell viability (Figure 8.10). The dose-dependent decrease in cell viability was greater following 72 hours of treatment. At 72 hours, the $EC_{50}$ for SU1433 was 0.10μM. The combination of 3μM Enzalutamide with SU1433 appeared to decrease the effect on the viability of the MDA-MB-453 cell lines at 24 and 48 hrs, suggesting combination of these agents is not beneficial.

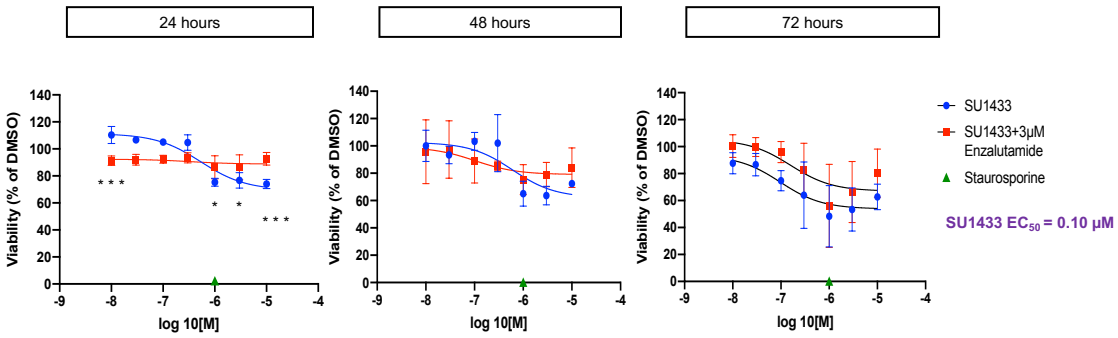


**Figure 8.10 Cell viability assay a combination of IKKα inhibitor (SU1433) and Enzalutamide in MDA-MB-453.** *A cell viability relative to DMSO control was determined by CellTiter-Glo after treatment with SU1433 (blue), SU1433+Enzalutamide (red). Results shown are means ± the standard error of mean (SEM). The symbols *, **, ***, and **** indicated P<0.05, <0.005, <0.0005 and <0.0001.*

In the MDA-MB-453 cell lines, no dose-dependent decrease in cell viability was observed after treated with SU1644 (Figure 8.11). However, the combination of 3μM Enzalutamide with SU1644 led to a dose-dependent decrease in cell viability similar to that seen with enzalutamide alone. The dose-dependent decrease in cell viability was larger following 48 and 72 hours of the treatment with an IC50 of 0.99uM. This suggest enzalutamide alone is sufficient.

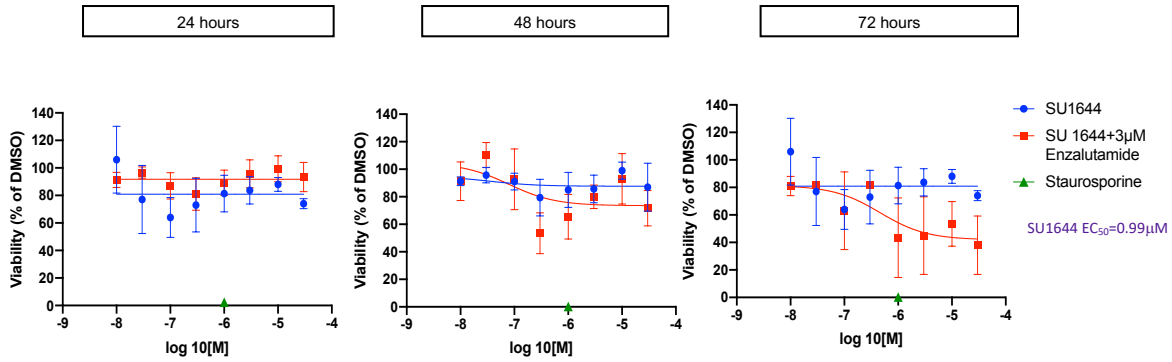


**Figure 8.11 Cell viability assay a combination of IKKα inhibitor (SU1644) and Enzalutamide in MDA-MB-453.** *A cell viability relative to DMSO control was determined by CellTiter-Glo after treatment with SU1644 (blue), SU1644+Enzalutamide (red). Results shown are means ± the standard error of mean (SEM). The symbols *, **, ***, and **** indicated P<0.05, <0.005, <0.0005 and <0.0001.*

