Phenotypic and Molecular Characterisation of Breast Ductal Carcinoma *in situ*

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Declaration

I declare that this thesis is the result of my own work and has not in this, or any other form, been presented to this or any other university in support of an application for any degree other than that for which I am now a candidate.

Islam Abdelaziz

Approval

This work attained ethics approval from the North West – Greater Manchester Central Research Ethics Committee under the title; Nottingham Health Science Biobank (NHSB), reference number 15/NW/0685.

Abbreviations

ADH	Atypical ductal hyperplasia
ALH	Atypical lobular hyperplasia
AURKA	Aurora kinase A
BRCA1	Breast Cancer gene 1
BRCA2	Breast Cancer gene 2
CC-DCIS	Comedo-carcinoma *in situ*
CGH	Comparative genomic hybridisation
CI	Confidence interval
CN	Copy number
CNAs	Copy-number aberrations
CNVs	Copy-number variations
COX2	Cyclo-oxygenase 2
DCIS	Ductal carcinoma *in situ*
DIN	Ductal intraepithelial neoplasia
ECM	Extracellular matrix
EPC	Encapsulated papillary carcinoma
ER	Oestrogen receptor
FEA	Flat epithelial atypia
FFPE	Formalin fixed paraffin embedded
FFS	Full-face sections
FISH	Fluorescent *in situ* hybridisation
GEP	Gene expression profiling
H&E	Haematoxylin & Eosin
HER2	Human epidermal growth factor receptor 2
HG-DCIS	High-grade DCIS
IBC	Invasive breast cancer
IDC	Invasive duct carcinoma
IHC	Immunohistochemistry
IL	Interleukin
LCIS	Lobular carcinoma *in situ*
LG-DCIS	Low-grade DCIS
LOH	Loss of heterozygosity
LR	Local recurrence
LRFI	Local recurrence free interval
MECs	Myoepithelial cells
MI	Micro-invasion
MMPs	Matrix metalloproteases
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NCCN	National Comprehensive Cancer Network
NSABP	National Surgical Adjuvant Breast and Bowel Project
PR	Progesterone receptor
ROS	Reactive oxygen species
RT	Radiotherapy
SCNAs	Somatic copy-number alterations
SLNB	Sentinel lymph node biopsy
SMA	Smooth muscle actin

SMM	Smooth muscle myosin
SRC	Non-receptor tyrosine kinase
TDLU	Terminal duct lobular unit
TMAs	Tissue Microarrays
TN	Triple negative
TPX2	Targeting protein for Xenopus kinesin-like protein 2
TRX	Thioredoxin
TXNIP	Thioredoxin interacting protein
UDH	Usual ductal hyperplasia
UK/ANZ	United Kingdom, Australia and New Zealand
USC	University of Southern California
VNPI	Van Nuys Prognostic Index

Abstract

Background

Ductal carcinoma in situ (DCIS) comprises more than 20% of screen-detected breast cancer cases. The current understanding of the biology and clinical behaviour of DCIS and prediction of its progression to invasive disease remains incomplete. Treatment modalities vary; however, in most cases it involves surgical excision with or without adjuvant radiotherapy and endocrine therapy. To date, predictors of DCIS progression are not well defined; therefore, the aim of the current study was to characterise a large cohort of DCIS with long term follow-up data and identify possible predictors of DCIS outcome.

Patients and Methods

In this study, 1,249 pure DCIS cases and a control group of 239 DCIS cases mixed with invasion were identified between 1987–2017. Collection of the clinicopathological, management and outcome data was performed. Cases were reviewed and representative tumour blocks were selected and retrieved. Tissue microarrays (TMAs) were constructed (776 pure DCIS and 239 DCIS mixed with invasion), followed by molecular characterisation of DCIS for oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and proliferation marker (Ki67), using immunohistochemistry (IHC) and chromogenic *in situ* hybridisation (CISH). Mining for biomarkers with potential prognostic and predictive values in DCIS was performed with the identification of thioredoxin

interacting protein (TXNIP), aurora kinase A (AURKA), a targeting protein for Xenopus kinesin-like protein 2 (TPX2), and a non-receptor tyrosine kinase (SRC) and their expression was assessed in the TMA using IHC. In addition, whole tissue sections of DCIS were used to assess tumour infiltrating lymphocytes (TILs) as a component of the tumours' microenvironment.

Results

Analysis of the clinicopathological data of the DCIS patients revealed a significant increase in incidence of screen-detected DCIS during the period of the study. This was associated with a significant increase in the rate of breast conserving surgery (BCS) and the use of adjuvant radiotherapy (RT), along with a marked decline in the rate of re-excisions and recurrence. Screen-detected DCIS, localised DCIS lesions, small tumour size and negative surgical margins were the contributing factors to the reduced rate of re-excisions. Large tumour size, high tumour grade, and no adjuvant radiotherapy were independent predictors of the development of ipsilateral local recurrence.

Molecular characterisation of DCIS using ER, PR, HER2 and Ki67 revealed that the highest proportion (60%) of cases were of Luminal A subtype, however the lowest attribution (11%) was for HER2 positive (+) tumours. Tumours of HER2+, Luminal B/HER2+ and Triple Negative subtypes were of larger tumour size (>40mm), higher tumour grade, and more frequently treated with mastectomy. When the molecular

classes were investigated against the development of local recurrence, HER2 positivity was associated with ipsilateral DCIS local recurrence.

The assessment of the selected tissue marker panel with potential role in predicting DCIS progression revealed a significant difference in their protein expression between pure DCIS tumours and those associated with invasion. An association between low expression of TXNIP, and high expression of AURKA, TPX2 and SRC with the development of local recurrence was detected and that AURKA, TPX2 and SRC were predictors of invasive recurrence independent of other predictors of recurrence, including tumour size, grade, and radiotherapy.

Analysis of tumour infiltrating lymphocytes (TILs) showed that the most reproducible and prognostically significant method of TILs evaluation in DCIS is the assessment of touching TILs (defined as lymphocytes touching or within one lymphocyte cell thickness from the DCIS basement membrane), and that dense TILs predicted the development of local recurrence.

Conclusions

The outcome of DCIS improved significantly during the period of the study. This study identified several predictors of DCIS progression. In addition to some clinical and pathological variables including tumour size, grade and radiotherapy use, expression of TXNIP, AURKA, TPX2 and SRC, together with TILs density were independent predictors of DCIS outcome. These variables can potentially be used in combination to refine the prognostic stratification of DCIS patients in routine practice.

Publications

A: Thesis-related Peer-Reviewed Publications

1- Thioredoxin-interacting protein is an independent risk stratifier for breast ductal carcinoma *in situ*.
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2- Targeting Protein for Xenopus kinesin-like Protein 2 (tPX2) expression is associated with Poor outcome in Breast ductal carcinoma *in situ* (DCIS).
Miligy IM, Gaber A, Toss MS, Al-Kawaz A, Nolan CC, Rodriguez MA, Ellis IO, Green AR, Rakha EA.

Poster Presentation, Winter Joint Meeting with the Royal Society of Medicine 209th Scientific Meeting of the Pathological Society of Great Britain & Ireland 25 – 26 January 2018, London.

3- Characterisation of HER2 status in DCIS Using Immunohistochemistry (IHC) and Chromogenic *in situ* Hybridisation (CISH).
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Podium Talk, USCAP 107th Annual Meeting, March 17 – 23, 2018 in Vancouver, BC, Canada.

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1 General Introduction

1.1 Ductal Carcinoma *in situ* of the Breast

1.1.1 Definition

Ductal carcinoma *in situ* (DCIS) of the breast is defined as malignant epithelial cells confined to the duct system of the breast without evidence of invasion through the basement membrane (BM) into the surrounding stroma (Lester et al., 2009). It is considered to be a non-obligate precursor of invasive carcinoma that does not fully envelope the hallmarks of malignancy: unlimited growth, invasiveness, angiogenesis, and metastatic capacity (Lippman 2002). It is noteworthy that some cancers seem to evolve from a pre-invasive lesion referred to as carcinoma *in situ*, in which cells display all cytologic features of malignancy without invasion of the BM, to an overt invasive stage. The latter is considered one step prior to invasive cancer; with time, cells penetrate the BM, infiltrate the sub-epithelial tissue and subsequently have the ability to metastasise to remote organs (Rakha et al., 2018). From the differences between these neoplastic entities, it is hypothesised that invasion and metastasis are prominent at the clinical level; however, they represent a continuum of changes at the molecular level (Duffy et al., 2008, Virnig et al., 2010).

1.1.2 Epidemiology

Cancer is a major public health problem in many parts of the world (Siegel et al., 2015), with breast cancer as the leading cause of cancer-related deaths among women. In 2015, more than 54,800 women were diagnosed with breast cancer and about 11,400 women have died from this (Office of National Statistics 2015). Although its absolute rates are

relatively low, breast cancer is the second most common cause of cancer death in females in the UK (Office of National Statistics 2015).

In 2015, 7,900 women in the UK were diagnosed with *in situ* breast carcinoma, with 10–20% of DCIS cases seen bilaterally and 65–69 years peak incidence (Office of National Statistics 2015).

Before the emergence of the UK breast screening programme in 1988, DCIS represented only 2–5% of symptomatic breast cancers; currently, it accounts for 20–25% of all cancers and up to half of screen-detected breast cancers (Kopans 2003, Benson and Wishart 2013).

1.1.3 Risk factors

DCIS is a precursor of invasive carcinoma and therefore they share the same risk factors. Older age, a family history of breast cancer in first-degree relatives, and proliferative breast disease are recognised risk factors. For hormone receptor positive cancer, over-exposure to oestrogen, such as reproductive factors including nulliparity, early menarche and late menopause and older age at the time of the first full-term pregnancy and long-term use of postmenopausal hormone-replacement therapy are reported risk factors associated with an increased incidence of DCIS and invasive carcinomas (O'Brien et al., 2015). Genetic factors, such as germline mutations in breast cancer susceptibility genes, BRCA1 and BRCA2, increase the likelihood of developing invasive cancer and, to some degree, DCIS (O'Brien et al., 2015).

Not all DCIS cases progress to invasive carcinoma, but an estimated risk of 25–50% of DCIS cases progress to invasion if untreated (Bartlett et al., 2014). The long-term natural history of DCIS remains poorly understood and is ambiguous in the literature. The potential for progression into invasive carcinoma differs among the histological types of DCIS, and the current understanding of the biology and clinical behaviour of these lesions is incomplete, making it difficult to understand the actual relationship between DCIS and invasive breast cancer (IBC). To date, neither the histopathological classification nor the conventional biomarkers can accurately predict whether DCIS lesions can invade the surrounding tissue and consequently progress to metastatic disease (Carraro et al., 2014).

1.1.4 Clinical presentations

In past years, DCIS was detected clinically as a palpable mass (Schnitt and Collins 2009). Although such presentation is occasionally seen, the majority of DCIS cases are detected by mammogram in the form of microcalcifications in more than three quarters (76%) of the cases (Stomper and Margolin 1994), as a non-palpable mass in 11%, and as a combination of the above in 13% (Harris et al., 2004). Infrequently, DCIS presents clinically with a haemorrhagic nipple discharge, which is often seen in association with Paget's disease of the nipple. Moreover, DCIS can also be detected incidentally during breast evaluation for an unconnected problem (Schnitt and Collins 2009).

DCIS is accurately detected with magnetic resonance imaging (MRI), particularly the histopathologic extent of DCIS (Benveniste et al., 2017);

however, overestimation due to inflammatory or benign lesions commonly occurs (Baek et al., 2017). Although an improved imaging of DCIS could improve the preoperative staging, the identification of a more extensive disease by MRI could lead to over-treatment (Preibsch et al., 2018). Therefore, it is recommended that MRI should be carefully used and in specialised and experienced centres. To date, there is a controversy regarding the role of MRI in predicting the outcome of DCIS (Schouten van der Velden et al., 2009, Elder et al., 2017, Kuhl et al., 2017).

1.1.5 Gross pathology

DCIS is not a mass-forming lesion that can be recognised on macroscopic examination of the surgical specimen (Liu and Peng 2012). However, cases such as high-grade DCIS with comedo necrosis, where widened duct spaces with ‘soft centres’ can be observed, and some cases of solid papillary DCIS, may result in a mass-forming lesion of varying size within a confined margin (Scripcaru and Zardawi 2012).

1.1.6 Histological features and classifications

1.1.6.1 Histopathological classification

Diagnosis of DCIS depends partly on the degree of cytological atypia, but generally includes cyto-nuclear and architectural features, clonality of the cell population and extent of the lesion (Siziopikou 2013) (**Figure 1-1)**.

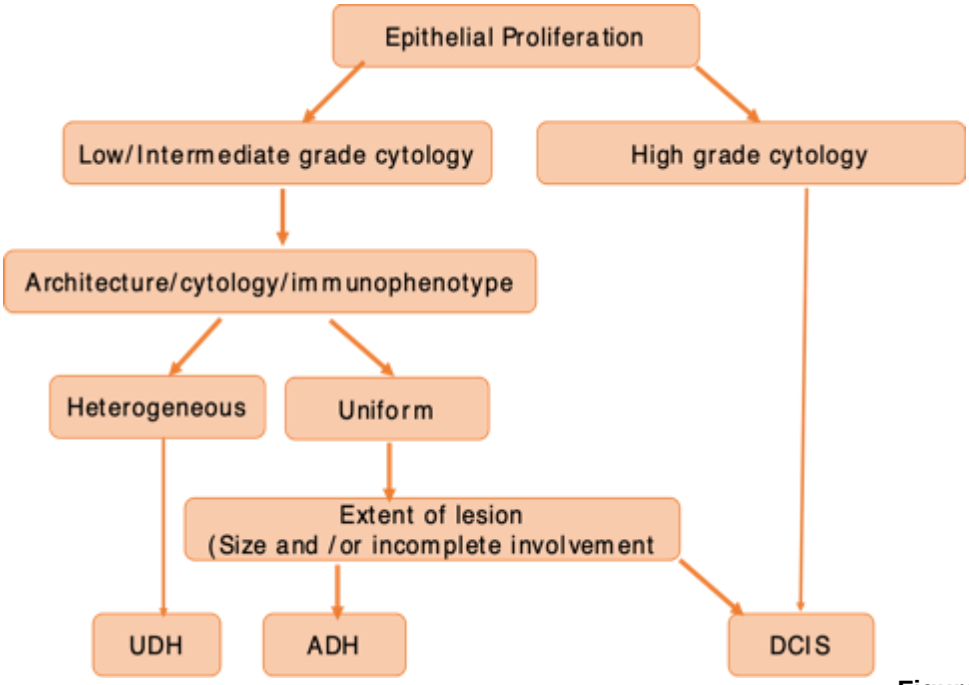


Figure 1-1: **Diagnostic criteria used for usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH) and DCIS (Siziopikou 2013).**

In spite of the prevalence of DCIS, there is no consistent accepted histological classification system; however, a growing consensus admits the importance of grade over morphology.

The conventional method of classifying DCIS was based on the architecture and growth pattern of the disease, with lesions classified as comedo, solid, cribriform, and micro-papillary (Douglas-Jones et al., 2000). Unfortunately, individual lesions can show architectural heterogeneity that moderates the use of this strategy. Systems of classification based on nuclear grade and necrosis show good reproducibility among pathologists, and therefore have prognostic value (Douglas-Jones et al., 2000).

The subtypes of DCIS were also previously divided into:

1. Comedo DCIS, large cell: more aggressive form;

2. Non-comedo DCIS, small cell: less aggressive form, which is further divided into cribriform, micropapillary, and solid DCIS (Page and Rogers 1987).

Histologically, DCIS is generally classified into three grades – low, intermediate and high grade (**Figure 1-2**). Grade is determined by cyto-nuclear features, including nuclear size, cellular polarisation, the presence and size of nucleoli and the frequency of mitotic figures (Lester et al., 2009). The features used in the assessment of nuclear grade are summarised in **Table 1-1**.

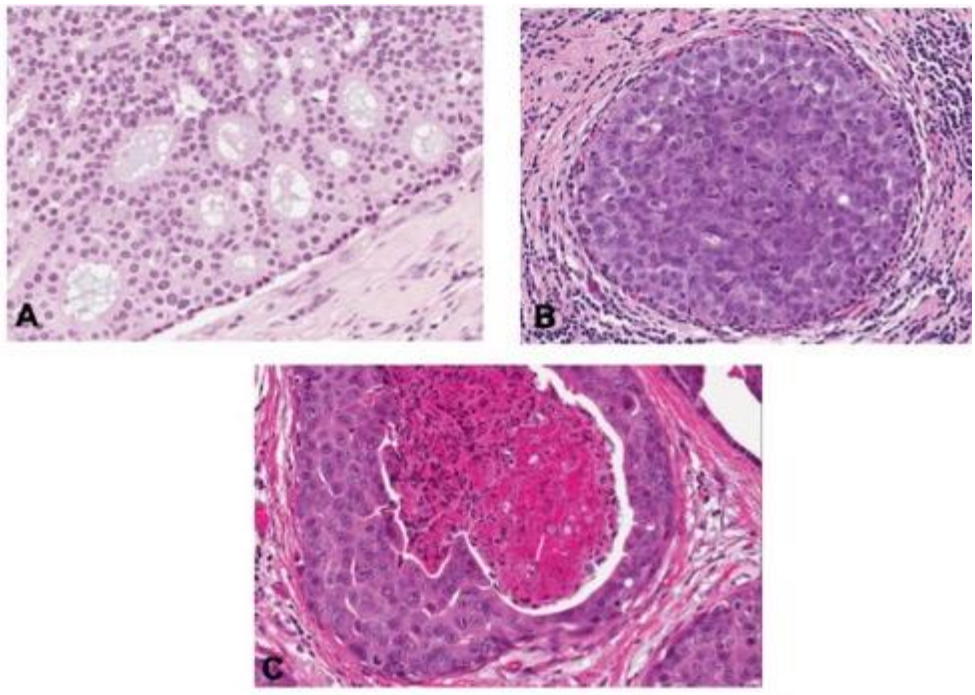


Figure 1-2: **Different grades of DCIS. A) Low-grade: the malignant cells show uniform small nuclei with fine chromatin and are polarised around secondary lumina. B) Intermediate grade: the neoplastic cells show moderately enlarged nuclei with lack of monotony. C) High-grade: the neoplastic cells display remarkably enlarged nuclei, with marked pleomorphism, coarse chromatin, loss of polarity, and central necrosis. All images are at 20x magnification.**

Table 1-1: Features determining nuclear grade in DCIS (Lester et al., 2009)

Feature	Low	Intermediate	High
Pleomorphism	Monomorphic	Moderate degree of pleomorphism	Pleomorphic
Size	1.5–2.0 the size of normal RBCs or a normal epithelial cell nucleus	2.0–2.5 the size of normal RBCs or a normal epithelial cell nucleus	>2.5 the size of normal RBCs or a normal epithelial cell nucleus
Chromatin pattern	Finely dispersed	Intermediate	Irregular chromatin distribution
Nucleoli	Rarely visible	Occasional nucleoli	Prominent and multiple
Mitoses	Occasional	Intermediate	Frequent
Orientation	Polarised towards the lumen	Preserved some degree of polarisation	Loss of polarity

RBCs: Red blood cells

However, it was not simple to classify DCIS into comedo and non-comedo types, and different classification systems have been suggested. One such system is the Van Nuys classification (Silverstein et al., 1995a). It combines three significant predictors of local recurrence: tumour size, margin width, and tumour grade [low grade (nuclear grade 1 or 2), which is further divided into two types based on the presence or absence of necrosis and high grade (nuclear grade 3)] (**Figure 1-3**).

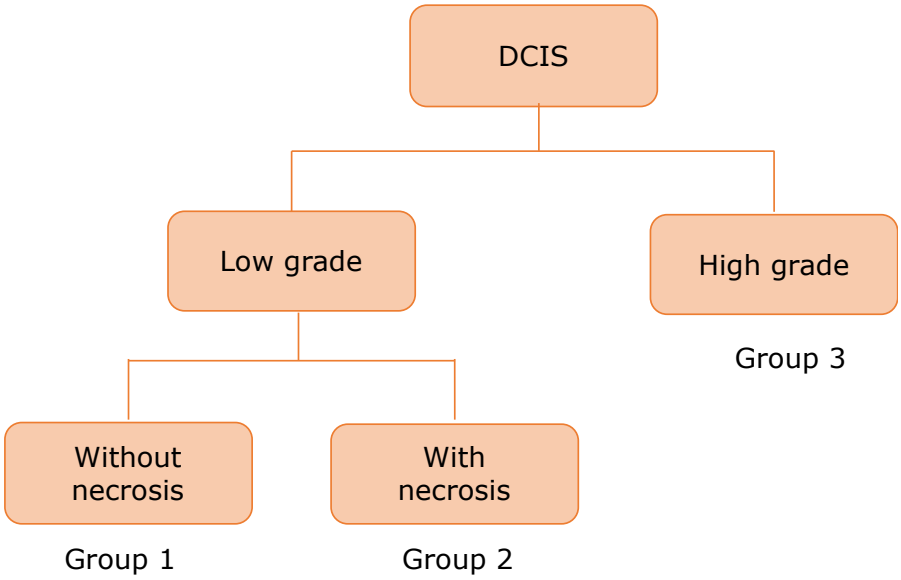


Figure 1-3: **Illustration of Van Nuys ductal carcinoma *in situ* (DCIS) classification (Silverstein et al., 1995a).**

It is rare to find low-grade DCIS (LG-DCIS) and high-grade DCIS (HG-DCIS) together, and LG-DCIS rarely proceeds to HG-DCIS (Carraro et al., 2014, Gorringe et al., 2015). Comedo-carcinoma *in situ* (CC-DCIS) is a distinctive type of HG-DCIS. CC-DCIS is the only DCIS that is observed grossly; therefore, it was over-diagnosed before the emergence of mammography. The term 'comedo' refers to a creamy yellowish necrotic material secreted from the ducts that resemble comedones. Histologically, this subtype is characterised by widened ducts filled with necrotic debris surrounded by a few layers of highly atypical epithelial cells and multiple mitotic figures (Shekhar et al., 2008). Necrosis usually occupies more than 90% of the duct cross section. The necrotic material calcifies and appears as large branching calcifications on mammography (Yamada et al., 2010).