## 8.10 Discussion

IKKα is a key kinase in the non-canonical NF-κB pathway, involved in phosphorylation of p100 and processing of p100 to p52 to regulate transcription of many genes that are involved in tumour growth (7). The results from the chapters on patient tissue studies demonstrated a complex relationship between IKKα and survival outcomes in TNBC patient dependent on phosphorylation site and potentially AR status. Therefore, we performed an investigation to establish the impact of pharmacologically inhibiting IKKα in two ER-negative breast cancer cells with differing AR status. The combination of IKKα inhibitor and AR antagonist was also investigated in order to establish the impact of dual targeting in these cell lines. Impact of inhibition on p52 processing was assessed by western blotting, and phenotypic response was assessed by changes in cell viability.

In the present study, lymphotoxin was used to stimulate activation of the non-canonical pathway in both ER-negative/AR-negative MDA-MB-231 cells and ER-negative/AR-positive MDA-MB-453 cells. Activation of non-canonical pathway after stimulation with lymphotoxin was observed in one experiment but not in the other experiments. This finding was inconsistence with the other studies, which showed lymphotoxin can induce activation of this pathway in both cell lines (253, 254). As only one experiment was completed per treatment protocol due to COVID-19, it difficult to draw any conclusion for this result. Future work that could perform three experiments in triplicate might be able to demonstrate consistent result with other studies.

In ER-negative/AR-negative MDA-MB-231, no inhibition effect on p52 expression was observed after treatment with SU1433 or SU1644. An effect on cell viability of this cell line after 48-hour treatment with SU1433 and SU1644 was observed, indicating an inhibitory effect of SU1433 and SU1644. Since the protein was extracted at 24 hours after treatment, it might be too soon to demonstrate the inhibitory effect on western blot. Therefore, this may explain the inconsistent result was observed between the two types of experiments and would warrant future western blot experiments to extract protein at 48 hours. As expected, in an ER-negative/AR-negative MDA-MB 231 cell line, no enhanced effect on the viability assay was observed after treatment with the combination of enzalutamide with either SU1433 or SU1644.

A small inhibition effect on p52 on western blots in ER-negative/AR-positive MDA-MB-453 cells was seen after treatment with SU1433. Since I was unable to complete all the experiments as planned due to the impact of COVID-19, statistical analysis was not performed. Therefore, it was unable to conclude the meaning of these results. Future experiments need to repeat three times in triplicate to demonstrate if there is a statistical significance.

In MDA-MB-453 cell, a dose-dependent decrease in cell viability was observed after treatment with SU1433 and was greater following 72 hours of treatment. However, this effect on the viability was not increased following treatment with the combination of enzalutamide with SU1433, suggesting the combination was not advantageous over SU1433 alone. Similarly, a dose-dependent decrease in cell viability was observed following 48 and 72 hours of the treatment with a combination of SU1644 and enzalutamide but not with SU1644 alone, suggesting enzalutamide alone was responsible for this effect.

Although these experiments demonstrated evidence of inhibiting IKKα might benefit in ER-negative breast cancer, it needs to perform in three experiments in triplicate to demonstrate statistically significant, which was not possible due to COVID-19 restrictions.

# Chapter 9 General Discussion

## 9.1 Tissue work

The NF-κB pathways regulate transcription of genes that involve in tumour progression and metastasis in many types of cancers, including prostate, melanoma, colon, breast and pancreatic cancer (134, 135) (149) (147, 151). The role of IKKα, a key kinase for NF-κB activation, has been widely investigated in cancer (149) (162). It has also been implicated in tumour progression, apoptosis, immune functions and chromatin remodelling in an NF-κB independent manner (162) (165, 166). We hypothesised that IKKα and its phosphorylation are associated with the development of breast cancer, resistance to treatment and is potentially a novel therapeutic target for breast cancer treatment.

A previous study has demonstrated crosstalk between the NF-κB pathway and AR to promote tumour progression and induce tumour resistance to treatment in prostate cancer (231, 232). However, it is still inconclusive in TNBC breast cancer. Thus, we also hypothesise that AR might potentially crosstalk with NF-κB through interacting with IKKα to affect tumour development and resistance to treatment in breast cancer. Therefore, we also hypothesise that AR might potentially crosstalk with NF-κB through interacting with IKKα to affect tumour development and resistance to treatment in breast cancer.

To address these hypotheses, we aimed to establish whether IKKα, pIKKα-Ser176, pIKKα-Thr23, and AR were associated with clinical outcomes utilising patient tissue cohorts and investigate the potential therapeutic option of an IKKα inhibitor alone or in combination with an AR antagonist in breast cancer cell lines.

Overall, the findings reported here suggest that the prognostic role of IKKα, pIKKα-Ser176 and pIKKα-Thr23 was differed within each subtype of breast cancer and differed depending on their cellular locations. There were inconsistent results of a prognostic role of AR and a combined expression of AR with IKKα, pIKKα-Ser176, and pIKKα-Thr23 did not provide additive prognostic power over high AR alone. Although this study did not confirm the prognostic role of the proteins of interest, several questions remain to be answered regarding the role of IKKα and its phosphorylation in breast cancer.

Several limitations need to be noted regarding the prognostic roles of IKKα and its phosphorylation in this study. One issue with the current study was using the IHC-based classification instead of gene expression subtyping to classify breast cancer subtype, which might lead to an appropriated subtypes classification that might affect the outcomes measure.

Although using the IHC-based classification is common in clinical practice, information on their prognostic capacity and comparative validity differs from those using gene expression-based molecular subtypes (255). The previous study has investigated and found that the concordance between IHC-based classification and PAM50 subtyping ranges from poor to moderated (255). Kaplan-Meier curves of distant recurrence-free survival were differed between using IHC-based and PAM50 (255). Therefore, a further study should assess the subtype using gene expression-based molecular subtypes to provide more accurate subtype classification.

An additional uncontrolled factor was the possibility of using old TMAs, which might lead to different expressions of the proteins of interest that might affect the IHC results. Previous studies have found that storage of paraffin sections for more than two months leads to epitope degradation and antigenic loss (256, 257). To avoid this problem, IHC should be done on newly cut sections.

Another limitation was that the optimal antibody to the phosphoproteins at serine 176 and threonine 23 on IKKα was unavailable at that time. Therefore, it was unable to perform an experiment to demonstrate if co-expression of these two phosphorylation sites would be adding more information.

The other limitation of this study was the multiple cut-off values to classify tumours with low and high expression of each protein. As it was a range in the expression of the proteins, the median was used at the cut-off value for low and high expression. To date, there is no currently standardised method for biomarkers cut-off determination (258, 259). Many efforts have been established the ways to determine cut-off points, including the minimum p-value approaches and the ROC curve analysis (258-260). The minimum p-value approach is the method that all measured values are analysed as provisional cut-offs, and the value that has maximum relative risk, minimum p-value and maximum chi-squared statistic is selected (259). The ROC curve analysis is another method that has been widely used. However, the cut-off drawn from the ROC curves analysis does not consider censoring of the data. Using dichotomising as “death” or “alive/censored” without the follow-up time could be suboptimal for prognostic study (258, 259). Using the minimum p-value approach may have been more appropriate for the determination of optimal cut-off values and should be considered in future work.

Another aim of the present study was to investigate differentially expressed gene profiles for tumours with low versus high cytoplasmic IKKα, cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23 in ER-negative tumours. We hypothesis that such DEGs are associated with distinct biological functions, representing a common mechanism of tumours with high expression of IKKa, pIKKα-Ser176 and pIKKα-Thr23.