An alternative grading system for atypical and intraductal malignant proliferations, including DCIS, was proposed by Tavassoli and Devilee

(Tavassoli and Devilee 2003). In this system, the term 'ductal intraepithelial neoplasia (DIN)' evolved and a numerical and alphabetical nomination was utilised to assign lesion severity; for instance, DIN 1C refers to low-grade DCIS, DIN II points at intermediate grade DCIS, and high-grade DCIS is referred to as DIN III (Veronesi et al., 2006). However, although this system has many advantages, it has not been widely accepted in clinical practice (Tavassoli 2005).

1.2 Morphological variants of DCIS

Different morphological variants of DCIS are known including solid, cribriform, comedo, micropapillary, papillary, solid variant of papillary DCIS, neuroendocrine, apocrine, cystic secretory, clinging and Paget's disease (Makki 2015). Over the past two decades, the classical classification of DCIS based on the morphological architecture has changed and current classification systems use nuclear grade and necrosis instead (Lester 2014).

The solid pattern may show micro-acini in which cells are polarised around small extra-cellular lumina in a rosette-type arrangement. Solid DCIS is often of low-grade; microcalcifications occur and are often of the psammomatous type (Rosser 1998). The cribriform pattern shows multiple adjacent ducts distended by a sieve-like proliferation of monotonous uniform cells. The multiple spaces within the proliferation are rounded and well-distributed in organised pattern (Rosser 1998).

A great number of DCIS lesions have more than one histological variant present, and the majority of such variants are well-recognised by

pathologists but some are uncommon and therefore deserve mention (Bane 2013).

1.2.1 Micropapillary DCIS

Micropapillary carcinoma is a variant of DCIS that is characterised by intraluminal tufts of malignant cells, which lack a true fibro-vascular core (Lee et al., 2011). It can be mixed with cribriform DCIS, but when present in a ‘pure’ form, it tends to involve multiple breast quadrants (Evers 2011).

1.2.2 Solid papillary carcinoma and encapsulated papillary carcinoma (EPC)

Although these entities have been regarded as variants of DCIS, recent studies have shown that the myoepithelial cells (MECs) are deficient in some lesions around the periphery of the tumour, evidenced by the immunohistochemical markers (Guo et al., 2016). Rakha et al. have proposed considering those lesions with obvious MECs as DCIS and those lacking as a special type of invasive carcinoma. In their study, these lesions showed a very low incidence of stromal invasion, lymph node metastasis, and a rare development of regional or distant recurrence. Therefore, they inferred that these lesions have indolent behaviour and good prognosis. They emphasised that local excision without post-operative chemotherapy is an adequate treatment of such lesions (Rakha et al., 2011).

1.2.3 Apocrine DCIS

Distinguishing apocrine DCIS from atypical apocrine proliferations is challenging as suggested by Scott et al. and the difficulty is in defining accurate grade (Scott et al., 1997). Characteristic cellular features best define apocrine DCIS include large cells with abundant eosinophilic cytoplasm and large nuclei with prominent nucleoli (Seo and Whang 2017). However, many morphological variants of apocrine DCIS have been recognised including solid, cribriform, and comedo subtypes (Scripcaru and Zardawi 2012). Nuclear grading of apocrine DCIS is essentially not easy, because apocrine cells classically are enlarged with prominent nucleoli (Leal et al., 2001). Therefore, it is suggested that the diagnosis and grading of apocrine DCIS should rely on the size of the lesion in addition to the nuclear grade and the presence or type of necrosis (O'Malley and Bane 2008, Visscher 2009).

1.2.4 Paget's disease

Paget's disease of the nipple is clinically defined as inflammation of the skin of the nipple, and pathologically is adenocarcinoma of the nipple epidermis (Ooi et al., 2018). Therefore, patients usually present the disease by an eczematous change in the nipple and areola and these are frequently associated with high-grade DCIS, with or without invasive disease in the underlying dermis. This variant of *in situ* disease is usually HER2 positive (Sandoval-Leon et al., 2013).

1.2.5 DCIS with micro-invasion (MI)

DCIS with MI is defined by malignant epithelial cells that have breached the BM and invaded the adjacent stroma up to a depth of 1mm. This

can be in the form of a single cell or clusters of cells (Kim et al., 2018). Multi-focal MI is defined when the size of the scattered single foci cannot be added together, and in this case, the lesion is still staged as T1mic. MI is most commonly seen with high-grade DCIS, although it may be identified in association with other DCIS grades (Wang et al., 2015). Exact clear diagnosis may be variable in cases associated with high-grade DCIS and extensive cancerisation of lobules or cases with a prominent stromal lymphocytic infiltrate and marked stromal distortion (Sopik et al., 2018). These difficulties can be overcome by the application of combinatory cytokeratin stains to highlight the epithelial cells and myoepithelial markers to highlight the presence of malignant cells beyond the boundaries of the DCIS duct (Momin et al., 2016). MI, particularly in association with high-grade DCIS often necessitates a sentinel lymph node biopsy as the ratio can be up to 20% in cases with lymph node metastases, predominantly micrometastases or isolated tumour cells (Orzalesi et al., 2015).

1.3 Molecular pathology of DCIS

1.3.1 Intrinsic ''molecular'' subtyping of DCIS

DCIS can be classified based on histological pattern and nuclear grade in addition to other features such as the presence of necrosis. Molecular profiling by gene array and its translation into a surrogate immunohistochemistry profile is likely to have a great impact on breast cancer classification, management and understanding of DCIS (Clark et al., 2011), particularly as the molecular profile of DCIS is generally

similar to invasive disease, which may reflect being the cell of origin (Dawson et al., 2010).

Each of the intrinsic subtypes identified in IBC are also recognised in DCIS, though there are differences in the relative frequencies of the groups in the two stages of the disease (Clark et al., 2011, Mardekian et al., 2016). Using a surrogate panel of immunohistochemical antibodies, oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), Luminal A, Luminal B, HER2 positive and Triple Negative (TN) are well characterised subgroups of both DCIS and invasive disease (Kurebayashi et al., 2007). Using additional antibodies, Tamimi et al. also reported significant differences between molecular phenotypes in DCIS and invasive breast cancer showing that the basal subtype (defined as negative for ER/PR/HER2 and positive for EGFR and CK5/6) was less frequent in DCIS compared with invasive cancers being more when restricted to IBC. Furthermore, the Luminal B HER2 positive (defined as ER/HER2 positive) subtypes are more frequent in DCIS compared with invasive disease (Tamimi et al., 2008). Additionally, the molecular subtypes correlate with grade. LG-DCIS is associated with ER positivity and luminal phenotypes, while HG-DCIS is associated with negative ER, positive HER2, and the basal-like phenotype (Lari and Kuerer 2011). The basal-like subtype is associated with a higher risk of local recurrence (LR) than is the case with luminal subtypes, and may even have stronger and more consistent associations than some of the conventional histopathological factors (Benson and Wishart 2013).

Owing to the relatively low rate of recurrence of DCIS, either *in situ* or as an invasive disease (Duggal et al., 2013), there are few studies that relate molecular phenotype or biomarker expression of DCIS to patient outcome (Clark et al., 2011). Thus, it is well recognised that the triple negative, basal subtype is associated with more aggressive disease in invasive cancers; therefore, it is postulated that DCIS with a basal phenotype is likely to exhibit more aggressive behaviour (Cyr and Margenthaler 2014). This is not a final conclusion and other factors may contribute, such as microenvironmental changes that may be of more importance in DCIS than established invasive disease (Duggal et al., 2013).

1.3.2 Molecular and genetic features of DCIS and its progression to invasive carcinoma

Many studies have been conducted to study the genetic aberrations underpinning the development of DCIS and its progression to invasive carcinoma (Lopez-Garcia et al., 2010). These studies have modified the original model of breast cancer evolution proposed by Wellings and Jensen (Wellings and Jensen 1973). In their model, breast cancer develops over an extended period of time from a normal terminal duct lobular unit (TDLU) via stages of UDH, ADH, DCIS and finally invasive cancer by the accumulation of genetic alterations (Wellings and Jensen 1973) (**Figure 1-4)**.

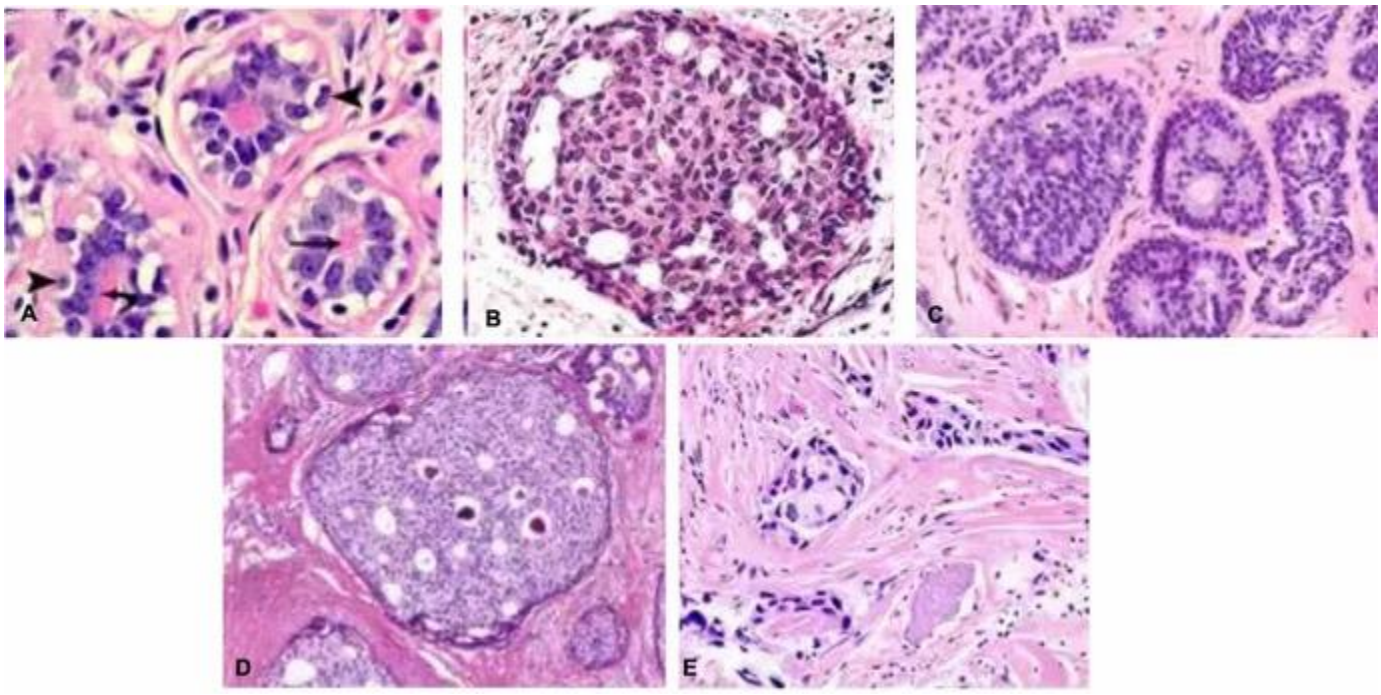


Figure 1-4: **Model of progression from normal (A), usual ductal hyperplasia (B), atypical ductal hyperplasia (C), ductal carcinoma *in situ* (D) to invasive breast carcinoma (E) (Wellings and Jensen 1973).**

The current hypothesis proposes two models: low-grade DCIS is the precursor of low-grade invasive carcinoma and high-grade DCIS is the precursor of high-grade invasive disease (Simpson et al., 2005). To corroborate this view, low-grade *in situ* and invasive lesions and many of their supposed precursor lesions – flat epithelial atypia (FEA), ADH, atypical lobular hyperplasia (ALH), and lobular carcinoma *in situ* (LCIS) – are frequently ER positive, HER2 negative lesions and are predominantly characterised by the shared deletion of the long arm of chromosome 16 (16q) and 1q gain (Roylance et al., 2006). On the other hand, high-grade DCIS and invasive lesions frequently show ER negativity, HER2 amplification, have more frequent alterations and

regions of amplifications, as identified by comparative genome hybridisation (CGH) and fluorescent *in situ* hybridisation (FISH) studies. The evolution of intermediate grade disease is less clear and currently no specific genetic alteration can robustly predict the progression from DCIS to invasion (Roylance et al., 2006).

1.4 Progression to invasive carcinoma

The molecular subtypes revealed by comprehensive gene expression profiling of invasive breast cancer (Sotiriou and Pusztai 2009) are present at the DCIS stage (Vincent-Salomon et al., 2008), suggesting the existence of distinct tumour progression pathways for each tumour subtype (Buerger et al., 1999). Either cell of origin or tumour subtype-specific genetic and／or epigenetic events, or a combination of both, are possible explanations of the presence of specific molecular subtypes from the early stages of breast cancer development (Weigelt and Reis-Filho 2009) and the particular maintenance of the phenotypic characteristics from DCIS to invasive cancer and from invasive cancer to metastasis in the majority of cases (Allred et al., 2008).

Phenotypic and genomic analysis comparing DCIS and invasive ductal carcinomas demonstrated strong similarities between these lesions and have failed to identify tumour-specific signatures (Ma et al., 2003, Johnson et al., 2012). These results corroborate the hypothesis that the progression from DCIS to invasive cancer may not be necessarily determined by specific genetic alterations or their consequences on gene expression profile (Buerger et al., 2000). An alternative explanation for the lack of a genetic aberration that could be considered

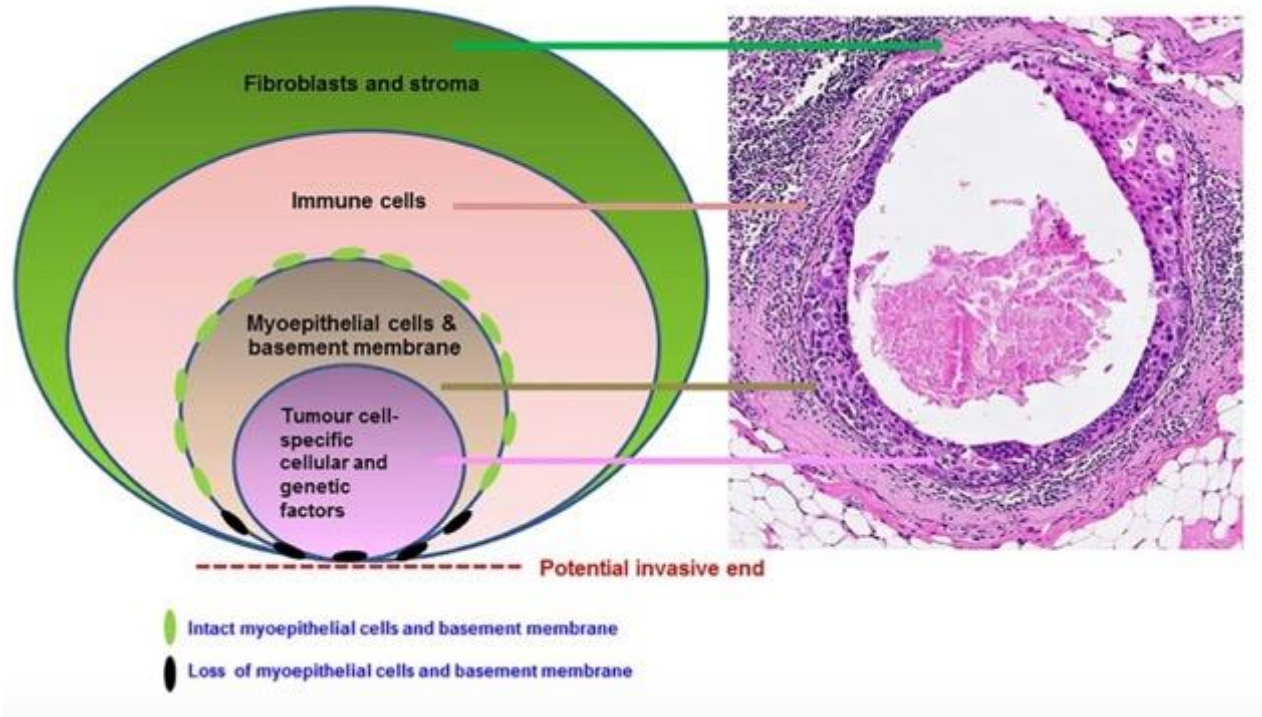
a common denominator in the progression from DCIS to invasive cancer is that there are multiple genetic or epigenetic aberrations that might drive progression, and that in each case, specific aberrations occur (Lopez-Garcia et al., 2010). In addition, molecular changes in the microenvironment may play a role in the progression from *in situ* to invasive disease (Lopez-Garcia et al., 2010). Distinct epigenetic alterations exist in all cell types during breast tumourigenesis, including tumour epithelial and stromal cells (Hu et al., 2005). Epigenetic changes observed in both the stroma and tumour cells may also be different depending on the tumour subtype, supporting the concept of co-evolution of tumour epithelial and stromal components (Lesurf et al., 2016).

The identification of novel genetic alterations and molecular pathways underlying the progression from DCIS to IBC may help to establish biomarkers that have the potential to distinguish low-risk patients who do not require aggressive treatment and high-risk patients who are likely to progress to IBC (Doebar et al., 2016b). In clinical practice, a proportion of patients present with extensive involvement of the breast with DCIS without signs of invasion (Yeong et al., 2017). Although it cannot be precluded that these cases would progress over time, the DCIS growth pattern suggests that these cases have a limited invasive potential (Pinder 2010a). On the other hand, other patients have a limited amount of DCIS adjacent to an invasive component or multiple foci of invasion, suggesting a high invasive potential. These differences

in the biological behaviour of DCIS display different alterations at the molecular level (Yeong et al., 2017).

1.4.1 Factors affecting the transition from DCIS to invasive carcinoma

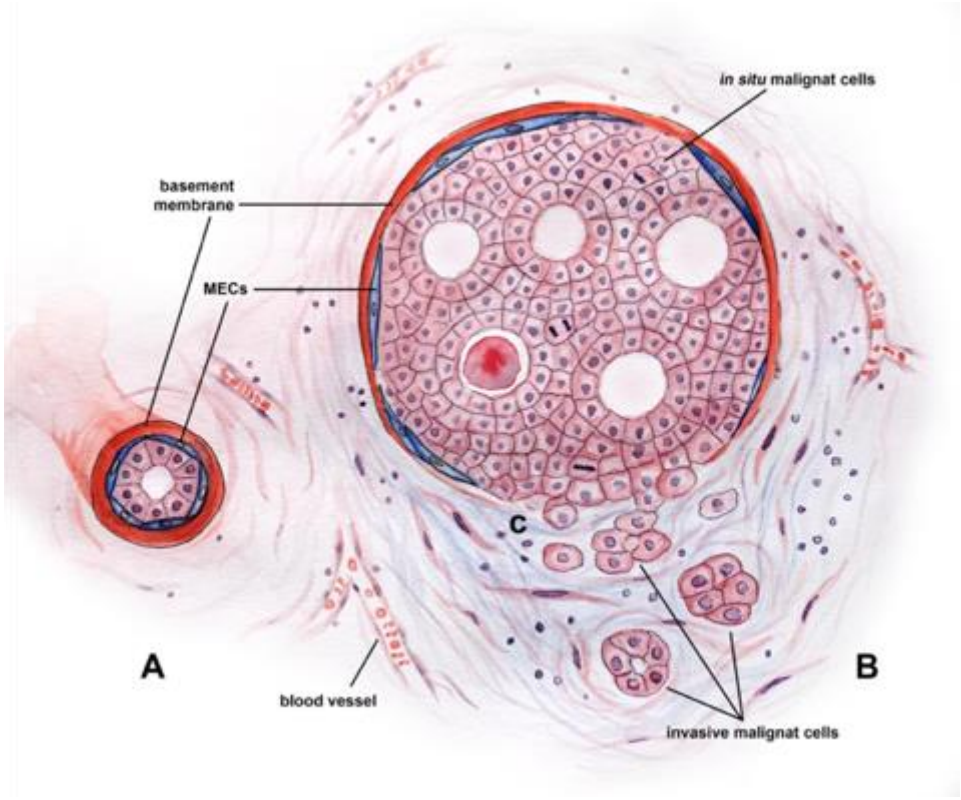
The study of the cells' ability to invade the stroma and acquire metastatic potential has been one of the areas of interest in recent years in patients with DCIS (Muggerud et al., 2010). Both of these steps are crucial, since predicting DCIS behaviour and preventing the tumour cells from becoming invasive is one of the principal objectives in the treatment of DCIS (Barrio and Van Zee 2017). MECs, stromal cells, vascular supply, and cell-mediated immune mechanisms all play a role in the transition from DCIS to invasive cancer (Yeong et al., 2017)



(**Figure 1-5)**.

Figure 1-5: **Factors contributing to the progression of ductal carcinoma *in situ* (DCIS) to invasive cancer including genetics of tumour cells, MECs, BM, immune cells, fibroblasts and stroma (Yeong et al., 2017).**

One of the main morphological distinctions between DCIS and IBC is the preservation of the MEC layer in DCIS; the lack of MECs is considered one of the hallmarks of invasion (Rakha et al., 2018)



(**Figure 1-6)**.

Figure 1-6: **Schematic representation of normal TDLU of the breast (A) and DCIS with foci of early invasion (B). Normal ducts are cuffed by a continuous MEC layer and a well-defined BM. MECs surrounding normal ducts are different from those around malignant *in situ* lesions. Invasion via breaks in the BM (C). (Rakha et al., 2018).**

1.4.1.1 Myoepithelial cells and their role in DCIS progression to invasive carcinoma

MECs are biologically different in normal and neoplastic breast tissue. In DCIS, they act as tumour suppressors, inhibitors of the invasive properties of the epithelial cells and angiogenesis (Pandey et al., 2010).

Loss of MECs is classically assessed using immunohistochemical markers such as p63, CD10, CK 5/6, S100 protein smooth muscle actin (SMA), and smooth muscle myosin (SMM) (Mardekian et al., 2016). Because of the heterogeneous staining patterns seen with these markers, pathologists frequently use combination of these markers to exclude invasive carcinoma. Abnormal expression of these markers has been observed in the MECs of DCIS lesions, high-grade DCIS with necrosis, and pleomorphic LCIS, supporting the concept that these MECs are phenotypically abnormal (Hilson et al., 2009, Mardekian et al., 2016).

It has been proposed that not only do DCIS MECs lack their normal tumour suppressive abilities, they may instead promote tumour progression (Rakha et al., 2018).

1.4.1.2 Role of stromal microenvironment in DCIS progression

The profound lack of genomic evolution between DCIS and IBC has led some researchers to focus on the potential role of the stromal microenvironment in mediating the transition to invasion (Sanati 2018). It is known that the tumour-associated stromal cells secrete factors such as vascular endothelial growth factor, interleukin (IL)-6, and IL-8, which promote angiogenesis and breast cancer progression (Rakha et al., 2018). In contrast to tumour epithelial cells, tumour-associated stromal cells demonstrate specific gene expression changes during the transition from *in situ* to invasive carcinoma. In stromal cells, the transition to invasive growth is accompanied by increased expression of several matrix metalloproteases (MMPs) including MMP2, MMP11, and

MMP14 (Ma et al., 2009). The majority of stromal gene expression changes are observed during normal to *in situ* transition and extensive gene expression changes occur in all cell types during cancer progression (Hu et al., 2008). The most consistent and striking changes affect MECs (Pang et al., 2017) with the contribution of both the epithelial and stromal compartments to the scenario of progression from DCIS to invasive duct carcinoma (IDC) (Rohilla et al., 2015).

1.5 Prognostic and predictive factors in DCIS

A variety of clinicopathological factors have been demonstrated to impact the rate of LR following current treatment modalities. Poor prognostic factors include the following (Pang et al., 2016, Gorringe and Fox 2017) (**Table 1-2**).

Table 1-2: **Clinicopathological factors and biomarkers associated with increased risk of recurrence in DCIS (Pang et al., 2016)**

Clinical Features	Young age (<40 years) High breast density (mammogram) Symptomatic presentation Multifocal disease Large tumour size
Histopathological Features	High nuclear grade Comedo necrosis Positive or close surgical margins
Protein Markers	ER HER2 Cell cycle markers Ki67, p16, p21, p53, COX-2

ER: Oestrogen receptor; HER2: Human epidermal growth factor receptor 2; COX-2: Cyclo-oxygenase 2.

It has been suggested that the final pathology report must include the following features as a minimum: tumour size, margins' width, tumour grade, and necrosis presence/type, to allow decision-making for

patients' management (Harrison et al., 2017). All of the mentioned parameters together with patient age are included in the University of Southern California/Van Nuys Prognostic Index (USC/VNPI). This scoring index gives a scoring range from 1 to 3 for each of the four clinicopathological tumour parameters, as demonstrated in **Table 1-3**. The authors claim that patients with a tumour featuring a low USC/VNPI score (defined as a score of 4, 5 or 6) are candidates for surgery alone (LR rate of 5.4% at 12 years), whereas tumours with a higher score (7 or more) are likely to develop LR (>20% at 12 years) if they are treated with excision alone, and therefore adjuvant radiotherapy (RT) or mastectomy is required (Silverstein and Lagios 2015).

Table 1-3: **USC/VNPI scoring system (Silverstein and Lagios 2015)**

Score	**1**	**2**	**3**
Size	≤ 15mm	16-40	> 40mm
Grade	1 or 2 without necrosis	1 or 2 with necrosis	Grade 3
Margin	≥ 10mm	1-9mm	<1mm
Age	>60	40-60	<40

Oncotype DX® is one of the commercially available molecular tests that have the potential to predict outcome in certain patients, particularly since thorough histopathological analysis alone does not seem to be sufficient for evaluating recurrence risk (Rath et al., 2018). Recently, a modified form of the Oncotype DX® recurrence score for IBC has been developed for DCIS to guide RT recommendations after BCS (Nofech-Mozes et al., 2018). This multigene expression assay score is generated from an algorithm that includes 12 genes (seven cancer

related genes and five reference genes) of the 21 genes in the Recurrence Score assay (**Figure 1-7**), to generate a score that quantifies the likelihood of cumulative LR (DCIS or invasive carcinoma) at ten years and predicts the risk of an invasive carcinoma local event at ten years (Solin et al., 2013).

Three risk categories are identified by the score: low risk (DCIS Score <39), intermediate risk (DCIS Score 39–54), and high risk (DCIS Score ≥55). The DCIS Score provides a trial of estimates of the recurrence risk that may help reduce under-treatment for women with a high risk of recurrence and over-treatment in those patients with a low risk (Lin et al., 2018). However, to date, the clear-cut difference between intermediate- and high-risk groups is not well established (Knopfelmacher et al., 2015).

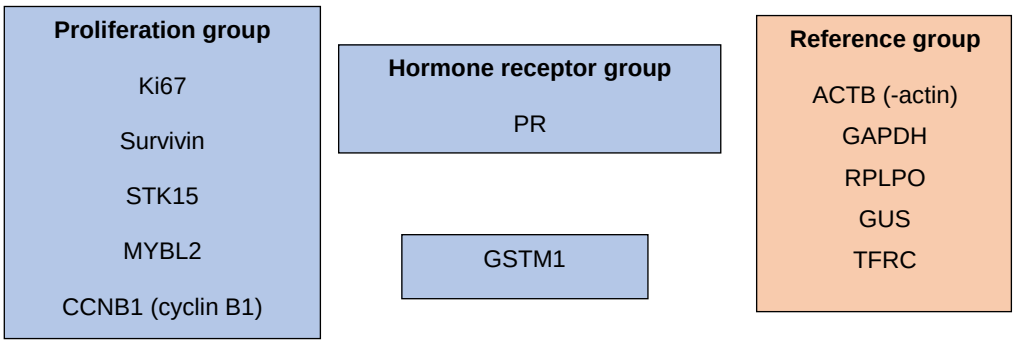


Figure 1-7: **Panel of 12 genes included in the DCIS Score. Seven cancer-related genes: Ki67 = MKI67; STK15 = aurora kinase A; survivin = BIRC5; CCNB1 = cyclin B1; MYBL2 = v-myb myeloblastosis viral oncogene homolog (avian)-like 2; PR = progesterone receptor; and GSTM1 = glutathione S-transferase M1. Five reference genes: ACTB = beta-actin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; RPLPO = large ribosomal protein; GUS = beta-glucuronidase; and TFRC = transferrin receptor (Solin et al., 2013).**

Certainly, the impact of molecular test costs and their application to a wide spectrum of patients with DCIS requires thorough analysis to maximise their effect on patient management (Mardekian et al., 2016).

It will be better to stratify patients with DCIS using a combination of pathologic examination, immunohistochemical studies and molecular analysis (Gorringe and Fox 2017). For this reason, identification of strategies to minimise the consequence of overdiagnoses has led to new research aiming at recognising potential low-risk DCIS and establishing whether surgical treatment is necessary for all patients with DCIS. Recent clinical trials were aimed at comparing the safety of observation alone versus standard surgical excision (Patel et al., 2018).

The National Comprehensive Cancer Network (NCCN) recommended that reporting of ER status should be an essential mandatory requirement in the near future because reporting of ER status is currently variable in clinical practice (Daly et al., 2017). They postulated that the evaluation of ER has a predictive value in addition to providing prognostic information.

1.6 Management and risk stratification of DCIS

1.6.1 The standard management options

The standard management options for the treatment of DCIS in the UK currently are:

(1) lumpectomy without lymph node surgery with whole breast irradiation;

(2) mastectomy with sentinel lymph node biopsy +/− reconstruction;

(3) lumpectomy without lymph node surgery or radiation.

Mastectomy has long been considered the standard management for DCIS and remains so in certain situations (e.g. extensive disease, those

with failed breast conservation, or where there are contraindications to RT in high-risk patients) (Rutter et al., 2015). It provides an effective and near total cure rate; however, for most women it is an aggressive over-treatment of a lesion with low mortality risk (Mathew et al., 2017). Breast-conserving surgery (BCS), with or without RT, is offered when DCIS can be removed with an acceptable cosmetic outcome and is considered equivalent to mastectomy in terms of overall survival or breast-cancer-specific survival (Sakorafas et al., 2008). However, studies report that women treated with BCS have increased risk of recurrence within ten years and half of these recurrences are invasive disease with subsequent mortality risk (Silverstein et al., 1995b).

The appropriate treatment option will depend on a variety of clinicopathologic factors such as the patient's age and the extent of the disease. The third option is the most conservative, and is only considered for patients regarded to be at very low risk of LR (<5% at ten years) or for those with significant co-morbidity factors that may argue against the use of RT (Siziopikou 2013).

1.6.2 Radiotherapy

Recently, whether RT can be avoided in a specific patient group of DCIS without affecting recurrence risk has been investigated (McCormick et al., 2015). It was shown that histopathological parameters can be used to define the low-risk DCIS group, whose members have a low five years ipsilateral recurrence rate if treated with lumpectomy alone. Whether or not these patients with small-size, low/intermediate-grade DCIS with wide surgical margins continue to

have low LR rates without RT has not been established. Even in this selected, low-risk DCIS population, adjuvant RT is associated with a significant reduction in ipsilateral breast events; however, there was no difference in overall survival (McCormick et al., 2015).

1.6.3 Endocrine therapy

It is suggested that tamoxifen for five years may be considered in the context of adjuvant setting for those patients treated with BCS with or without adjuvant RT, and with ER positive disease (Carlson et al., 2010). The benefit of adjuvant tamoxifen in DCIS patients treated with lumpectomy was investigated in two clinical trials: the United Kingdom, Australia and New Zealand DCIS (UK/ANZ DCIS) trial (Cuzick et al., 2011) and the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-24 trial (Wapnir et al., 2011). A meta-analysis of the two trials (Staley et al., 2014) and some individual research studies (Fausto and Barni 2012, Hwang et al., 2018) reported an overall reduction in ipsilateral *in situ* recurrence risk and lower risk of subsequent invasive disease (Margolese et al., 2016).

1.7 DCIS outcome and risk assessment

Since the overall risk of DCIS progression to IBC ranges between 25% and 50%, perhaps due to different risk assessment and treatment modalities, the goals of treatment are set to minimise the risk of LR while trying to maximise breast preservation. LR is defined as developing IBC or DCIS in the ipsilateral breast six months after DCIS diagnosis (Rakovitch et al., 2015). Surgical intervention may be in the

form of BCS or mastectomy with or without immediate reconstruction (Choy and Mokbel 2014). The optimal treatment of DCIS requires enough surgical excision of the lesion with tumour-free surgical margins (2mm tumour-free margin), so most women with recently diagnosed DCIS are treated by BCS with or without RT (Rakovitch et al., 2015). Clinical trials show that adding RT after surgical treatment helps reduce the risk of LR by 50% (Correa et al., 2010, Cuzick et al., 2011, Formenti et al., 2011, Amichetti and Vidali 2012).

Patients undergoing mastectomy for DCIS have excellent prognosis and usually do not require additional treatment; only half of DCIS patients treated by BCS receive RT (Lambert et al., 2012). This may be a reflection of clinicians' assumptions that women with a low risk of recurrence following treatment by BCS alone can be precisely identified. Numerous studies have demonstrated that patient age, the size of the lesion, nuclear grade, presence or absence of necrosis, margin width and comedo architecture are factors affecting the recurrence risk (Zhang et al., 2015). Multifocal DCIS, defined as the presence of at least two DCIS foci separated by at least 5mm, is a strong predictor of LR because multifocality carries the risk of a greater burden of residual disease, or it could be an indicator of molecular heterogeneity (Rakovitch et al., 2015).

Until now, reproducible and robust methods using clinicopathological features to distinguish patients of low risk of LR following BCS alone have not been well established and it is necessary to improve personalised treatment decisions to reduce both over- and under-

treatment. Moreover, clinicopathological criteria alone are deficient to clearly and reliably identify individuals with a low recurrence risk (Rakovitch et al., 2013).

1.8 Role of the genetic studies in DCIS risk assessment

The process of recurrence in DCIS is not well understood, especially progression to invasive recurrence. It is assumed that all ipsilateral recurrences arise as a result of residual carcinoma cells inadvertently left behind after surgery and, therefore, re-establishing a local tumour (Gorringe and Fox 2017). In contrast, contralateral recurrences are assumed to be independent primary tumours. Clinical trial data shows that there are four to five times as many ipsilateral recurrences, ten years after diagnosis of DCIS, compared with contralateral tumours when treated with surgery alone (Cuzick et al., 2011, Wapnir et al., 2011, Donker et al., 2013).

On the assumption that ipsilateral recurrences are derived from residual primary tumour cells and therefore share a genetic common ancestor (i.e. primary and recurrence are clonally related), biomarker studies mostly seek tumour-intrinsic features to predict recurrence (Gorringe and Fox 2017). These studies have not been fully successful in identifying robust predictors; this could be because some recurrences are in fact independent primary tumours. Such cases will ‘contaminate’ tumour-intrinsic biomarker studies, reducing the power to detect predictive features (Gorringe et al., 2015).

The ipsilateral recurrence rate after DCIS suggests that women with recurrences have inadequate treatment and/or a breast tissue environment favouring tumour development. Patient management in the latter situation should focus on prevention of new tumours rather than aggressive treatment of the new primary. To solve this clinically significant dilemma, the clonal relationship between recurrence and original DCIS are crucial to be established.

1.9 Hypothesis

The hypothesis of this study is that the progression of DCIS and the development of recurrences is multifactorial, and that there are as yet unknown factors and/or biomarkers associated with adverse clinical outcome in DCIS, in particular development of ipsilateral LR. It is also hypothesised that tumour microenvironment particularly tumour infiltrating lymphocytes (TILs) are contributing to the progression of DCIS. Identifying such factors and characterisation of DCIS will explain at the translational-level DCIS behaviour and act as a useful prognostic and/or predictive tool.

1.10 Aim of the study

1.10.1 General aim

The general aim of this study is to identify potential predictors of DCIS outcome. This is via characterisation of the phenotypic and molecular features of DCIS and correlate them with the clinicopathological and

outcome data in order to accurately predict DCIS outcome in routine clinical practice. The ability to distinguish patients with DCIS at high or low risk of recurrence, would facilitate personalised patient management.

In this context, the present study aims to determine the clinicopathological and molecular characteristics associated with recurrence rates in women with DCIS.

1.10.2 Specific aims

1- Collect a large series of DCIS diagnosed in Nottingham over a 20-year period with linked clinicopathological, treatment and follow-up information;
2- Construct tissue microarrays (TMAs) from representative sections of pure DCIS lesions and a control group of lesions associated with invasion;
3- Correlate the clinicopathological characteristics of the study population with patients' outcomes;
4- Characterise the DCIS cohort according to the molecular classification of breast cancer using IHC (for ER, PR, Ki67 and HER2) and chromogenic in situ hybridisation (CISH) (to confirm HER2 status);
5- Perform quantitative assessment to scrutinise the expression pattern of selected biomarkers of adverse clinical outcome in DCIS and IBC to assess the likely predictive biomarkers that might predict the outcome of DCIS;

6- Assess the role of tumour infiltrating lymphocytes in DCIS as part of the tumour microenvironment.

2 Patients and Methods

2.1 Patient cohort and data collection

All primary pure DCIS cases diagnosed between 1987–2017 at the Nottingham Breast Institute were identified (n=1,249). Cases associated with micro-invasive carcinoma were excluded. Patients with multiple specimens from one operation – primary excision and immediate re-excision specimen(s) – were considered as a single surgical episode and size of any DCIS identified in the re-excision specimens was added to the size of tumour in the primary excision specimen to calculate the final size. Clinicopathological data, including age at diagnosis, mode of DCIS presentation (screen-detected or symptomatic), size of the lesion, nuclear grade, presence of comedo type necrosis, DCIS morphological type and associated Paget's disease, were retrieved from patient records. Management options including primary operation type (BCS vs mastectomy), margin status, re-excision surgery and its type, presence of residual DCIS in the re-excised specimens and the final operation together with RT data were also retrieved.

Resection margin status data was further categorised based on recent recommendations, i.e. positive margin (tumour on ink), close margin (<2mm) and negative margin (≥2mm) (Morrow et al., 2016, Toss et al., 2017). For comparison purposes, the whole cohort was further split into two groups: those diagnosed between 1987–2008 (n=803) and 2009–2017 (n=446). This was based on the guideline change with regard to margin of excision, soon after 2008, with acceptable free margin after BCS in DCIS of 5mm instead of 10mm free margin (Mathew et al., 2017). Although, the service currently follows the new guidelines

published in 2016 that recommend 2mm as the optimal free margin in DCIS, cases managed in 2016 and 2017 were included in the latter group to avoid bias in statistical analysis. VNPI was assessed for all cases treated with BCS after the first operation (n=824) and the risk score was estimated.

Data on ipsilateral tumour recurrence including type of recurrence and recurrence free interval (RFI) were also collected. Local recurrence free interval (LRFI) was estimated (in months) and defined as any event of ipsilateral tumour recurrence (either as DCIS or invasive disease) that occurred after six months from the first DCIS surgery. Contralateral breast carcinoma, either DCIS or invasive, were not considered as recurrence. Data collection was performed mainly by the author.

A control group of patients diagnosed as invasive carcinoma associated with DCIS was also identified (n=239).

Frequencies for clinical and pathological parameters of pure DCIS cases are included in **Table 2-1**.

Table 2-1: Clinicopathological parameters of pure DCIS cases

Clinicopathological parameter	Number (%)
Age (years)*	
<40	32 (2.6)
40 to 60	688 (55.1)
>60	529 (42.3)
Presentation	
Screening	748 (59.9)
Symptomatic	501 (40.1)
Site**	
Localised	929 (85.3)
Diffuse	160 (14.7)
Size*	
<16mm	457 (36.8)
16 to 40mm	484 (39.0)
>40mm	301 (24.2)
Grade	
Low	156 (12.5)
Intermediate	333 (26.7)
High	760 (60.8)
DCIS histologic type	
Single type	550 (44.0)
Mixed types	699 (66.0)
Associated LCIS	
Yes	120 (9.6)
No	1129 (90.4)
Associated Paget's	
Yes	56 (9.2)
No	550 (90.8)
Management***	
BCS	642 (51.4)
Mastectomy	606 (48.6)
Axillary surgery	
Sample	559 (44.8)
Clearance	4 (0.4)
No surgery	685 (54.8)
Radiotherapy	
Yes	263 (21.1)
No	986 (78.9)
Recurrence	
Yes	93 (7.5)
No	1156 (92.5)
Type of recurrence	
DCIS	33 (35.5)
Invasive disease	60 (64.5)

DCIS: Ductal carcinoma *in situ,* LCIS: Ductal carcinoma *in situ*
BCS: Breast conserving surgery
*Age and size are according to Van Nuys Prognostic Index (VNPI)
**Site; localised: DCIS is in one quadrant, Diffuse: DCIS is in more than one quadrant,
***Management of DCIS as a final operation.

2.2 TMA construction

The TMA technique brings revolutionary changes in high-throughput analysis by being able to handle up to several hundred samples in one slide with subsequent application of techniques such as immunohistochemistry to allow a diverse range of biomarkers to be studied (Ilyas et al., 2013). Using TMAs saves a considerable amount of time, and reagents, while attaining more standardised laboratory conditions (Dancau et al., 2010). TMAs can make working with and evaluation of IHC and other staining protocols faster and easier (Shergill and Arya 2004, Jawhar 2009). The steps of the TMA production included: collection of the study cohort to be arrayed; preparation of the recipient array blocks; designing the array grids; construction of the individual array blocks; and preparation of the array block for sectioning (Dancau et al., 2016). The initial tasks of preparing donor blocks and TMA design are the most critical parts of TMA construction and are therefore quite time consuming. TMA construction was performed mainly by the author.

2.2.1 Collection of the study cohort, preparation of the donor blocks and their corresponding slides

Searching for pure DCIS cases and those associated with invasion was carried out through different data storage pathology systems. Pulling and reviewing slides for DCIS cases was executed to confirm the diagnosis and select suitable cases for TMA construction. Using light microscopy, Haematoxylin & Eosin (H&E) tissue sections were

thoroughly scanned to select the most appropriate area of the DCIS tumour, avoiding necrotic tumour zones.

A representative formalin-fixed paraffin-embedded (FFPE) tumour block (donor) for each patient's specimen was retrieved from the tissue block archives. Priority was given to blocks containing viable tumour tissue and that were not frequently sectioned for previous studies (not less than 4mm in depth). A fresh full-face section of 4 micrometres (μm) thickness was cut from each block and stained with H&E and each slide was reviewed by the author using light microscopy to confirm the presence of the tumour and to identify the most representative areas suitable for array sampling. In cases where the block did not contain any tumour, insufficient tumour, or was thinner than 4mm, an alternative archive block for the patient was retrieved. Some cases were omitted from the arrays because either the block was thin or contained no/insufficient tumour with no alternative blocks in the archive.