Among the total of DEGs, 14 and 3 significant DEGs were observed in the IKKα and the pIKKα-Thr23 group, respectively. None of the DEGs was found to be a significant difference among the pIKKα-Ser176 group. The cluster analysis of tumours with high versus low expression of cytoplasmic IKKα, cytoplasmic pIKKα -Ser176 and nuclear pIKKα-Thr23 was performed to cluster the top 20 DEGs. Although the samples did not perfectly cluster together, there were notable different gene profiles observing for cytoplasmic IKKα. However, there were no clear clustering of the top 20 DEGs for cytoplasmic pIKKα-Ser176 and nuclear pIKKα-Thr23.

Among the significantly DEGs in the IKKα group, several genes have been reported to involve in tumour progression in breast cancer, including ACTA2, ALCAM, PDCD6IP, CD9, MVP, and PRSS8 (1-6). Among those three DEGs in the pIKKα-Thr23, none was observed in breast cancer (190). As the DEGs analysis was based on a small sample size, the present study did not confirm the hypothesis regarding DEGs between tumours that have high and low expression of the proteins of interest. The study should be repeated using an adequate number of samples.

## 9.2 *In vitro* studies

The prognostic role of IKKα and pIKKα-Ser176 were found to differ among TNBC tumour from the cohort of mixed breast cancer subtype and the Belfast cohort. Expression of pIKKα-Thr23 was associated with worse prognosis in TNBC tumour from the mixed cohort but this was not validated in the Belfast TNBC cohort, but does show similar results in AR-positive tumours in this cohort. Generally, this finding demonstrated a complex relationship between IKKα and survival in TNBC tumours dependent on phosphorylation site and potentially AR status. Therefore, an *in vitro* study was performed to establish the impact of pharmacologically inhibiting IKKα in two ER-negative breast cancer cells with differing AR status.

The cell lines were ER-negative/AR-negative MDA-MB-231 and ER-negative/AR-positive MDA-MB-453 cells. Proprietary selective preclinical IKKα inhibitors, SU1433 and SU1644 were used to investigate the sensitivity of the two cell lines to IKKα inhibitors. These inhibitors were generated through CRUK and MRC-DPFS programme awards, by Professor Simon Mackay at the University of Strathclyde. Impact of IKKα inhibition on p52 processing was assessed by western blotting and phenotypic response was assessed by changes in cell viability.

In ER-negative/AR-negative MDA-MB-231, an inhibition effect on p52 expression was observed using western blotting after treatment with SU1433 but was not seen with SU1644. Since the protein was extracted at 24 hours after treatment, this might be too soon to show the inhibitory effect. The effect on cell viability was observed after treatment with 48 hours SU1433 and SU1644. As expected, the combination of enzalutamide with either SU1433 or SU1644 had no apparent enhanced effect on the viability assay.

In ER-negative/AR-negative MDA-MB-453, the small inhibition effect on p52 was observed after treatment with either SU1433 or SU1644. A dose-dependent decrease in cell viability was observed after treatment with SU1433 and was greater following 72 hours of treatment.

However, an additive effect on the viability at 24 and 48 hours was not found following treatment with the combination of 3mM Enzalutamide with either SU1433 or SU 1644, suggesting disadvantageous of the combination. Each treatment has been planned to repeat three time in triplicate. However, due to time restrictions and the impact of COVID-19, I was unable to complete all the experiments as planned. Therefore, statistical analysis was not performed. However, these experiments demonstrated evidence that inhibiting IKKα might be of benefit in ER-negative breast cancer.

## 9.3 Future work

Breast cancer is a heterogeneous disease controlled by the coordination between tumour cells and their tumour microenvironment (TME), including extracellular matrix (ECM), immune cells, myoepithelial and endothelial cells (261). Much of the literature has emphasised among breast cancer subtypes, TNBC and HER2-positive breast cancer have a highly inflammatory microenvironment, which is composed of the infiltrating immune cells, growth factors and cytokines (261, 262). There is a large volume of published studies