Digital slide scanning with a 'Pannoramic' digital slide scanner (250 Flash III, 3DHISTECH®, Budapest, Hungary), followed by marking the representative tissue regions, was carried out (**Figure 2-1**), using 'Pannoramic Viewer' software v 1.15.4 (3D HISTECH®, Budapest, Hungary). The Pannoramic Viewer enabled me to view the scanned slides and to mark the areas of interest (those non-necrotic with high tumour burden). Block layout design using the TMA Grand Master software v 2.8 (3D HISTECH®, Budapest, Hungary), followed by digital slide overlay and marker transfer, and finally punching from donor block into recipient block, was performed.

776 cases of pure primary DCIS with available FFPE tumour blocks were included in the TMA construction together with a series of 239 cases diagnosed as synchronous DCIS and invasive tumours (mixed DCIS and IBC) collected for comparison. The former steps were performed by the author.

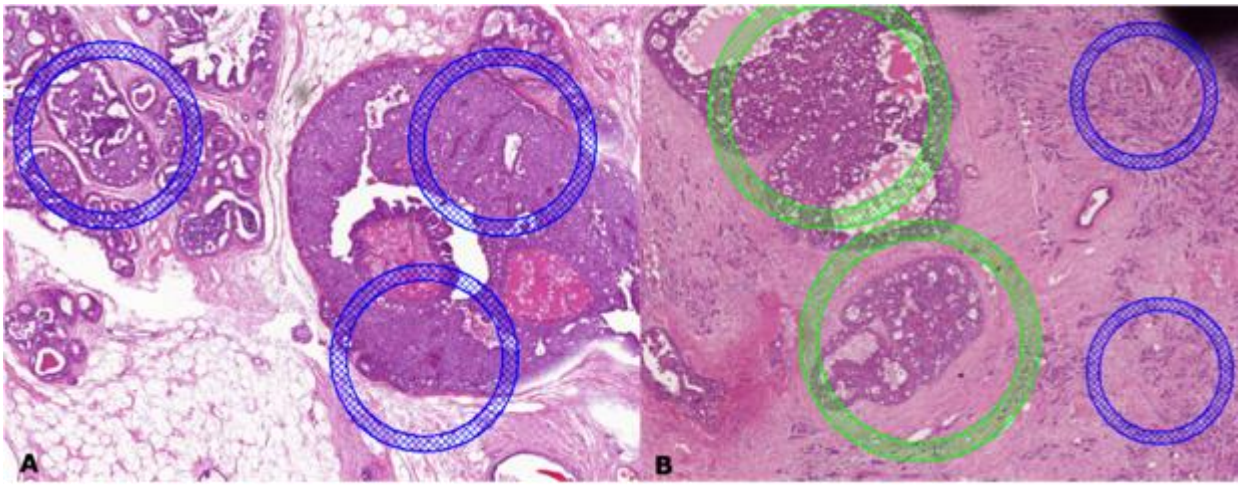


Figure 2-1: **A): Marking of DCIS lesions using 1mm circle diameter using Pannoramic Viewer software. B) Marking of a mixed DCIS lesion (green 1mm circle diameter) and invasive carcinoma (blue 0.6mm circle diameter).**

2.2.2 Preparation of the recipient array blocks

Recipient blocks were prepared by using freshly poured empty paraffin blocks (5–10 mm deep) into which the tissue array cores were extruded. The recipient block was prepared by pouring molten (56–58°C) regular paraffin wax into a mould, after which a tissue cassette was placed on top (Dancau et al., 2016). The complex was cooled on ice until hardened. The wax was checked for presence of air bubbles, striations or stress marks, and fault line, and it was ensured that wax covered the entire base of the cassette without surplus wax on the edges or back of the cassette (Lacombe et al., 2015). The block was trimmed smooth to ensure a flat surface using a microtome (Lacombe

et al., 2015). This step was performed with the help of the group technicians.

2.2.3 Construction of the individual array blocks

The 'TMA Grand Master® (3D HISTECH®, Budapest, Hungary) was used for automated TMA production with a punch set of 0.6 and 1mm (Lacombe et al., 2015). Donor blocks were loaded, and automatic donor block labelling and aligning with the image was used.

This was done by the author.

2.2.4 Designing the array grids

Selection of the TMA layout was made through selecting layout editor in the TMA grandmaster software v 2.8 (3D HISTECH®, Budapest, Hungary). Creation of a new layout followed by insertion of a new layout name was performed. Block size was inserted as a standard of 24x35mm. Core size was set up to 0.6mm (for invasive tumours) and 1mm (for DCIS) with 1mm insertion gap. Six replicates were prepared where possible, to allow for backup and compendsate for core loss if needed. Blocks were distinguished by version number, manually added to the block later.

Each array was designed to contain 70 samples arranged in two groups (subarrays). The first group representing the upper half of the block contained 42 cores (six rows, seven columns), while the second/lower half of the block held 28 cores (four rows, seven columns). Cores in each subarray were arranged at a centred distance and the subarrays were separated by a single gap for orientation and macroscopic

identification of the TMA block. Additional orientation cores from normal kidney, liver and tonsil tissues were included outside of the geometric margin of the array on the top and bottom left-hand side. The orientation cores served as positive internal experimental controls (**Figure 2-2**).

Great care was taken during documentation of both the donor block and the TMA position of each individual core. An array map grid was produced for each array block. Each grid contained the patients' unique identifier and was arranged in the same sequence corresponding to its position in the array block. All the former steps were performed by the author.



Figure 2-2: Block holding 1mm DCIS cores. Blue arrows point for the identification cores.

2.2.5 Preparation of the array block for sectioning

TMA blocks were heated at 37°C for 20 minutes to facilitate adherence and annealing of the tissue cores to the walls of the recipient holes and to make the wax flexible to handle. After warming the block, a clean

glass microscope slide was placed on top of TMA blocks and gently pushed all tissue cores into the block for paving them with the surface and to smoothen the block surface. Using a microtome, 4µm sections were cut from TMA blocks on to permanent positively-charged glass slides (Surgipath, X-tra™ Adhesive, Peterborough, UK), using the standard microtome sectioning technique. The initial section from each block was stained in H&E for quality control purposes to ensure histology and efficient construction steps (**Figure 2-3**). This step was performed with the help of the group technicians.

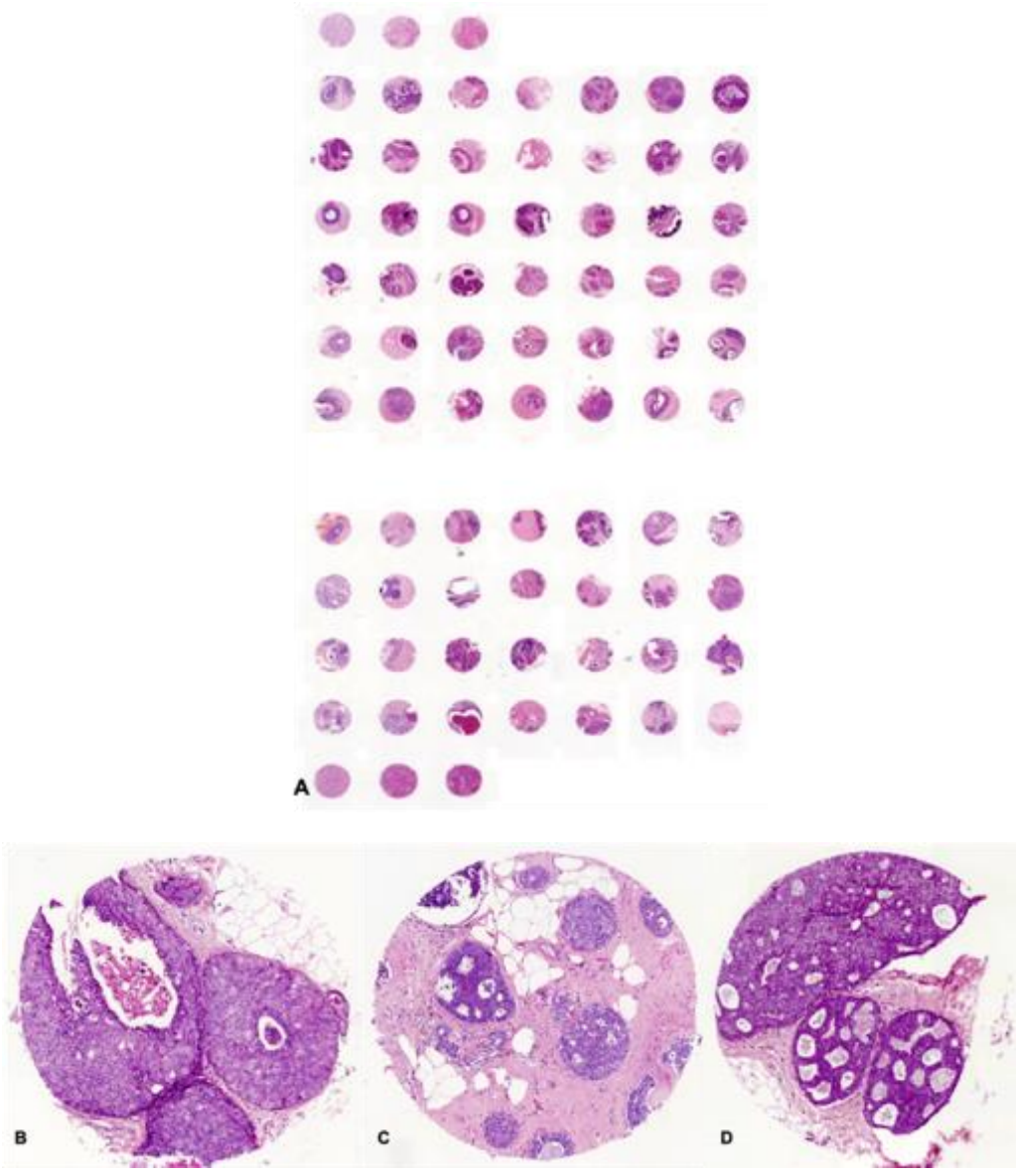


Figure 2-3: H&E sections of DCIS TMA. A) Overview of 1mm needle core diameter TMA, (B, C and D) DCIS TMA cores representing different histopathological patterns; cribriform, solid and associated necrosis.

2.3 Literature-based selection of biomarkers

A plethora of studies have been conducted to unravel the diverse molecular and biological nature of DCIS. A thorough review of the literature was performed regarding different proteins and molecular markers closely relevant to DCIS progression in published studies, which used different techniques and tissue platforms. Studies conducted using gene microarrays, *in vitro* studies utilising *in situ* and IBC cell lines, *in-vivo* animal models for inoculates from breast cancer cell lines of varying malignant/aggressive potentials (Johnson et al., 2012, Gorringe et al., 2015, Rane et al., 2015), as well as those studies using conventional FFPE clinical specimens were reviewed (Elias et al., 2016, Meng et al., 2016).

The human proteome is composed of approximately 20000 different proteins resulting from translation of protein-coding genes (Clamp et al., 2007). Following translation, many proteins are subject to post-translational modifications. The development of cancer is driven by the acquisition of somatic genetic alterations, including single-base substitutions, translocations and CNAs (Futreal et al., 2004, Stuart and Sellers 2009). The genomic instability of cancer cell genome fuels this diversity in cellular proteome.

A powerful way to discover key genes playing causal roles in oncogenesis is to identify genomic regions that undergo frequent alteration in human cancers. Somatic copy-number alterations (SCNAs) are extremely common in cancer (Baudis 2007, Beroukhim et al., 2010). Therefore, proteins involved in tumour progression are anticipated to be highly diverse, integrated in complex interrelated networks of proteins and molecular pathways. Bearing in mind that some proteins may play a key role in carcinogenesis and tumour progression makes these proteins 'master regulatory' proteins. Therefore, a panel of molecular markers of potential roles in DCIS progression has been proposed and subject to scrutiny.

Accordingly, the rationale behind choosing certain biomarkers out of a myriad in the literature, while omitting others, was based on: A) molecules used to determine the hormonal status of the patients (ER and PR); B) a well-known oncogene used for characterisation of IBC (HER2); C) molecules reflecting the proliferative fraction of the tumour, e.g. Ki67; D) molecules that might have a role in tumour progression or being involved in the carcinogenic cascade with copy number aberrations (CNAs), which may function as tumour suppressor and transcription factor, e.g. Thioredoxin Interacting Protein (TXNIP); cell cycle regulator, e.g. aurora kinase A (AURKA), microtubule nucleation factor, TPX2 and oncogene, e.g. SRC proto-oncogene.

2.4 Validation of antibody specificity: Western blot

Western blotting (WB) was performed to determine the specificity of the antibodies used in IHC; it was performed by the author. A mixture of cell

lysates from six cell lines (MCF-7, MD-MBA-231, 293, SKBR3, HeLa, and Jurkat) was used to reflect the expression of the selected proteins. All western blot steps were performed by the author.

2.4.1 Preparation of samples, electrophoresis and transfer

Cell line lysate was prepared in a maximum volume of 30 µL (19.5µl of lysate, 7.5µl of NuPAGE LDS sample buffer (4x), and 3µl of reducing agent, 10x); all reagents were from Novex Life Technologies. This mixture was heated for five minutes at 100°C and left to cool on ice. The prepared sample was loaded on to 10% SDS–NuPAGE (4–12%) or (3–8% Tris-Acetate gels for detection of high molecular weight (HMW) proteins (Novex Life Technologies) in 20x MOPS running buffer (Novex Life Technologies) or Tris-Acetate SDS Running buffer 20x (for HMW proteins). The pre-stained Thermo Scientific PageRuler Plus Protein Ladder 26619 was used as a molecular weight standard and gels were run at 150 volts for 90 minutes. Proteins were transferred to a Hybond-P PVDF nitrocellulose membrane by wet transfer and electro-blotting at 30 volts for one hour in transfer buffer. For blocking, the membranes were incubated in Phosphate Buffered Saline (PBS, Sigma) containing 0.1% Tween-20 (Sigma, P 2287), and 5% non-fat dry milk (Marvel dried skimmed milk, UK) for 60 minutes. The membrane was washed three times with PBS Tween-20 (TPBS) and incubated with the primary antibody overnight at 4°C using the optimal concentration as instructed by the manufacturer (**Table 2-2**); β-actin was used as a positive control.

Membranes were washed with TPBS (3×five minutes) and incubated with the secondary antibody; florescent secondary antibody was used

(anti-mouse or anti-rabbit, Licor Biosciences), in a concentration of (1:15000). The membrane was washed three times with TPBS. For fluorescence, the membrane was visualised using the Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).

Table 2-2: Specificity of the selected antibodies for the project

Antibody	Molecular Weight (KDa)	Concentration	Supplier
TXNIP	44	1:1000	Abcam
AURKA	23,40	1:500	Abcam
TPX2	86	2ug/ml	Abcam
SRC	69, 59	1:250	Sigma
β-actin	42	1:5000	Sigma

TXNIP: Thioredoxin interacting protein, AURKA: Aurora kinase A, TPX2: Targeting protein for Xenopus kinesin-like protein 2, SRC: Non-receptor tyrosine kinase.

2.5 Immunohistochemistry (IHC)

2.5.1 Optimisation

Optimisation of the staining conditions for each antibody (**Table 2-3**) was performed by changing several variables in the staining protocol until the optimal result was obtained. These variables include: 1) dilution of the antibody, starting with the dilution recommended by the manufacturer plus dilutions above and below (e.g. in case of recommended 1: 100 dilution, I tried 1:100, 1:50, 1:25, 1:150, 1:200); 2) antigen retrieval solution: microwave pre-treatment in citrate buffer at pH 6.0, or water bath pre-treatment in EDTA buffer solution EDTA Buffer (AR9640, Bond^{TM} Epitope Retrieval Solution 2), pH 9.0.

Ideally, an antibody with specific staining of the cells showing immunoreactivity within the specific cellular location (membranous, cytoplasmic, or nuclear) with no or minimal background staining would allow the most accurate evaluation of its protein expression status. This was obtained through finding the optimum antibody concentration by applying serial dilutions of the antibody to sequential sections of breast tissue (**Table 2-3**). All the staining procedures including optimisation were performed by the author except ER staining, which was performed by the Histopathology department, Queen's Medical Centre, Nottingham.

2.5.2 IHC technique

IHC was performed on 4µm tissue or TMA sections mounted on permanent positively-charged glass slides (Surgipath, X-tra™ Adhesive, Peterborough, UK). Unless otherwise stated, for antibodies studied, sections were stained using Novolink™ Polymer Detection System (RE7150-K). To confirm adhesion of tissue sections with the slide surface, sections were placed on a hot plate (60°C) for ten minutes and left to cool. Slides were dewaxed in xylene (Genta Medica, York, UK) twice for five minutes, rehydrated thrice in Industrial Methylated Spirit (IMS) or methanol (two minutes/each) followed by five minutes in running tap water.

Heat-induced retrieval of antigen epitopes was performed, when required, by Whirlpool JT359 microwave (1000 W) treatment of the slides in one litre of 10mM sodium citrate buffer (pH 6.0), using full power for 20 minutes. Slides were cooled for ten minutes under a

continuous stream of tap water and were rinsed with Tris Buffered Saline (TBS) (0.05 mol/L Tris HCL, 0.15 mol/L NaCl; pH 7.6). Endogenous peroxidase activity was blocked by incubation of the slides in peroxidase block (RE7101, 3–4% Hydrogen peroxide) for five minutes, protein block (RE7102, 0.4% casein in phosphate-buffered saline, with stabilisers, surfactant, and 0.2% Bronidox L as a preservative) for five minutes and primary antibodies, optimally diluted using Leica antibody diluent (AR9352), were added. Each of these steps was followed by washing 3×five minutes with TBS-Tween 20 0.1%. Post primary block (RE7111, Rabbit anti-mouse IgG (<10 µg/mL) in 10% animal serum in TBS/0.09% ProClin™ 950) was incubated for 30 minutes followed by 3×five minutes washing with TBS-Tween-20 0.1% and then Novolink Polymer (RE7112, Anti-rabbit Poly-HRP-IgG (<25µg/mL) containing 10% animal serum in TBS/0.09% ProClin™ 950) was added for 30 minutes and followed by the same washing. 3,3'-Diaminobenzidine (DAB) working solution [(DAB chromogen, RE7105, 1.74% w/v 3,3'-diaminobenzidine, in a stabiliser solution) and DAB substrate buffer (RE7143, buffered solution containing ≤0.1% hydrogen peroxide and preservative) of a ratio 1:20] was added for five minutes, 3×five minutes washing then was followed by counterstaining with Haematoxylin (RE7107, <0.1% haematoxylin) for six minutes. The slides were dehydrated with alcohol and cleared with xylene (Genta Medica, York, UK). Finally, slides were mounted with Distyrene, a Plasticizer, and Xylene (DPX).

For ER, sections were stained using the Ventana Benchmark® ULTRA system (Tucson, Arizona, USA) with the Ventana anti-ER (SP1) rabbit monoclonal primary antibody as per the recommended protocol. The primary antibody was applied for 16 minutes at 37°C followed by the OptiView HQ Linker for eight minutes and the OptiView HRP Multimer for eight minutes.

Negative as well as positive controls were included with every staining run during staining of each antibody. A negative control was obtained by omitting the primary antibody and incubating slides in antibody diluent only. Additionally, tissues with known reactivity to each antibody according to the supplier's data sheet were used as a positive control (**Table 2-3**).

Table 2-3: Optimum conditions, expression localisation and cut-off values of the selected antibodies

Antibody	Supplier	Species	Clone	Dilution	Pre-treatment	Incubation time	Positive control	IHC Localisation	Cutoff values	Method for cut-off determination
ER	Ventana	Rabbit monoclonal	SP1	N/A	N/A	16 minutes	Internal/ normal breast ducts	Nuclear	Negative (0), Positive (≥1%)	(Hammond et al., 2010a)
PR	Agilent	Mouse monoclonal	PgR636	1:150	Microwave	30 minutes	Internal/normal breast ducts	Nuclear	Negative (0), Positive (≥1%)	(Hammond et al., 2010a)
HER2	DAKO	Rabbit polyclonal	-	1:400	-	30 minutes	BC tissue	Membranous	Negative (<10%), Positive (≥10%)	(Wolff et al., 2013)
Ki67	DAKO	Mouse Monoclonal	MIB-1	1:100	Microwave	1 hour	Tonsil tissue	Nuclear	Negative (<14%), Positive (≥14%)	(Cheang et al., 2009)
P53	Leica Novocastra	Mouse monoclonal	DO7	1:50	Microwave	30 minutes	BC tissue	Nuclear	Inactivating mutation (0), Normal (1-50%), Missense mutation (>50%)	(Kobel et al., 2010)
TXNIP	abcam	Rabbit monoclonal	EPR14774	1:3000	Microwave	30 minutes	Kidney/Tonsil tissue	Cytoplasmic	≥185 (H score)	X-Tile
AURKA	abcam	Rabbit polyclonal	-	1:50	Microwave	Overnight/4°C	Tonsil tissue	Nuclear	Negative (<30%), Positive (≥30%)	X-Tile
TPX2	abcam	Mouse monoclonal	18D5-1	1:150	Microwave	30 minutes	Testis tissue	Nuclear	Negative (<25%), Positive (≥25%)	X-Tile
SRC	Sigma	Rabbit polyclonal	-	1:75	Microwave	Overnight/4°C	Testis tissue	Cytoplasmic	≥100 (H score)	X-Tile

ER: Oestrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2, P53: Tumour suppressor p53, TXNIP: Thioredoxin interacting protein, AURKA: Aurora kinase A, TPX2: Targeting protein for Xenopus kinesin-like protein 2, SRC: non-receptor tyrosine kinase.

2.5.3 Assessment of immunoreactivity

Prior to the application of antibodies to the TMA using IHC, I performed staining of ten full-face sections (FFS) from FFPE invasive breast cancer tissues mixed with DCIS with each primary antibody to assess the homogeneity of immunoreactivity in terms of staining intensity and distribution. For all the studied biomarkers, I only proceeded to apply each of the candidate antibodies on TMA when staining homogeneity was ensured. Therefore, all candidate biomarkers were studied on TMA sections except Ki67, which showed heterogeneous distribution of staining positivity, and poor concordance between Ki67 assessed on TMA and FFS. Accordingly, Ki67 was assessed on three replicates of the TMA, by quantifying positively stained nuclei in 100 tumour cells/core, to compensate for the discrepancy and the final score was taken as average (Muftah et al., 2017). Previous studies have validated the use of one TMA core to study the expression of tumour markers in a large series (Torhorst et al., 2001).

High-resolution digital images (0.45µm/pixel) of stained slides were obtained using a NanoZoomer slide scanner (Hamamatsu Photonics, Welwyn Garden City, UK) and accessed using Pannoramic viewer software v 1.15.4 (3D HISTECH®, Budapest, Hungary). Immunoreactivity was manually assessed at 20x magnification using a minimum of a 24” high-resolution screen (1920x1080).

Each TMA core was assessed as to whether DCIS/invasive tumour occupied >15% of the total area of the core, to ensure that the TMA

core is representative of the whole tumour. If this was the case, the core was eligible for scoring. Histo-score (H-score) (McCarty et al., 1985), was the main scoring system used, taking into consideration the intensity (0=negative, 1=weak, 2=moderate and 3=strong staining) and the percentage of stained tumour cells (0-100%). The intensity was multiplied by the percentage and added together to obtain the final H-score with a range from 0–300. Cases with multiple cores were scored and the average H-score was used as the final H-score. All cases were scored without prior knowledge of patients' pathological or outcome data.

Scoring TMA cores stained by HER2 was performed using HercepTest (Wolff et al., 2013), according to the manufacturer's recommendation: DAKO protocol (DAKO Corporation, Glostrup, Denmark). Each core was placed into one of four categories (0, 1+, 2+, or 3+). Tumours with a complete absence of membrane staining, or in which membrane staining is observed in <10% of the tumour cells, were scored as 0. Those with faint/barely perceptible, incomplete membranous staining detected in >10% of tumour cells were classified as 1+. Tumours with either weak to moderate complete membranous staining in >10% of the tumour cells were classified as 2+, while those with strong, complete membranous staining in >10% of the tumour cells were classified as 3+. Aberrant p53 staining was determined based on eyeballing the percentage and the staining pattern of p53 in tumour cell nuclei. Assessment of the other selected biomarkers was done by the semi-

quantitative H score (for TXNIP and SRC) or eyeballing the percentage of the positively stained tumour cell nuclei (for AURKA and TPX2).

2.5.4 Determination of the cut-off points

Cut-off values for the dichotomisation of biomarkers were individually assessed and determined by several methods: 1) using cut-offs from previous studies that assessed a large amount of data if available; 2) using either the mean or median according to the distribution of data, whether normally or not normally distributed, respectively; or 3) using a non-commercial research tool: X-tile software (version 3.6.1, 2003-2005, Yale University, USA http://x-tile.software.informer.com). This software randomly divides the total patient cohort into two separate equal training and validation sets by producing separate lists of 'censored' and 'uncensored' observations, ranked by patients' follow-up time. Those patients who developed ipsilateral local recurrence were considered as uncensored, while patients who did not develop local recurrence or lost follow-up during this study were censored during the analysis. The optimal cut-off points were determined by locating the brightest pixel on the X-tile plot diagram of the training set. Statistical significance was tested by validating the obtained cut-point to the validation set (Camp et al., 2004) with corrected *p* value and relative risk against local recurrence-free survival (Camp et al., 2004, Dolled-Filhart et al., 2006). Information regarding the stained antibodies in this study, their expression and their cut-offs is given in **Table 2-3**.

2.6 Chromogenic *in situ* hybridisation for HER2

HER2 gene amplification is primarily detected by *in situ* hybridisation and CISH is an introduced alternative method to FISH (Zhao et al., 2002). The performance of CISH in HER2 testing has rarely been tested in series studies, so it is aimed to characterise HER2 status using IHC and CISH in DCIS and assess its prognostic value in a large and well-annotated cohort. CISH staining and assessment was performed by the author.

HER2 gene amplification was determined by CISH using the ZytoDot 2C SPEC ERBB2/CEN 17 Probe Kit, (ZytoVision®, Germany). TMA sections were deparaffinised and incubated for ten minutes (at 70°C on thermal plate) followed by dewaxing in xylene (2×five minutes), and then serial ethanol dilutions (each five minutes). Incubation of the slides with hydrogen peroxide (H_2O_2) (3%, for five minutes) is followed by washing in distilled water (D.W) Treatment of the slides by pre-heated pre-treatment solution EDTA (PT2) for 15 minutes was performed, followed by immediate transfer into D.W (2×two minutes each). Enzymatic digestion was carried out by drop-wise application of pepsin solution (ES1) to the tissue sections and incubation for five minutes at room temperature (RT) in a humidity chamber. After a wash in D.W, slides were dehydrated with graded ethanol followed by air drying. A ready to use DNA probe (ZytoDot 2C SPEC ERBB2/CEN 17 Probe, PD12) was applied to the slides (10µl per slide) and cover-slipped (22x22mm) then sealed with a layer of rubber cement. The sections were denatured on a thermal plate (80°C for five minutes), and

hybridisation was carried out overnight at 37°C in a humidity chamber. After hybridisation, the cover slip was removed, and slides were washed twice (each five minutes) with wash buffer SSC (WB1) (first wash at room temperature, and the second heated to 75°C). After two washes in D.W (one minute each), slides were immersed into 1x wash buffer TBS (prepared using WB5). The HER2 probe was detected by sequential incubation with anti-digoxigenin DIG/DNP-Mix (AB14) for 15 minutes (at 37°C in a humidity chamber), peroxidase polymer horseradish peroxidase HRP/AP-Polymer-Mix (AB13) for 15 minutes (at 37°C in a humidity chamber), AP-Red Solution B (SB6b) for 15 minutes (at room temperature) and HRP-Green Solution A (SB7a) for 15 minutes (at room temperature). Counter-staining was achieved by Nuclear Blue Solution (CS2) for two minutes followed by washing and dehydration in absolute ethanol followed by xylene. Covering the slides (22x22mm cover slips) using Alcoholic Mounting Solution (MT4) and air-dry slides for 30 minutes was the final step.

2.7 Conventional statistical approach

Statistical analysis was performed using SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA) and distribution of the biomarkers' graphs are generated by R studio software (v 1.1.463 – © 2009-2018 RStudio, Inc). For all statistical tests, a *p* value <0.05 (two-sided) was considered significant. The following statistical methods were used:

2.7.1 Univariate analysis with clinicopathological parameters

Analysis of continuous variables was performed using the appropriate statistical test (e.g. t-test, Mann-Whitney U test, ANOVA test). Chi-squared analyses were used for inter-relationships between categorical variables (e.g. biomarker expression and clinicopathological variables). Inter-class correlation coefficient was used for inter-rater concordance (as continuous values) and Kappa test for the concordance rate as a categorical values.

2.7.2 Univariate analysis with outcome

Survival estimates for patients' local recurrence-free survival (LRFS) were analysed by the Kaplan-Meier method with significance determined by the log rank test.

2.7.3 Multivariate analysis with patients' outcome

Multivariate analysis was performed by Cox proportional hazard analysis to test for confounders and prognostic or predictive independency of the studied biomarker from standard prognostic/predictive factors.

All statistical analyses were performed by the author.

3 Morphological and Clinical Characterisation of DCIS

3.1 Introduction

3.1.1 Incidence of DCIS

Pure DCIS represents approximately 10% of all breast carcinomas (Sakorafas et al., 2008). The breakthrough of molecular techniques shows that DCIS is not a single disease; rather, it is a heterogeneous group of diseases. Such heterogeneity is reflected in tumour behaviour and, ideally, the management approach needs to be designed accordingly to decease risk of progression and/or recurrence (Fallowfield et al., 2016, Mathew et al., 2017).

3.1.2 Treatment of DCIS

RT following BCS decreases the risk of recurrence although clear guidelines for its indications in specific risk groups continue to be debated (McCormick et al., 1991, Rampaul et al., 2001, Dodwell et al., 2007, Holmberg et al., 2008, Donker et al., 2013, Farante et al., 2014, Kim et al., 2014, McCormick et al., 2015). Over- or under-treatment of DCIS remains a problem and numerous trials have been performed to assess more personalised therapy (Elshof et al., 2015, Francis et al., 2015).

3.1.3 Factors affecting the outcome of DCIS

Various factors affecting outcome of treated DCIS have been analysed and different risk groups can be stratified accordingly, aiding treatment decisions (Silverstein 2003a). Age at diagnosis, clinical presentation, nuclear grade and tumour size, resection margin status are well-

recognised factors used for this purpose. The VNPI is a popular risk assessment tool combining patient age, lesion size, nuclear grade and margin status for treatment decisions (**Table 3-1**) (Boland et al., 2003, Silverstein 2003b).

Table 3-1: Modified Van Nuys Prognostic Index (VNPI) suggested treatment options

Score	Treatment option
Low (score 4, 5, 6)	Excision alone
Intermediate (score 7, 8, 9)	Excision with RT
High (score 10, 11, 12)	Mastectomy

Optimal extent of free surgical resection margins after BCS is still controversial. A UK survey in 2007 identified that approximately half of surgeons aim for a free margin of more than 2mm (Young et al., 2007). In 2016, the Society of Surgical Oncology, American Society for Radiation Oncology and American Society of Clinical Oncology published consensus guidelines on margins for BCS with whole-breast irradiation in DCIS is a 2mm free surgical margin (Morrow et al., 2016). Positive or close margins are the main indication for surgical re-excision or completion mastectomy after primary surgery for DCIS (Holland et al., 1998, Dillon et al., 2007, Wei et al., 2012).

3.2 Hypothesis and aims

In this chapter, it is hypothesised that different clinicopathological factors of DCIS patients are important predictors of DCIS behaviour and

could predict patient outcome, particularly if they are studied on a large number of patients. Therefore, the aim was to collect a large cohort of DCIS patients treated in a single institution and correlate their clinicopathological characteristics with the outcome.

3.3 Patients and methods

Pure DCIS cases diagnosed between 1987–2017 at the Nottingham Breast Institute were identified and included in this chapter (n=1,249). Clinicopathological characteristics together with patient outcome data was collected (**Chapter 2**). Correlations between different clinicopathological parameters of DCIS patients and factors contributing to patient outcome were studied.

3.4 Results

3.4.1 Data analysis

A total of 1,249 primary DCIS cases were included into the analysis; 748 women (60%) were diagnosed by screening. High nuclear grade was observed in 61% of cases, while comedo type necrosis was recorded in two thirds of cases (67%). Solid DCIS was the predominant histological type, either in pure form or mixed with other morphological types, and represented 58% of cases. Paget's disease was observed in 56 cases (9%) (**Table 3-2** and **Figure 3-1**).

As the most significant change in the margin status was introduced in 2008, this time point was used to compare the old and recent series of DCIS in this study. After 2008, a significant reduction in the rate of

mastectomy as the first operative choice for DCIS management was observed (p=0.015), along with a marked decrease in the rate of re-excision to 37% vs 58% prior to 2008 (p<0.0001).

3.4.1.1 Correlation between clinicopathological factors associated with the selection of type of primary surgery for DCIS management

With regard to DCIS management, 824 cases (66%) were treated primarily with BCS, while mastectomy was performed in 424 cases (34%). The former was the first choice for those who were diagnosed by screening (p<0.0001), with localised DCIS lesions (p<0.0001), DCIS size less than 16mm (p<0.0001), with no associated solid, cribriform, papillary or micropapillary DCIS (p=0.030, p=0.002, p=0.002 and p=0.008, respectively). However, mastectomy was the choice for high grade tumours and DCIS associated with comedo necrosis (both p<0.0001) (**Table 3-3**).

3.4.1.2 Factors affecting re-excision of DCIS

After primary BCS, 306 cases (39%) showed positive margins (tumour on ink), while 75 cases (10%) showed close margins less than 2mm. Margin of more than 10mm was observed in 20% of cases. Over the entire study period, half of the DCIS cases treated with BCS underwent re-excision (n=414). The re-operation was either in the form of further conservation surgery (n=232) or completion mastectomy (n=182) (**Table 3-4**).

Several factors influenced the rate of re-excision, including patient's age (p=0.028), patient presentation (p<0.0001), tumour localisation (p=0.003), DCIS size (p<0.0001), comedo necrosis (p=0.009), solid DCIS (p<0.0001), micropapillary DCIS (p<0.0001), resection margins' width (p<0.0001) and VNPI (p<0.0001) (**Table 3-5**). **Figure 3-2a** shows the rate of re-excision with regard to margin status. It is noteworthy that 196 (47%) of those who underwent re-excision were of low and intermediate grade. Moreover, 10% of those who had a second operation were of low-risk VNPI (**Figure 3-2b** and **Figure 3-2c** show the rate of re-excision in the context of tumour grade and risk groups).

Completion mastectomy was more likely to be recommended for younger women (p=0.018), with symptomatic DCIS (p<0.0001), multiple DCIS lesions or involving more than one breast quadrant (p=0.005), with DCIS more than 4cm in maximum diameter (p<0.0001), positive surgical margin (p<0.0001) and high-risk DCIS according to VNPI (p<0.0001). (**Table 3-6**).

3.4.1.3 Clinicopathological factors associated with presence of residual tumour in the re-excision operation

Residual tumour tissue was detected in 218/414 cases (53%) who had re-excision surgery. Presence of residual tumour tissue in the re-excised specimens was associated with DCIS presenting symptomatically (p<0.0001), larger tumour size (p<0.0001), positive surgical margin (p=0.015) and high-risk VNPI (p<0.0001). Patients who underwent a second re-excision operation showed a higher rate of

residual tumour tissue than those who had completion mastectomy (p<0.0001). **Table 3-7** shows the different factors associated with the presence of residual tumour tissue in the re-excision and completion mastectomy specimens.

VNPI was assessed for all cases treated with BCS after the first operation (n=824) and the risk score was calculated (mean score was 7.7±1.7, range 4–12). Low-risk DCIS was observed in 195 cases (25%), with 470 cases (60%) assigned as moderate risk and 118 cases (15%) being high-risk DCIS. VNPI could not be assessed in 41 cases due to one or more of the index parameters being missing.

3.4.1.4 Association of adjuvant radiotherapy and the clinicopathological factors

263 out of 642 patients (41%) treated with BCS as a final surgery received post-operative RT. Its use was associated with aggressive DCIS features including large tumour size (p<0.0001), higher tumour grade (p<0.0001), presence of comedo type necrosis (p<0.0001) and close surgical margins (p=0.015) (**Table 3-8**).

Management of DCIS showed significant changes with acceptance of smaller margins, improving quality of imaging detection of DCIS and more frequent use of local RT. **Figure 3-3** shows details of the trends of primary and final BCS, RT, re-excision and local recurrence rates.

3.4.2 Axillary lymph node surgery

Axillary lymph node surgery was performed for 563 (45%) patients (559 axillary sample while axillary clearance was performed in four cases). Eighty-five per cent of axillary lymph node surgery was carried out for patients treated with mastectomy compared to 15% for patients treated with BCS. Large DCIS size, high nuclear grade and presence of comedo necrosis (all p <0.0001) were associated with an increased rate of axillary lymph node surgery (**Table 3-9**).

3.4.3 Factors influencing the development of local recurrence

Within the median follow-up period of 103 months, 93 cases were reported to develop recurrence. Recurrence as DCIS occurred in 35% (n=33) of recurrent cases while approximately two thirds (n=60; 65%) recurred as invasive disease.

Patients treated with BCS alone had the worst prognosis in terms of tumour recurrence compared to patients treated with either mastectomy or BCS with adjuvant RT (p<0.0001), **Figure 3-4**.

In patients treated with BCS, local recurrence-free survival was associated with tumour site; localised or diffuse (p=0.023), tumour size (p=0.041), grade (p=0.020), cribriform and papillary DCIS (p=0.042 and 0.046, respectively) and adjuvant radiotherapy (p=0.004) (**Table 3-10**).

Multivariate analysis showed that diffuse tumours occupying more than one breast quadrant (p=0.040), large tumour size (p=0.045), high

nuclear grade (p=0.009), papillary DCIS (p=0.004) and no adjuvant radiotherapy (p=0.038) are independent predictors of tumour recurrence in patients treated with BCS (**Table 3-11**).

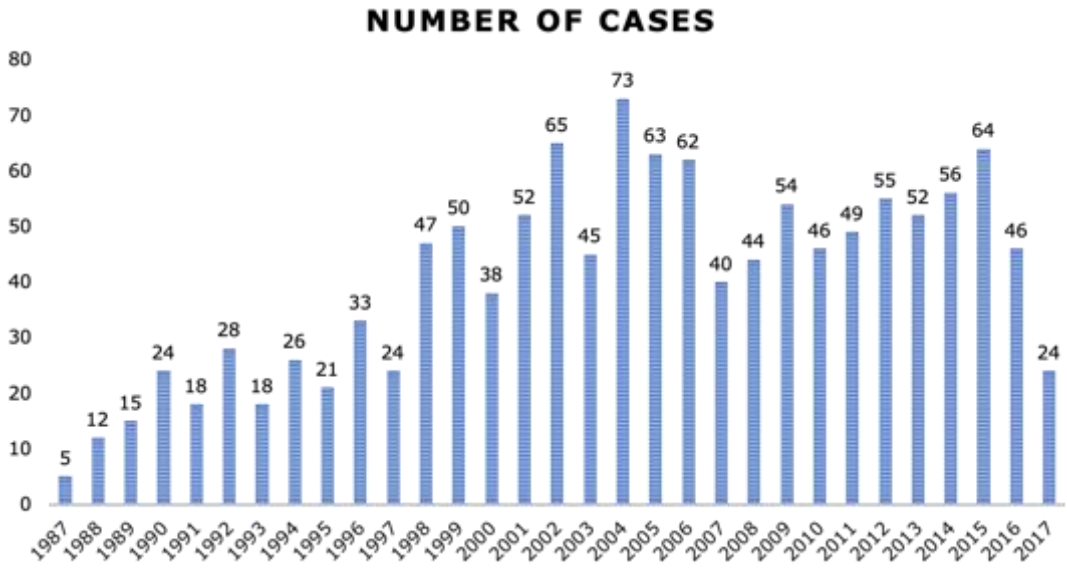


Figure 3-1: Distribution of DCIS cases diagnosed from 1987–2017.

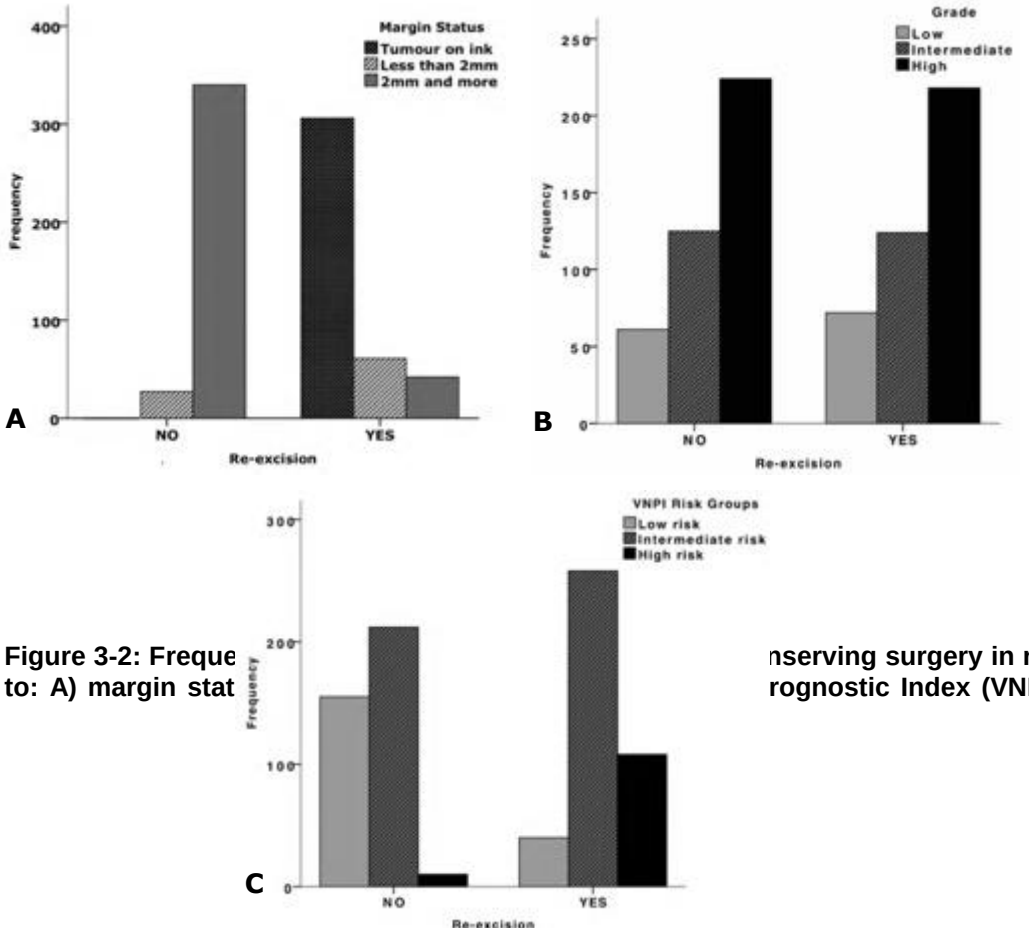


Figure 3-2: Freque nserving surgery in relation to: A) margin stat rognostic Index (VNPI) risk

groups. A considerable percentage of low and intermediate grade as well as low-risk DCIS underwent re-excision.