describing the molecular mechanisms of the interaction between tumour and the TME that further allows immunotherapy as a new therapeutic option for breast cancer (261-263). Up to now, several prognostic and predictive biomarkers for immunotherapy has been widely investigated in breast cancer, including programmed death-ligand 1 (PD-L1) and tumour infiltrating lymphocytes (TILs) (264, 265). In a clinical trial, patients with PD-L1-positive have a better response to immunotherapy (265). Similarly, TILs have been established as a predictive marker for response to chemotherapy and the prognosis for all breast cancer subtypes, particularly TNBC and HER2-positive breast cancer (264, 266). Not only the number of TILs but also the phenotype of TILs affects the clinical outcomes (267). A previous study has reported that Type 1 CD4+ T-helper 1 (Th1) are associated with favourable prognosis, and CD8+ cytotoxic T-cells (CTL) are essential for tumour destruction (268). In contrast, Type 2 CD4+ T-helper 2 (Th2) such as Forkhead box P3 (FOXP3) CD4+ regulatory T-cells has been reported to suppress CTL function, inhibit host immune response that could promote tumour growth (269).

Recently, there has been growing recognition of the crucial links between the NF-κB pathway and TME (270). NF-κB can be activated by cytokines such as IL-1, IL-6, IL-8 and TNFα, produced by TILs (271, 272). In turn, NF-κB regulated transcription of genes involved in tumorigenesis and induced cytokines and chemokines production to attract inflammatory and immune cells lead to a positive feedback loop to maintain tumour-associated inflammation (271, 272).

High levels of NF-κB in the plasma breast cancer patients has been reported to correlate with an increased inflammatory marker, including IL-6 and TNFα (270). A previous study has reported that activation of NF-κB and STAT3 in tumour and stromal immune cells leads to the secretion of tumour-promoting cytokines such as IL-1, IL-6, IL-8, TNFα and VEGF (273). NF-κB also binds to the promotor of various genes, including the C-X-C motif chemokine receptor 2 (CXCR2) and vascular endothelial growth factor (VEGF) results in stimulating angiogenesis in TME (274, 275). NF-κB also induces an expression of MMP-2 and MMP-9 to facilitate epithelial-mesenchymal transition (EMT) and regulated transcription of cyclooxygenase-2 (COX-2), which is an essential factor of the inflammatory TME (276).

Programmed cell death protein 1 (PD-1) and T-lymphocyte-associated protein 4 (CTLA-4), which is expressed on an activated T cell, are key immune checkpoints that maintain immune

homeostasis (277). The binding to its ligand PD-L1 leads to suppression of T cell activation. Interactions of CTLA-4 and its ligands B7 inhibit T cell response leading to evading the host immune system of tumours (278). A study has reported that NF-κB regulates PD-L1 expression in repones to TNFα activation in breast cancer cell lines and tumour specimens from breast cancer patients (279). In TNBC, oncogene Mucin1 (MUC1) promote Mync and NF-κB to bind to the PD-L1 promoter and further enhance PD-L1 gene expression (280). Although the interaction between CTLA-4 and NF-κB is incompletely understood, it has been hypothesized that engagement of CTLA-4 and NF-κB might contribute to the suppression of T cell functions (281).

Currently, immune checkpoint blockade has provided promising results in breast cancer treatment, and there are still some patients that do not benefit from such treatment alone (265, 282). NF-κB has been demonstrated to regulate PD-L1 expression results in tumour immune suppression. Inhibition of PD-L1 expression with the NF-κB inhibitors in combination with PD-1/PD-L1 inhibitors could be beneficial to reverse T cell suppression and enhance host defence immunity.

Therefore, future works are required to better understand the complex linkage between the NF-κB pathway and the crucial components in TME in breast cancer, such as PD-L1 and CTLA-4. This will serve as a therapeutic potential of inhibition at NF-κB directly or molecules involved in NF-κB activation such as IKKα inhibitors, in combination with immune checkpoint blockade for patients with breast cancer, especially TNBC.

## 9.4 Conclusion

We hypothesised that high expression of IKKα, pIKKα-Ser176 and pIKKα-Thr23 would result in poorer outcomes in patients with breast cancer. Furthermore, combining these proteins with AR expression would contribute to unfavourable outcomes in patients with TNBC. The result in this thesis using three patients tissue cohorts suggest that IKKα, pIKKα-Ser176 and pIKKα-Thr23 may be associated with clinical outcome. However, the prognostic roles of these proteins are different depending on tumour subtypes and cellular locations.

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