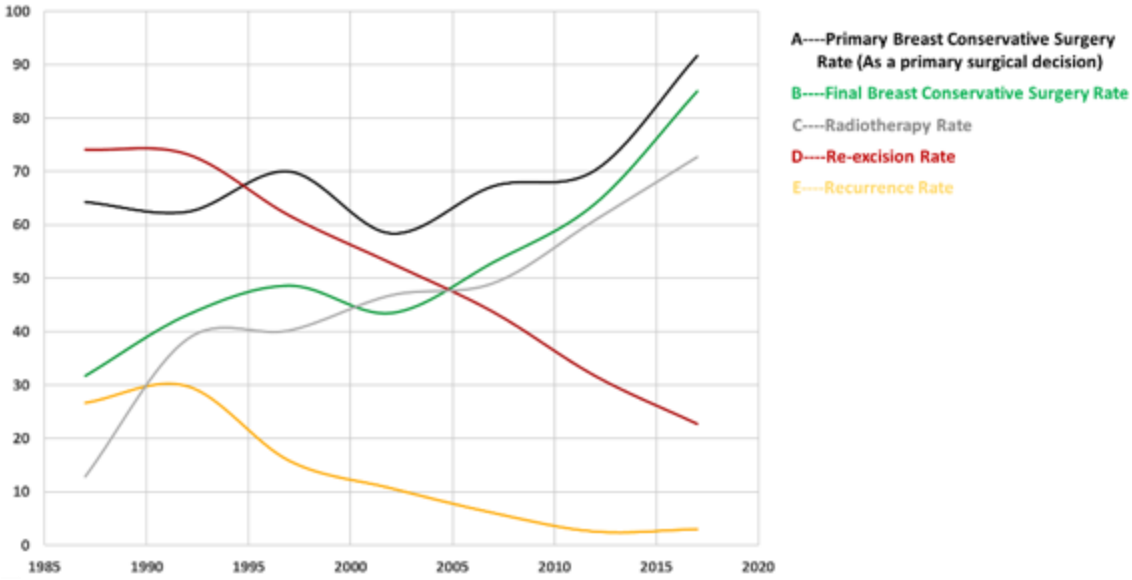


Figure 3-3: The annual rates, over the period between 1987–2017, of: A) breast conserving surgery (BCS), showing the higher rates of BCS as a primary surgical choice over the years; B) shows the comparable rates of final BCS after re-excisions – obviously, over the last ten years, the rates of BCS exceed the rates of mastectomy; C) post-operative adjuvant radiotherapy – a significant increase of radiotherapy use post-operatively after 2000 is observed; D) re-excision; and E) recurrence after primary BCS (a significant decrease in both re-excision and recurrence is observed over the last ten years).

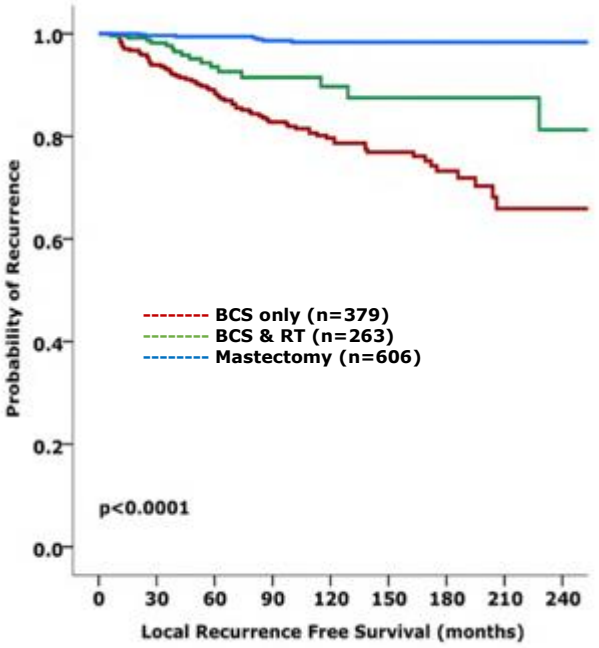


Figure 3-4: Kaplan-Meier curve shows the effect of different treatment modalities on the development of local recurrence. Patients treated with breast conserving surgery (BCS)

without adjuvant radiotherapy (RT) have the worst prognosis compared to those who received radiotherapy or were treated with mastectomy.

Table 3-2: Clinicopathological parameters of the primary DCIS cohort

Parameter	Total (n=1249) N (%)	DCIS Diagnosed 1987-2008 (n=803) N (%)	DCIS diagnosed 2009-2017 (n=446) N (%)	X^2 *p*-value
Age (years)*				
<40	32 (2.6)	24 (3.0)	8 (1.8)	11.9
40 to 60	688 (55.1)	467 (58.2)	221 (49.6)	**0.003**
>60	529 (42.3)	312 (38.8)	217 (48.6)	
Presentation				
Screening	748 (59.9)	452 (56.3)	296 (66.4)	11.8
Symptomatic	501 (40.1)	351 (43.7)	150 (33.6)	**0.001**
Site**				
Localised	929 (85.3)	590 (88.2)	339 (80.7)	11.5
Diffuse	160 (14.7)	79 (11.8)	81 (19.3)	**0.001**
Size*				
<16mm	457 (36.8)	308 (38.7)	149 (33.4)	3.6
16 to 40mm	484 (39.0)	304 (38.2)	180 (40.4)	0.160
>40mm	301 (24.2)	184 (23.1)	117 (26.2)	
Grade				
Low	156 (12.5)	109 (13.6)	47 (10.5)	16.8
Intermediate	333 (26.7)	185 (23.0)	148 (33.2)	**<0.0001**
High	760 (60.8)	509 (63.4)	251 (56.3)	
Comedo necrosis				
No	409 (32.7)	280 (34.9)	129 (28.9)	4.6
Yes	840 (67.3)	523 (65.1)	317 (71.1)	**0.032**
Solid DCIS				
No	529 (42.4)	406 (50.6)	123 (27.6)	62.3
Pure	291 (23.3)	164 (20.4)	127 (28.5)	**<0.0001**
Mixed with other types	429 (34.3)	233 (29.0)	196 (43.9)	
Cribriform DCIS				
No	649 (52.0)	444 (55.3)	205 (46.0)	12.1
Pure	114 (9.1)	75 (9.3)	39 (8.7)	**0.002**
Mixed with other types	486 (38.9)	284 (35.4)	202 (45.3)	
Papillary DCIS				
No	1127 (90.2)	729 (90.8)	398 (89.2)	0.8
Pure	42 (3.4)	26 (3.2)	16 (3.6)	0.663
Mixed with other types	80 (6.4)	48 (6.0)	32 (7.2)	
Micropapillary DCIS				
No	916 (73.4)	594 (74.0)	322 (72.2)	1.3
Pure	79 (6.3)	53 (6.6)	26 (5.8)	0.520
Mixed with other types	254 (20.3)	156 (19.4)	98 (22.0)	
Associated Paget's				
No	550 (90.8)	390 (90.1)	160 (92.5)	0.7
Yes	56 (9.2)	43 (9.9)	13 (7.5)	0.361
Management***				
Mastectomy	424 (34.0)	292 (36.4)	132 (29.6)	5.9
BCS	824 (66.0)	510 (63.6)	314 (70.4)	**0.015**
Margin width†				
Positive (Tumour on ink)	306 (38.9)	232 (48.5)	74 (23.9)	72.5
<2mm	75 (9.5)	31 (6.5)	44 (14.3)	**<0.0001**
≥2mm	406 (51.6)	215 (45.0)	191 (61.8)	

Re-excision†				
Yes	414 (50.2)	298 (58.4)	116 (36.9)	35.9
No	410 (49.8)	212 (41.6)	198 (63.1)	**<0.0001**
Type of re-excision†				
Re excision	232 (56.0)	159 (53.4)	73 (62.9)	3.1
Mastectomy	182 (44.0)	139 (46.6)	43 (37.1)	0.078
Residual Tumour†				
No	196 (47.3)	150 (50.3)	46 (39.7)	3.8
Yes	218 (52.7)	148 (49.7)	70 (60.3)	0.051
Management****				
Mastectomy	606 (48.6)	431 (53.7)	175 (39.2)	24.1
BCS	642 (51.4)	371 (46.3)	271 (60.8)	**<0.0001**
Radiotherapy§				
Yes	263 (41.0)	92 (24.8)	171 (63.1)	127.1
No	379 (59.0)	279 (75.2)	100 (36.9)	**<0.0001**
VNPI†				
Low risk	195 (24.9)	105 (22.2)	90 (29.1)	7.1
Moderate risk	470 (60.0)	288 (60.8)	182 (58.9)	**0.030**
High risk	118 (15.1)	81 (17.0)	37 (12.0)	

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, *p* values in **bold** are significant

*Age and size were categorised according to Van Nuys Prognostic Index (VNPI)

** Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant

*** Management of DCIS as a first operation

† Refers to patients treated primarily with BCS (first operation)

**** Management of DCIS as a final operation

§ Refers to patients treated finally by BCS (final operation)

Table 3-3: Clinicopathological factors associated with the selection of type of primary surgery for DCIS management

Parameter	DCIS Diagnosed 1987-2008 N (%)		X^2 *p*-value	DCIS diagnosed 2009-2017 N (%)		X^2 *p*-value	Total N (%)		X^2 *p*-value
	BCS (n=510)	**Mastectomy** (n=292)		**BCS** (n=314)	**Mastectomy** (n=132)		**BCS** (n=824)	**Mastectomy** (n=424)	
Age (years)*									
<40	13 (2.5)	11 (3.8)	0.9	5 (1.6)	3 (2.3)	1.8	18 (2.2)	14 (3.3)	2.6
40 to 60	297 (58.2)	170 (58.2)	0.610	150 (47.8)	71 (53.8)	0.412	447 (54.2)	241 (56.8)	0.271
>60	200 (39.3)	111 (38.0)		159 (50.6)	58 (43.9)		359 (43.6)	169 (39.9)	
Presentation									
Screening	306 (60.0)	146 (50.0)	7.6	230 (73.2)	66 (50.0)	22.5	536 (65.0)	212 (50.0)	26.4
Symptomatic	204 (40.0)	146 (50.0)	**0.006**	84 (26.8)	66 (50.0)	**<0.0001**	288 (35.0)	212 (50.0)	**<0.0001**
Site**									
Localised	365 (92.4)	224 (82.1)	51.7	250 (84.7)	89 (71.2)	10.5	615 (74.6)	313 (73.8)	48.6
Diffuse	30 (7.6)	49 (17.9)	**<0.0001**	45 (15.3)	36 (28.8)	**0.005**	75 (25.4)	85 (26.2)	**<0.0001**
Size*									
<16mm	256 (50.6)	52 (17.9)	96.9	133 (42.3)	16 (12.1)	78.1	389 (47.4)	68 (16.1)	162.5
16 to 40mm	175 (34.6)	129 (44.5)	**<0.0001**	134 (42.7)	46 (34.8)	**<0.0001**	309 (37.7)	175 (41.5)	**<0.0001**
>40mm	75 (14.8)	109 (37.6)		47 (15.0)	70 (53.1)		122 (14.9)	179 (42.4)	
Grade									
Low	92 (18.0)	17 (5.8)	39.1	41 (13.1)	6 (4.5)	18.3	133 (16.1)	23 (5.4)	58.6
Intermediate	133 (26.1)	51 (17.5)	**<0.0001**	116 (36.9)	32 (24.2)	**<0.0001**	249 (30.2)	83 (19.6)	**<0.0001**
High	285 (55.9)	224 (76.7)		157 (50.0)	94 (71.3)		442 (53.7)	318 (75.0)	
Comedo necrosis									
No	215 (42.1)	64 (21.9)	33.5	109 (34.7)	20 (15.1)	17.3	324 (39.3)	84 (19.8)	48.4
Yes	295 (57.9)	228 (78.1)	**<0.0001**	205 (65.3)	112 (84.9)	**<0.0001**	500 (60.7)	340 (80.2)	**<0.0001**

Solid DCIS									
No	273 (53.5)	132 (45.2)	6.3	95 (30.3)	28 (21.2)	4.3	368 (44.7)	160 (37.7)	7.1
Pure	103 (20.2)	61 (20.9)	**0.043**	89 (28.3)	38 (28.8)	0.115	192 (23.3)	99 (23.3)	**0.030**
Mixed with other types	134 (26.3)	99 (33.9)		130 (41.4)	66 (50.0)		264 (32.0)	165 (39.0)	
Cribriform DCIS									
No	274 (53.7)	169 (57.9)	8.1	143 (45.5)	62 (47.0)	4.2	417 (50.6)	231 (54.5)	12.1
Pure	59 (11.6)	16 (5.5)	**0.017**	33 (10.5)	6 (4.5)	0.120	92 (11.2)	22 (5.2)	**0.002**
Mixed with other types	177 (34.7)	107 (36.6)		138 (43.9)	64 (48.5)		315 (38.2)	171 (40.3)	
Papillary DCIS									
No	458 (89.8)	271 (92.8)	4.7	270 (86.0)	128 (97.0)	11.7	728 (88.3)	399 (94.1)	12.6
Pure	21 (4.1)	4 (1.4)	0.095	15 (4.8)	1 (0.7)	**0.003**	36 (4.4)	5 (1.2)	**0.002**
Mixed with other types	31 (6.1)	17 (5.8)		29 (9.2)	3 (2.3)		60 (7.3)	20 (4.7)	
Micropapillary DCIS									
No	391 (76.7)	202 (69.2)	6.3	236 (75.2)	86 (65.2)	5.9	627 (76.1)	288 (67.9)	9.6
Pure	33 (6.5)	20 (6.8)	**0.043**	14 (4.5)	12 (9.1)	0.052	47 (5.7)	32 (7.5)	**0.008**
Mixed with other types	86 (16.8)	70 (24.0)		64 (20.3)	34 (25.7)		150 (18.2)	104 (24.6)	

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, BCS: Breast conserving surgery, *p* values in **bold** are significant.
*Age and size according to Van Nuys Prognostic Index (VNPI), **Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant.

Table 3-4: Detailed surgical management of the DCIS cases as a whole cohort

<table>
<tr><th colspan="4">Surgical management</th></tr>
<tr><td>Primary operation</td><td>Mastectomy
(n=424)
34.0%</td><td colspan="2">BCS
(n=824)
66.0%</td></tr>
<tr><td rowspan="3">Re-excision</td><td rowspan="3"></td><td colspan="2">Yes (n=414)</td><td rowspan="3">No re-excision
(n=410)</td></tr>
<tr><td>Mastectomy</td><td>Re-excision</td></tr>
<tr><td>182</td><td>232</td></tr>
<tr><td>Final operation</td><td colspan="2">Mastectomy
(n=606)
48.6%</td><td colspan="2">BCS
(n=642)
51.4%</td></tr>
</table>

BCS: Breast conserving surgery; N: Number.

Table 3-5: Clinicopathological factors associated with re-excision after primary treatment with BCS (n=824)

Parameter	DCIS Diagnosed 1987-2008 N (%)		X^2 *p*-value	DCIS diagnosed 2009-2017 N (%)		X^2 *p*-value	Total N (%)		X^2 *p*-value
	No (n=212)	**Yes** (298)		**No** (198)	**Yes** (116)		**No** (410)	**Yes** (414)	
Age (years)*									
<40	3 (1.4)	10 (3.4)	6.7	3 (1.5)	2 (1.7)	0.1	6 (1.5)	12 (2.9)	7.2
40 to 60	113 (53.3)	184 (61.7)	**0.034**	95 (48.0)	55 (47.4)	0.987	208 (50.7)	239 (57.7)	**0.028**
>60	96 (45.3)	104 (34.9)		100 (50.5)	59 (50.9)		196 (47.8)	163 (39.4)	
Presentation									
Screening	147 (69.3)	159 (53.4)	13.2	157 (79.3)	73 (62.9)	9.9	304 (74.1)	232 (56.0)	29.7
Symptomatic	65 (30.7)	139 (46.6)	**<0.0001**	41 (20.7)	43 (37.1)	**0.002**	106 (25.9)	182 (44.0)	**<0.0001**
Site**									
Localised	157 (92.4)	208 (92.4)	1.6	169 (88.9)	81 (77.1)	10.9	326 (79.5)	289 (69.8)	11.5
Diffuse	13 (7.6)	17 (7.6)	0.459	21 (11.1)	24 (22.9)	**0.004**	34 (20.5)	41 (30.2)	**0.003**
Size*									
<16mm	133 (63.6)	123 (41.4)	37.1	112 (56.6)	21 (18.1)	47.8	245 (60.2)	144 (34.9)	69.1
16 to 40mm	66 (31.6)	109 (36.7)	**<0.0001**	69 (34.8)	65 (56.0)	**<0.0001**	135 (33.2)	174 (42.1)	**<0.0001**
>40mm	10 (4.8)	65 (21.9)		17 (8.6)	30 (25.9)		27 (6.6)	95 (23.0)	
Grade									
Low	34 (16.0)	58 (19.5)	0.9	27 (13.6)	14 (12.1)	1.6	61 (14.9)	72 (17.4)	0.9
Intermediate	57 (26.9)	76 (25.5)	0.610	68 (34.4)	48 (41.4)	0.459	125 (30.5)	124 (30.0)	0.614
High	121 (57.1)	164 (55.0)		103 (52.0)	54 (46.5)		224 (54.6)	218 (52.6)	
Comedo necrosis									
No	81 (38.2)	134 (45.0)	2.3	62 (31.3)	47 (40.5)	2.7	143 (34.9)	181 (43.7)	6.7
Yes	131 (61.8)	164 (55.0)	0.128	136 (68.7)	69 (59.5)	0.098	267 (65.1)	233 (56.3)	**0.009**

Solid DCIS									
No	98 (46.3)	175 (58.7)	9.8	59 (29.8)	36 (31.1)	9.1	157 (38.3)	211 (51.0)	22.1
Pure	55 (25.9)	48 (16.1)	**0.007**	67 (33.8)	22 (19.0)	**0.011**	122 (29.8)	70 (16.9)	**<0.0001**
Mixed with other types	59 (27.8)	75 (25.2)		72 (36.4)	58 (50.0)		131 (31.9)	133 (32.1)	
Cribriform DCIS									
No	108 (50.9)	166 (55.7)	1.2	97 (49.0)	46 (39.7)	4.5	205 (50.0)	212 (51.2)	0.9
Pure	27 (12.8)	32 (10.7)	0.543	23 (11.6)	10 (8.6)	0.103	50 (12.2)	42 (10.1)	0.646
Mixed with other types	77 (36.3)	100 (33.6)		78 (39.4)	60 (51.7)		155 (37.8)	160 (38.7)	
Papillary DCIS									
No	188 (88.7)	270 (90.6)	0.5	168 (84.8)	102 (87.9)	3.9	356 (86.8)	372 (89.9)	3.2
Pure	10 (4.7)	11 (3.7)	0.766	13 (6.6)	2 (1.7)	0.141	23 (5.6)	13 (3.1)	0.204
Mixed with other types	14 (6.6)	17 (5.7)		17 (8.6)	12 (10.4)		31 (7.6)	29 (7.0)	
Micropapillary DCIS									
No	175 (82.6)	216 (72.5)	10.1	160 (80.8)	76 (65.5)	9.2	335 (81.7)	292 (70.5)	16.8
Pure	6 (2.8)	27 (9.1)	**0.006**	7 (3.5)	7 (6.0)	**0.010**	13 (3.2)	34 (8.2)	**<0.0001**
Mixed with other types	31 (14.6)	55 (18.4)		31 (15.7)	33 (28.5)		62 (15.1)	88 (21.3)	
Margin width									
Positive (tumour on ink)	0 (0.0)	232 (78.4)	318.7	0 (0.0)	74 (65.5)	225.1	0 (0.0)	306 (74.8)	543.7
<2mm	4 (2.2)	27 (9.1)	**<0.0001**	10 (5.1)	34 (30.1)	**<0.0001**	14 (3.7)	61 (14.9)	**<0.0001**
≥ 2mm	178 (97.8)	37 (12.5)		186 (94.9)	5 (4.4)		364 (96.3)	42 (10.3)	
VNPI* **									
Low risk	73 (40.3)	32 (10.9)	108.9	82 (41.8)	8 (7.1)	71.3	155 (41.1)	40 (9.9)	168.9
Moderate risk	103 (56.9)	185 (63.2)	**<0.0001**	109 (55.6)	73 (64.6)	**<0.0001**	212 (56.2)	258 (63.1)	**<0.0001**
High risk	5 (2.8)	76 (25.9)		5 (2.6)	32 (28.3)		10 (2.7)	108 (27.0)	

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, *p* values in **bold** are significant
VNPI: Van Nuys Prognostic Index
*Age and size according to VNPI, **Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant, ***VNPI assessed after the primary operation.

Table 3-6: Clinicopathological factors associated with the choice of type of DCIS re-excision (n=414)

Parameter	DCIS Diagnosed 1987-2008 N (%)		X^2 *p*-value	DCIS diagnosed 2009-2017 N (%)		X^2 *p*-value	Total N (%)		X^2 *p*-value
	BCS (n=159)	**Mastectomy** (n=139)		**BCS** (n=73)	**Mastectomy** (n=43)		**BCS** (n=232)	**Mastectomy** (n=182)	
Age (years)*									
<40	2 (1.2)	8 (5.8)	4.6	0 (0)	2 (14.7)	4.6	2 (0.9)	10 (5.5)	7.9
40 to 60	101 (63.6)	83 (59.7)	0.098	38 (52.1)	17 (39.5)	0.099	139 (59.9)	100 (54.9)	**0.018**
>60	56 (35.2)	48 (34.5)		35 (47.9)	24 (45.8)		91 (39.2)	72 (39.6)	
Presentation									
Screening	99 (62.3)	60 (43.2)	10.9	53 (72.6)	20 (46.5)	7.9	152 (65.5)	80 (44.0)	19.2
Symptomatic	60 (37.7)	79 (56.8)	**0.001**	20 (27.4)	23 (53.5)	**0.005**	80 (34.5)	102 (56.0)	**<0.0001**
Site**									
Localised	98 (93.3)	110 (91.7)	16.7	55 (82.1)	26 (68.4)	2.9	153 (89.0)	136 (86.1)	10.7
Diffuse	7 (6.7)	10 (8.3)	**<0.0001**	12 (17.9)	12 (31.6)	0.233	19 (11.0)	22 (13.9)	**0.005**
Size*									
<16mm	94 (59.1)	29 (21.0)	61.6	19 (26.0)	2 (4.7)	18.7	113 (48.7)	31 (17.1)	73.8
16 to 40mm	54 (34.0)	55 (39.9)	**<0.0001**	44 (60.3)	21 (48.8)	**<0.0001**	98 (42.2)	76 (42.0)	**<0.0001**
>40	11 (6.9)	54 (39.1)		10 (13.7)	20 (46.5)		21 (9.1)	74 (40.9)	
Grade									
Low	32 (20.1)	26 (18.7)	0.1	10 (13.7)	4 (9.3)	0.6	42 (18.1)	30 (16.5)	0.2
Intermediate	40 (25.2)	36 (25.9)	0.952	29 (39.7)	19 (44.2)	0.754	69 (29.7)	55 (30.2)	0.911
High	87 (54.7)	77 (55.4)		34 (46.6)	20 (46.5)		121 (52.1)	97 (53.3)	
Comedo necrosis									
No	75 (47.2)	59 (42.4)	0.7	27 (37.0)	20 (46.5)	1.1	102 (44.0)	79 (43.4)	0.1
Yes	84 (52.8)	80 (57.6)	0.413	46 (63.0)	23 (53.5)	0.313	130 (56.0)	103 (56.6)	0.909
Solid DCIS									
No	87 (54.7)	88 (63.3)	2.3	24 (32.9)	12 (27.9)	0.4	111 (47.8)	100 (54.9)	2.2
Pure	29 (18.2)	19 (13.7)	0.306	14 (19.2)	8 (18.6)	0.824	43 (1.9)	27 (14.8)	0.333
Mixed with other types	43 (27.1)	32 (23.0)		35 (47.9)	23 (53.5)		78 (50.3)	55 (30.3)	

Cribriform DCIS									
No	90 (56.6)	76 (54.7)	1.9	30 (41.2)	16 (37.2)	1.9	120 (51.7)	92 (50.5)	2.7
Pure	20 (12.6)	12 (8.6)	0.389	8 (10.9)	2 (4.7)	0.388	28 (12.1)	14 (7.7)	0.251
Mixed with other types	49 (30.8)	51 (36.7)		35 (47.9)	25 (58.1)		84 (36.2)	76 (41.8)	
Papillary DCIS									
No	142 (89.3)	128 (92.1)	3.9	65 (89.0)	37 (86.0)	2.1	207 (89.2)	165 (90.7)	5.1
Pure	9 (5.7)	2 (1.4)	0.141	2 (2.7)	0 (0)	0.356	11 (4.7)	2 (11.0)	0.080
Mixed with other types	8 (5.0)	9 (6.5)		6 (8.3)	6 (14.0)		14 (6.1)	15 (8.3)	
Micropapillary DCIS									
No	121 (76.1)	95 (68.3)	3.6	51 (69.9)	25 (58.1)	2.6	172 (74.1)	120 (65.9)	5.1
Pure	15 (9.4)	12 (8.6)	0.165	5 (6.8)	2 (4.7)	0.269	20 (8.6)	14 (7.7)	0.079
Mixed with other types	23 (14.5)	32 (23.1)		17 (23.3)	16 (37.2)		40 (17.3)	48 (26.4)	
Margin width†									
Positive (Tumour on ink)	108 (68.8)	124 (89.2)	19.9	42 (58.3)	32 (78.0)	6.918	150 (65.5)	156 (86.7)	24.9
<2mm	21 (13.4)	6 (4.3)	**<0.0001**	25 (34.7)	9 (22.0)	0.075	46 (20.1)	15 (8.3)	**<0.0001**
≥ 2mm	28 (17.8)	9 (6.5)		5 (7.0)	0 (0)		33 (14.4)	9 (5.0)	
VNPI* **									
Low risk	25 (16.1)	7 (5.1)	31.8	7 (9.7)	1 (2.4)	4.8	32 (14.1)	8 (4.5)	30.9
Moderate risk	109 (70.3)	76 (55.1)	**<0.0001**	48 (66.7)	25 (61.0)	0.190	157 (69.2)	101 (56.4)	**<0.0001**
High risk	21 (13.6)	55 (39.8)		17 (23.6)	15 (36.6)		38 (16.7)	70 (39.1)	

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, BCS: Breast conserving surgery, *p* values in **bold** are significant.
*Age and size according to Van Nuys Prognostic Index (VNPI), **Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant, ***VNPI: assessed after the primary operation. **†** Refers to patients treated primarily with BCS (first operation).

Table 3-7: Clinicopathological factors associated with presence of residual tumour tissue in the re-excision specimens (n=414)

Parameter	DCIS Diagnosed 1987-2008 N (%)		X^2 *p*-value	DCIS diagnosed 2009-2017 N (%)		X^2 *p*-value	Total N (%)		X^2 *p*-value
	No (n=150)	**Yes** (n=148)		**No** (n=46)	**Yes** (n=70)		**No** (n=196)	**Yes** (n=218)	
Age (years)*									
<40	3 (2.0)	7 (4.7)	3.2	0 (0.0)	2 (2.8)	1.5	3 (1.5)	9 (4.1)	3.4
40 t0 60	89 (59.3)	95 (64.2)	0.205	21 (45.7)	34 (48.6)	0.462	110 (56.1)	129 (59.2)	0.182
>60	58 (38.7)	46 (31.1)		25 (54.3)	34 (48.6)		83 (42.4)	80 (36.7)	
Presentation									
Screening	95 (63.3)	64 (43.2)	12.1	39 (84.8)	34 (48.6)	15.6	134 (68.4)	98 (45.0)	22.9
Symptomatic	55 (36.7)	84 (56.8)	**0.001**	7 (15.2)	36 (51.4)	**<0.0001**	62 (31.6)	120 (55.0)	**<0.0001**
Site**									
Localised	105 (94.6)	103 (90.4)	1.8	33 (75.0)	48 (78.7)	2.5	138 (89.0)	151 (86.3)	0.6
Diffuse	6 (5.4)	11 (9.6)	0.403	11 (25.0)	13 (21.3)	0.281	17 (11.0)	24 (13.7)	0.719
Size*									
<16mm	82 (55.0)	41 (27.7)	37.1	11 (23.9)	10 (14.3)	9.2	93 (47.7)	51 (23.4)	47.9
16 to 40mm	54 (36.2)	55 (37.2)	**<0.0001**	30 (56.2)	35 (50.5)	**0.010**	84 (43.1)	90 (41.3)	**<0.0001**
>40	13 (8.8)	52 (35.1)		5 (10.9)	25 (35.7)		18 (9.2)	77 (35.3)	
Grade									
Low	33 (22.0)	25 (16.9)	1.2	5 (10.9)	9 (12.9)	0.9	38 (19.4)	34 (15.6)	1.9
Intermediate	37 (24.7)	39 (26.4)	0.538	17 (36.9)	31 (44.3)	0.616	54 (27.6)	70 (32.1)	0.378
High	80 (53.3)	84 (56.7)		24 (52.2)	30 (42.8)		104 (53.0)	114 (52.3)	
Comedo necrosis									
No	76 (50.7)	58 (39.2)	3.9	18 (39.1)	29 (41.4)	0.1	94 (47.9)	87 (39.9)	2.7
Yes	74 (49.3)	90 (60.8)	0.46	28 (60.9)	41 (58.6)	0.805	102 (52.1)	131 (60.1)	0.099
Solid DCIS									
No	84 (56.0)	91 (61.5)	1.1	19 (41.3)	17 (24.3)	7.1	103 (52.6)	108 (49.5)	3.4
Pure	27 (18.0)	21 (14.2)	0.566	11 (23.9)	11 (15.7)	**0.029**	38 (19.4)	32 (14.7)	0.178
Mixed with other types	39 (26.0)	36 (24.3)		16 (34.8)	42 (60.6)		55 (28.0)	78 (35.8)	

Cribriform DCIS									
No	85 (56.7)	81 (54.7)	4.6	19 (41.3)	27 (38.6)	2.3	104 (53.1)	108 (49.5)	7.9
Pure	21 (14.0)	11 (7.4)	0.098	6 (13.0)	4 (5.7)	0.313	27 (13.8)	15 (6.9)	0.089
Mixed with other types	44 (29.3)	56 (37.9)		21 (45.7)	39 (55.7)		65 (33.1)	95 (43.6)	
Papillary DCIS									
No	132 (88.0)	138 (93.2)	4.6	42 (91.4)	60 (85.7)	5.8	174 (88.8)	198 (90.8)	8.3
Pure	9 (6.0)	2 (1.4)	0.099	2 (4.3)	0 (0.0)	0.055	11 (5.6)	2 (0.9)	0.066
Mixed with other types	9 (6.0)	8 (5.4)		2 (4.3)	10 (14.3)		11 (5.6)	18 (8.3)	
Micropapillary DCIS									
No	114 (76.0)	102 (68.9)	2.9	32 (69.6)	44 (62.9)	2.3	146 (74.5)	146 (66.9)	5.5
Pure	14 (9.3)	13 (8.8)	0.236	4 (8.7)	3 (4.3)	0.318	18 (9.2)	16 (7.4)	0.064
Mixed with other types	22 (14.7)	33 (22.3)		10 (21.7)	23 (32.8)		32 (16.3)	56 (25.7)	
Margin width									
Positive (Tumour on ink)	107 (72.3)	125 (84.5)	9.2	24 (52.2)	50 (74.6)	9.5	131 (67.5)	175 (81.4)	12.3
<2mm	19 (12.8)	8 (5.4)	0.057	18 (39.1)	16 (23.9)	**0.023**	37 (19.1)	24 (11.2)	**0.015**
≥ 2mm	22 (14.9)	15 (10.1)		4 (5.7)	1 (1.5)		26 (13.4)	16 (7.4)	
Type of re-excision									
Re excision	85 (56.7)	74 (50.0)	20.4	32 (69.6)	41 (58.6)	2.9	117 (59.7)	115 (52.8)	21.0
Completion Mastectomy	65 (43.3)	74 (50.0)	**<0.0001**	14 (30.4)	29 (41.4)	0.086	79 (40.3)	103 (47.2)	**<0.0001**
VNPI* **									
Low risk	22 (15.1)	10 (6.8)	22.5	3 (6.5)	5 (7.5)	9.1	25 (13.1)	15 (6.9)	23.9
Moderate risk	100 (69.0)	85 (57.4)	**<0.0001**	36 (78.3)	37 (55.2)	**0.028**	136 (71.2)	122 (56.7)	**<0.0001**
High risk	23 (15.9)	53 (35.8)		7 (15.2)	25 (37.3)		30 (15.7)	78 (36.4)	

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, *p* values in **bold** are significant
*Age and size according to Van Nuys Prognostic Index (VNPI), **Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant, ***VNPI: assessed after the primary operation.

Table 3-8: Clinicopathological factors associated with adjuvant radiotherapy treatment after BCS (n=642)

Parameter	DCIS Diagnosed 1987-2008 N (%)		X^2 *p*-value	DCIS diagnosed 2009-2017 N (%)		X^2 *p*-value	Total N (%)		X^2 *p*-value
	No (n=279)	**Yes** (n=92)		**No** (n=100)	**Yes** (n=171)		**No** (n=379)	**Yes** (n=263)	
Age (years)*									
<40	5 (1.8)	0 (0.0)	7.9	0 (0.0)	3 (1.8)	2.1	5 (1.3)	3 (1.2)	0.2
40 t0 60	150 (53.8)	64 (69.6)	**0.019**	52 (52.0)	81 (47.3)	0.344	202 (53.3)	145 (55.1)	0.891
>60	124 (44.4)	28 (30.4)		48 (48.0)	87 (50.9)		172 (45.4)	115 (43.7)	
Presentation									
Screening	182 (65.2)	64 (69.6)	0.6	77 (77.0)	133 (77.8)	0.1	259 (68.3)	197 (74.9)	3.2
Symptomatic	97 (34.8)	28 (30.4)	0.446	23 (23.0)	38 (22.2)	0.882	120 (31.7)	66 (25.1)	0.071
Site**									
Localised	201 (93.5)	54 (90.0)	5.8	84 (89.4)	140 (85.9)	0.9	285 (92.2)	194 (87.0)	5.2
Diffuse	14 (6.5)	6 (10.0)	0.054	10 (10.6)	23 (14.1)	0.649	24 (7.8)	29 (13.0)	0.076
Size*									
<16mm	177 (63.9)	50 (54.9)	2.6	70 (70.0)	61 (35.7)	31.3	247 (65.5)	111 (42.4)	36.4
16 to 40mm	86 (31.0)	34 (37.4)	0.278	27 (27.0)	86 (50.3)	**<0.0001**	113 (30.0)	120 (45.8)	**<0.0001**
>40	14 (5.1)	7 (7.7)		3 (3.0)	24 (14.0)		17 (4.5)	31 (11.8)	
Grade									
Low	55 (19.7)	11 (12.0)	14.1	33 (33.0)	4 (2.4)	21.4	88 (23.2)	15 (5.7)	67.9
Intermediate	83 (29.8)	14 (15.2)	**0.001**	53 (53.0)	44 (25.7)	**<0.0001**	136 (35.9)	58 (22.1)	**<0.0001**
High	141 (50.5)	67 (72.8)		14 (14.0)	123 (71.9)		155 (40.9)	190 (72.2)	
Comedo necrosis									
No	136 (48.7)	20 (21.7)	20.7	66 (66.0)	23 (13.5)	30.3	202 (53.3)	43 (16.3)	80.8
Yes	143 (51.3)	72 (78.3)	**<0.0001**	34 (34.0)	148 (86.5)	**<0.0001**	177 (46.7)	220 (83.7)	**<0.0001**
Solid DCIS									
No	146 (52.3)	39 (42.4)	3.8	49 (49.0)	34 (19.9)	6.5	195 (51.5)	73 (27.8)	18.3
Pure	57 (20.4)	27 (29.3)	0.148	19 (19.0)	62 (36.3)	0.089	76 (20.1)	89 (33.8)	0.190
Mixed with other types	76 (27.3)	26 (28.3)		32 (32.0)	75 (43.8)		108 (28.4)	101 (38.4)	

Cribriform DCIS									
No	141 (50.5)	57 (62.0)	3.9	43 (43.0)	84 (49.1)	4.8	184 (48.5)	141 (53.6)	3.3
Pure	36 (12.9)	11 (12.0)	0.137	17 (17.0)	14 (8.2)	0.086	53 (14.0)	25 (9.5)	0.186
Mixed with other types	102 (36.6)	24 (26.0)		40 (40.0)	73 (42.7)		142 (37.5)	97 (36.9)	
Papillary DCIS									
No	243 (87.0)	87 (94.6)	4.1	74 (74.0)	159 (93.0)	4.8	317 (83.6)	246 (93.5)	6.4
Pure	18 (6.5)	1 (1.1)	0.089	12 (12.0)	3 (1.7)	0.078	30 (7.9)	4 (1.6)	0.098
Mixed with other types	18 (6.5)	4 (4.3)		14 (14.0)	9 (5.3)		32 (8.5)	13 (4.9)	
Micropapillary DCIS									
No	222 (79.5)	74 (80.4)	1.5	83 (83.0)	128 (74.9)	2.2	305 (80.5)	202 (76.8)	3.1
Pure	18 (6.5)	3 (3.3)	0.470	7 (7.0)	5 (2.9)	0.908	25 (6.6)	8 (3.0)	0.090
Mixed with other types	39 (14.0)	15 (16.3)		10 (10.0)	38 (22.2)		49 (12.9)	53 (20.2)	
Margin width* **									
Positive (Tumour on ink)	0 (0.0)	0 (0.0)	9.2	0 (0.0)	0 (0.0)	9.5	0 (0.0)	0 (0.0)	12.3
<2mm	1 (0.4)	8 (9.1)	0.057	0 (0.0)	5 (3.0)	**0.023**	1 (0.3)	13 (5.1)	**0.015**
≥ 2mm	251 (99.6)	80 (90.9)		100 (100.0)	164 (97.0)		351 (99.7)	244 (94.9)	

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, *p* values in **bold** are significant
*Age and size according to Van Nuys Prognostic Index, **Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant,
***Margin width is the width after BCS as a final operation.

Table 3-9: Clinicopathological factors associated with axillary node surgery (n=1248)

Parameter	DCIS Diagnosed 1987-2008 N (%)		X^2 *p*-value	DCIS diagnosed 2009-2017 N (%)		X^2 *p*-value	Total N (%)		X^2 *p*-value
	No (n=395)	**Yes** (n=407)		**No** (n=290)	**Yes** (n=156)		**No** (n=685)	**Yes** (n=563)	
Age (years)*									
<40	9 (2.3)	15 (3.7)	1.9	5 (1.7)	3 (1.9)	2.4	14 (2.0)	18 (3.2)	2.2
40 t0 60	237 (60.0)	230 (56.6)	0.371	136 (46.9)	85 (54.5)	0.291	373 (54.5)	315 (56.0)	0.326
>60	149 (37.7)	162 (39.7)		149 (51.4)	68 (43.6)		298 (43.5)	230 (40.8)	
Presentation									
Screening	145 (36.7)	205 (50.4)	5.1	218 (75.2)	78 (50.0)	2.8	363 (53.0)	283 (50.3)	1.3
Symptomatic	250 (63.3)	202 (49.6)	0.110	72 (24.8)	78 (50.0)	0.051	322 (47.0)	280 (49.7)	0.067
Site**									
Localised	293 (91.6)	296 (84.9)	9.7	235 (85.5)	104 (71.7)	12.1	528 (88.7)	400 (81.1)	12.9
Diffuse	27 (8.4)	52 (15.1)	**0.008**	40 (14.5)	41 (28.3)	**0.002**	67 (11.3)	93 (18.9)	**0.002**
Size*									
<16mm	207 (53.2)	101 (24.8)	80.8	132 (45.5)	17 (10.9)	104.1	339 (49.9)	118 (20.9)	150.4
16 to 40mm	138 (35.5)	166 (40.7)	**<0.0001**	124 (42.8)	56 (35.9)	**<0.0001**	262 (38.6)	222 (39.4)	**<0.0001**
>40	44 (11.3)	140 (34.5)		34 (11.7)	83 (53.2)		78 (11.5)	223 (39.7)	
Grade									
Low	73 (18.5)	36 (8.9)	25.1	39 (13.4)	8 (5.1)	13.1	112 (16.3)	44 (7.9)	39.4
Intermediate	104 (26.3)	80 (19.9)	**<0.0001**	104 (35.9)	44 (28.2)	**0.001**	208 (30.4)	124 (22.0)	**<0.0001**
High	218 (55.2)	291 (71.2)		147 (50.7)	104 (66.7)		365 (53.3)	395 (70.1)	
Comedo necrosis									
No	172 (43.5)	107 (26.6)	25.3	98 (33.8)	31 (19.9)	9.6	270 (39.4)	138 (24.7)	30.3
Yes	223 (56.5)	300 (73.4)	**<0.0001**	192 (66.2)	125 (80.1)	**0.002**	415 (60.6)	425 (75.3)	**<0.0001**

Solid DCIS									
No	207 (52.4)	198 (48.4)	3.4	88 (30.3)	35 (22.4)	3.9	295 (43.1)	233 (41.4)	2.8
Pure	85 (21.5)	79 (19.6)	0.182	83 (28.6)	44 (28.2)	0.142	168 (24.5)	123 (21.8)	0.238
Mixed with other types	103 (26.1)	130 (32.0)		119 (41.1)	77 (49.4)		222 (32.4)	207 (36.8)	
Cribriform DCIS									
No	210 (53.2)	233 (57.1)	2.4	132 (45.5)	73 (46.8)	4.0	342 (49.9)	306 (54.4)	5.5
Pure	43 (10.9)	32 (7.9)	0.294	31 (10.7)	8 (5.1)	0.113	74 (10.8)	40 (7.1)	0.061
Mixed with other types	142 (35.9)	142 (35.0)		127 (43.8)	75 (48.1)		269 (39.3)	217 (38.5)	
Papillary DCIS									
No	356 (90.1)	373 (91.6)	5.7	252 (86.9)	146 (93.6)	5.4	608 (88.8)	519 (92.2)	5.3
Pure	18 (4.6)	7 (1.7)	0.057	14 (4.8)	2 (1.3)	0.065	32 (4.7)	9 (1.6)	0.079
Mixed with other types	21 (5.3)	27 (6.7)		24 (8.3)	8 (5.1)		45 (6.5)	35 (6.2)	
Micropapillary DCIS									
No	301 (76.2)	292 (71.7)	3.7	221 (76.2)	101 (64.7)	2.4	522 (76.2)	393 (69.8)	3.2
Pure	28 (7.1)	25 (6.2)	0.154	14 (4.8)	12 (7.7)	0.079	42 (6.1)	37 (6.6)	0.088
Mixed with other types	66 (16.7)	90 (22.1)		55 (19.0)	43 (27.6)		121 (17.7)	133 (23.6)	
Management									
BCS	296 (74.9)	75 (18.6)	101.1	260 (89.7)	11 (7.1)	190.2	556 (81.2)	86 (15.3)	250.4
Mastectomy	99 (25.1)	332 (81.4)	**<0.0001**	30 (10.3)	145 (92.9)	**<0.0001**	129 (18.8)	477 (84.7)	**<0.0001**

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, *p* values in **bold** are significant
BCS: Breast conserving surgery
*Age and size according to Van Nuys Prognostic Index, **Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant.

Table 3-10: Correlation between the clinicopathological factors and the development of local recurrence (univariate analysis) for patients treated with BCS as a final operation (n=642)

Parameter	(n=642) N (%)	Recurrence (n=93) N (%)	*p*-value (Log rank test)	Recurrence		*p*-value (Log rank test)
				DCIS (n=33) N (%)	Invasive (n=66) N (%)	
Age (years)*						
<40	8 (1.2)	3 (3.3)	0.358	2 (6.1)	1 (1.7)	0.420
40 to 60	347 (54.0)	55 (59.1)		19 (57.6)	36 (60.0)	
>60	287 (44.8)	35 (37.6)		12 (36.3)	23 (38.3)	
Presentation						
Screening	456 (71.0)	49 (52.7)	0.051	17 (51.5)	32 (53.3)	0.158
Symptomatic	186 (29.0)	44 (47.3)		16 (48.5)	28 (46.7)	
Site**						
Localised	479 (90.0)	6 (10.2)	**0.023**	3 (13.6)	3 (8.1)	**0.012**
Diffuse	53 (10.0)	53 (89.8)		19 (86.4)	34 (91.9)	
Size*						
<16mm	358 (56.0)	48 (52.2)	**0.041**	17 (51.5)	31 (52.5)	**0.027**
16 to 40mm	233 (36.5)	34 (37.0)		14 (42.4)	20 (33.9)	
>40mm	48 (7.5)	10 (10.8)		2 (6.1)	8 (13.6)	
Grade						
Low	103 (16.0)	7 (7.6)	**0.020**	3 (9.1)	4 (6.7)	0.072
Intermediate	194 (30.2)	23 (24.7)		9 (27.3)	14 (23.3)	
High	345 (53.8)	63 (67.7)		21 (63.6)	42 (70.0)	
Comedo necrosis						
No	245 (38.2)	40 (43.0)	0.637	16 (48.5)	24 (40.0)	0.944
Yes	397 (61.8)	53 (57.0)		17 (51.5)	36 (60.0)	

Solid DCIS						
No	268 (41.7)	52 (55.9)	0.414	21 (63.6)	31 (51.7)	0.352
Pure	165 (25.7)	17 (18.3)		7 (21.3)	10 (16.7)	
Mixed with other types	209 (32.6)	24 (25.8)		5 (15.1)	19 (31.6)	
Cribriform DCIS						
No	325 (50.6)	61 (65.6)	**0.042**	25 (75.8)	36 (60.0)	0.500
Pure	78 (12.2)	7 (7.5)		3 (9.1)	4 (6.7)	
Mixed with other types	239 (37.2)	25 (26.9)		5 (15.1)	20 (33.3)	
Papillary DCIS						
No	563 (87.7)	76 (81.7)	**0.046**	25 (75.8)	51 (85.0)	0.805
Pure	34 (5.3)	8 (8.6)		4 (12.1)	4 (6.7)	
Mixed with other types	45 (7.0)	9 (9.7)		4 (12.1)	5 (8.3)	
Micropapillary DCIS						
No	507 (79.0)	81 (87.1)		30 (90.9)	51 (85.0)	0.184
Pure	33 (5.1)	5 (5.4)	0.134	1 (3.0)	4 (6.7)	
Mixed with other types	102 (15.9)	7 (7.5)		2 (6.1)	5 (8.3)	
Radiotherapy						
No	379 (59.0)	77 (82.8)	**0.004**	25 (75.8)	52 (86.7)	0.197
Yes	263 (41.0)	16 (17.2)		8 (24.2)	8 (13.3)	
Margin width						
Positive (Tumour on ink)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
<2mm	14 (2.3)	9 (10.2)	0.994	5 (17.2)	4 (6.8)	0.898
≥ 2mm	595 (97.7)	79 (89.8)		24 (82.8)	55 (93.2)	

DCIS: Ductal carcinoma *in situ*; N: Number
*Age and size according to Van Nuys Prognostic Index, **Site; Localised: DCIS is involving one quadrant, diffuse: DCIS is in more than one quadrant.

Table 3-11: Predictors of local recurrence following breast conserving surgery: multivariable analysis

Variables	HR	95% CI	Significance (*p* value)
Site*	1.745	1.031-2.952	**0.040**
Size	1.2	1.133-3.254	**0.045**
Grade	3.031	1.260-7.286	**0.009**
Cribriform DCIS	0.828	0.357-1.921	0.563
Papillary DCIS	0.463	0.293-0.731	**0.004**
Radiotherapy	0.560	0.323-0.969	**0.038**

HR: Hazard Ratio; CI: Confidence Interval
Site: Diffuse or localised tumour, where Localised is DCIS involving one quadrant and diffuse is DCIS in more than one quadrant

3.5 Discussion

Currently, after introduction of screening programme, the incidence of DCIS has dramatically increased and accounts for 20–25% of screen-detected breast cancers (Virnig et al., 2010). Variability in the figures of screen-detected versus symptomatic DCIS diagnosis is related to cancer centres' registration strategies, variable target screening age range between countries and time intervals between each screening, reporting of the discovered DCIS and women's compliance with the screening programme (Elshof et al., 2017). For instance, the targeted screening age in the USA ranges from 40–84 years old while in the UK and Australia it is between 50–69, with current trends to expand it (Ernster et al., 2002). In the current series, 60% of cases were screen-detected and the remaining were presented symptomatically either with

a mass forming lesion, nipple discharge or Paget's disease of the nipple. These figures are comparable to findings of the National Cancer Intelligence Network in 2007, where 61.9% of DCIS were screen-detected (Lawrence et al., 2007).

Prior to the early 1990s, the standard treatment for DCIS was mastectomy. However, BCS with adjuvant RT showed comparable outcome to mastectomy in terms of recurrence-free interval and overall survival and, as a result, the rate of mastectomies has declined and BCS predominates (Boyages et al., 1999, Zujewski et al., 2011). In Nottingham, over a 30-year period, approximately two thirds of cases were treated with BCS as a first surgical modality. Mastectomy was the preferred option for patients with high tumour grade, larger sized tumours (more than 40mm), showing comedo necrosis and tumours involving more than one quadrant of the breast. There was not only an increase in the rates of initial BCS but also a significant decline in the re-excisions, final mastectomy and the ipsilateral local recurrences rates. Although the change in local practice occurred at certain points of time – such as in 2008 in which a margin width of 5mm was accepted instead of 10mm, and a recent change of practice in 2016 in which a 2mm margin was adopted (Toss et al., 2017) – the frequency of BCS, re-excision rates and local recurrence rates seem to show a gradual change over time rather than a sudden transition at a certain time point. This may reflect the impact of several factors including the improvement of imaging quality with better assessment of DCIS size and extent, quality of surgery, and the growing use of local RT.

Sentinel lymph node biopsy (SLNB) is not routinely performed as a part of the surgical management of DCIS and current practice guidelines recommend offering SLNB for patients scheduled to be treated by mastectomy as they are usually at a higher risk of harbouring invasive foci within their DCIS (Park and Hwang 2016). For conservative breast surgery, SLNB might be performed if the lesion is larger than 5cm or forming a mass (Lyman et al., 2014), although positive SLNB rarely changes the adjuvant regimen therapy in these patients (Heymans et al., 2017). Approximately half of cases in the current series had axillary lymph node sampling, which was targeted mainly for patients treated with mastectomy. Symptomatically presented young patients treated with BCS for a large-size high-grade DCIS, with comedo necrosis, also underwent SLNB. There were four patients who underwent axillary clearance and those were presented symptomatically before 1995, older than 60 years old and had large-sized high-grade tumours showing comedo necrosis and managed by mastectomy. It can be postulated that such aggressive features were convenient to the surgeons to follow such an aggressive approach in treatment.

The results shown describe the radiological and pathological indications for attempting BCS and the factors associated with the decision to perform additional therapeutic operations. The predominant factor in deciding BCS as the surgical modality is the extent of the disease. Patient choice might also be a contributing factor, as evidenced by the NHS Breast Screening Programme, where patient choice accounts for about 11% of mastectomies for DCIS (Thomas et al., 2014). Recently,

in the UK and US, mastectomy rates have increased, particularly in young women perceived at high risk of further breast cancer events (Rutter et al., 2015, Shiyanbola et al., 2016). In a report on 8,000 DCIS cases included in the Sloane Project and treated between 2003 and 2012, the rate of BCS as an initial primary operation was 79% and successful BCS was 68% (Thomas et al., 2010, Thomas et al., 2014). Achieving success at BCS for DCIS remains a challenge and the issue is that a woman with DCIS is at least as likely to have a mastectomy similar to a woman with IBC. Re-excision rates after BCS as a treatment for DCIS are higher than re-excisions for IBC. Furthermore, invasive disease accompanying DCIS has a higher re-operation rate than invasive disease alone (Dieterich et al., 2012, Jeevan et al., 2012). The need to perform further surgery, re-excision or completion mastectomy is governed predominantly by what is regarded as a minimum free resection margin. Young patient age, symptomatic presentation, lesions involving more than one breast quadrant, DCIS size more than 40mm, and presence of comedo necrosis are associated with a positive surgical margin. A positive resection margin is likely due to radiologic under-estimation of DCIS size, which occurs in over 50% of cases (Thomas et al., 2010, Dixon et al., 2016). Other studies (Chakrabarti et al., 2006, Jacobson et al., 2008, Guidroz et al., 2011, Dieterich et al., 2012, Gurdal et al., 2012, McCahill et al., 2012, Dixon et al., 2016, Merrill et al., 2016) have shown that diffuse DCIS growth patterns, such as micropapillary and cribriform types, high nuclear grade DCIS with comedo type necrosis and high-risk VNPI, are factors associated with an increased rate of re-excisions.

Approximately half of the DCIS patients who underwent re-excision, due to positive or close surgical margins, were of low and intermediate grade. This might change pending the outcome of recent trials of more conservative management of low-risk DCIS (Elshof et al., 2015, Pilewskie et al., 2016).

In this study, half of the re-excised specimens were free of residual tumour tissue, although some cases were reported to have tumour on ink after the primary excision. Such a finding perhaps reflects the inconsistency in pathological evaluation. However, the presence of residual DCIS was associated with adverse clinicopathological parameters such as symptomatic presentation, tumour size, positive surgical margin, conservative treatment and high risk DCIS defined by VNPI. Some studies have tried to establish a margin index based on tumour size and the closest surgical margin to show the association with presence of residual tumour tissue after re-excision, but this did not show a significant association (Margenthaler et al., 2010, Fisher et al., 2011). Another study concluded that the extent of margin involvement as well as the number of involved ducts in that margin are key factors for predicting presence of residual DCIS in the re-excision specimen (Wei et al., 2012). In this study, it was shown that after 2008, there was a reduction in the rate of residual tumour in the re-excision specimen and this might reflect a better overall strategy for further surgery.

Determining the optimal free margin for BCS is challenging, particularly in pure DCIS. The Society of Surgical Oncology-American Society for

Radiation Oncology published new guidelines for the optimal surgical margin in invasive disease and defined negative margin as ‘no tumour on ink’ because there is no evidence that wider clear margins reduce the ipsilateral local recurrence (Moran et al., 2014). The same group has published consensus guidelines suggesting a 2mm free margin as a standard for adequate surgical resection of DCIS (Morrow et al., 2016).

Previous studies showed that negative surgical margin minimises the risk of ipsilateral local recurrence by 50% compared to involved margins. A 2mm margin minimises the risk of recurrence compared to narrow negative margins (Singletary 2002, Kell and Morrow 2005, Morrow 2009, Azu et al., 2010, Morrow et al., 2016). However, the agreement on the previous recommendations is different and the choice of optimal margins depends mainly on the surgeons’ practice and the pathologists’ methods of margin assessment, which is currently supported by the National Institute for Health and Care Excellence (NICE) guidelines (Hancock 2019). Adopting close margins of excision is a means of reducing reoperation rates, as it has been shown in invasive disease (Schulman et al., 2017); however, planning a wider macroscopic margin in higher-risk cases but tailoring the scrutiny of margin analysis to the risk factors should be performed. The current results have shown that the change in surgical practice with narrow free margin in patients treated conservatively did not increase the recurrence rate. This reflects better risk assessment, modest use of

adjuvant radiotherapy and individualised management for patients based on constellation of all clinicopathological factors.

Some trials have shown a 50% reduction in the recurrence rate using adjuvant RT after conservative DCIS surgery (Emdin et al., 2006, Ben-David et al., 2007, Dodwell et al., 2007, Holmberg et al., 2008, Donker et al., 2013). However, they could not identify a specific group of patients where RT could be safely avoided; hence, the continued debate over RT indication after BCS remains. In routine practice, approximately 30–50% of BCS-treated patients receive adjuvant RT (Ward et al., 2015). A common local practice before 2008 was not to offer further treatment for patients with a clear surgical margin of 10mm or more. However, in the current series, 41% of BCS-treated patients were offered post-operative RT regardless of their margin status. Outcome analysis of previous studies has shown that selective RT was introduced (Correa et al., 2010) and then it was recommended after BCS to those with high-grade DCIS, women younger than 50 and lesions larger than 30mm following a multidisciplinary team discussion (Mathew et al., 2017).

The current study reports a local recurrence rate of 7.5% regardless of the management modality and 14.5% following BCS, which is close to the reported figures with a range of 7–12% (Wadsten et al., 2016). Most of the previous trials reported that half of the local recurrences are invasive (Silverstein et al., 1999, Fisher et al., 2001, Bijker et al., 2006, Wapnir et al., 2011). In this series, approximately 64% of the recurrences were invasive.

Although factors of aggressive DCIS such as young patient age, symptomatic presentation, high grade, comedo necrosis and close surgical margins are associated with poor outcome, their results were inconsistent between various studies depending on the included sample size, local surgical management practice and DCIS pathological evaluation (Weng et al., 2000, Lagios and Silverstein 2015, Barrio and Van Zee 2017, Kanbayashi and Iwata 2017). The current study showed that diffuse tumours involving more than one quadrant, large tumour size, high nuclear grade and absence of adjuvant radiotherapy are the key independent factors that were associated with poor outcome in terms of shorter RFI in BCS-treated DCIS. Papillary DCIS was associated with shorter RFI in this series, which may be related to incomplete excision of these lesions, which show an extendable growth pattern. It is postulated that the high recurrence rate associated with high-grade DCIS might reflect the natural history of DCIS rather than being a true predictive factor of recurrence (Pinder et al., 2010b).

Ringberg et al. showed that patients with low/intermediate-grade DCIS had approximately half the risk of an ipsilateral recurrence compared to patients with high-grade DCIS (Ringberg et al., 2007). Although there is a great acceptance that low-grade tumours are associated with a lower risk of recurrence, few studies have examined the long natural history of low-grade DCIS. A study in the USA included 28 women with low-grade DCIS after biopsy alone, with follow-up over a 30-year period (Sanders et al., 2005). After the follow-up period, 40% of women developed an

ipsilateral invasive breast cancer, with all recurrences being in the same quadrant. The authors concluded that the long natural history of low-grade DCIS is distinct, with a different behaviour to higher-grade DCIS. They concluded that women with low-grade DCIS who did not receive definite treatment remained at risk of recurrence for a prolonged period after the diagnosis, particularly in the first 15 years. Analysis of risk factors in the European Organisation for Research and Treatment of Cancer (EORTC) study (Bijker et al., 2006) showed that patients with low-grade DCIS had a significantly lower risk of DCIS recurrence compared to patients with an intermediate or high-grade tumour. However, there was no significant difference in the incidence of invasive recurrences at ten years. The above data suggest that the risk of recurrence in patients with low-grade DCIS increases beyond five years of follow-up. Thus, sufficient follow-up time is crucial for evaluating the outcome of patients with low-grade DCIS. This should be taken into consideration for management decision-making in patients with low-grade DCIS (Boxer et al., 2013).

Although young patient age varied as a risk factor among the studies, it was shown to be significantly associated with an increased risk of ipsilateral local recurrence (Bijker et al., 2001, Pinder et al., 2010b, Wapnir et al., 2011). In a study of 615 patients treated with BCS for DCIS, multifocality (defined as the presence of more than one distinct focus of DCIS with at least 5mm of intervening healthy tissue confined to a single quadrant of the breast) was identified in approximately one third of patients. In a multivariate analysis, multifocality was a predictor

of local recurrence, independent of other studied factors. In addition, omission of RT, large tumour size and high nuclear grade were also predictors of local recurrence.

VNPI assesses DCIS risk for optimal management based on the previously described four clinicopathological parameters (Silverstein 2003b). Several studies have attempted to validate its value (Boland et al., 2003, Asjoe et al., 2007, MacAusland et al., 2007, Di Saverio et al., 2008). VNPI risk groups in the current series were categorised after the first operation and showed high correlation with the final treatment decisions. However, VNPI assessed after the final operation showed no association with tumour recurrence. Our findings were concordant with other studies that tried to verify using this index to predict tumour recurrence (Asjoe et al., 2007, MacAusland et al., 2007). Taken together, VNPI would be a reasonable risk index for choosing the management approach but might not be a powerful index for predicting tumour recurrence. So, other nomograms were developed in order to provide a risk index for tumour recurrence, but they need more validation (Rudloff et al., 2010, Mazouni et al., 2011, Lee et al., 2013).

In conclusion, this study showed the interplay between different clinicopathological factors and patient outcome and that some indices, like VNPI, may be helpful in deciding treatment but not in predicting patients' outcomes. Surgical margins should be thoroughly examined to eliminate their effect on the later development of local recurrence.

4 Molecular Taxonomy of DCIS Using Well-Established Biomarkers

4.1 Introduction

4.1.1 DCIS prognostic and predictive factors

4.1.1.1 Definitions

A useful prognostic factor has the ability to identify a group of patients whose prognosis is good and hence adjuvant therapy following local surgery would not be cost beneficial and/or even harmful, and a second group with poor prognosis for whom adjuvant therapy would be warranted (Clark et al., 1994). To be applicable, prognostic factors must show a wide separation in the outcome of the identified groups with adequate numbers of patients in each group. Nevertheless, for personalised medicine, no single prognostic factor satisfies all these criteria (Lee and Ellis 2008). In addition, the reliability of such prognostic factors using routine techniques of tissue and imaging diagnostic tools, besides cost limits are practically not fully applicable.

Predictive factors that predict the likelihood of a tumour to respond to a certain treatment are complimentary to prognostic factors and are used to select the most appropriate additional therapy for a patient when needed. Contrary to predictive factors for hormonal treatment, which are well-established, those predictive of chemosensitivity are less well defined. It is difficult to recognise those patients who are more likely to respond to a particular chemotherapy regimen based on traditional prognostic/predictive factors such as lymph node status and tumour size alone (Mauriac et al., 2005, Yamashiro and Toi 2008).

A biomarker is defined as ‘a detectable substance that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Vuong et al., 2014).

In DCIS, there is a current inability to predict patients’ risk to develop invasive cancer; therefore, the standard management for patients diagnosed with DCIS has long been considered as BCS followed by adjuvant RT (Nofech-Mozes et al., 2005). Even with RT, some risk of invasive progression remains, and therefore current management is to eradicate DCIS in order to prevent this event (Karlsson 2017). However, DCIS is a heterogeneous disease; where some cases will behave as indolent with no progression to invasive carcinoma during the patient’s lifetime, while others will follow an aggressive course (Pang et al., 2016). The events driving the progression of DCIS to invasive carcinoma have not been fully identified (Cowell et al., 2013).

4.1.1.2 Clinicopathological factors as prognostic factors

Certain clinical and pathological features of DCIS may be prognostic of local and/or invasive recurrence following surgical excision (Benson and Wishart 2013). For instance, some studies have suggested that tumour size is a strong predictor of local recurrence (Holmes et al., 2011, Kantor and Winchester 2014). Nuclear grade and margin status are other factors that appear to influence recurrence in DCIS (Carraro et al., 2014, Wong et al., 2014, Van Zee et al., 2015), though certainly there is a study that disproves these findings (Berman et al., 2010). Two major

clinical tools that aid in risk stratification and treatment planning – the USC/VNPI and the DCIS nomogram introduced by Rudloff et al. – utilise both clinical and pathological factors such as tumour size, necrosis, and margin status (Rudloff et al., 2010, Yi et al., 2012, Benson and Wishart 2013). Currently, no single factor or combination of factors are able to predict the behaviour of a DCIS lesion, leading to potential over- or under-treatment of the patient with associated morbidity, potential mortality, financial and social burden.

While the current ability to accurately predict recurrence on the basis of these parameters is limited, clearly the paradigm that one approach fits all in patients with DCIS is changing (Mardekian et al., 2016).

Research efforts have focused on classifying patients who may be managed conservatively and those who are at higher risk for disease progression and thus may require adjuvant therapy (Mardekian et al., 2016). The ideal classification scheme would be clinically useful and easy to adapt with the ability to stratify patients into prognostic groups. While not consistently identified in all studies, certain histopathological parameters and expression of various immunohistochemical markers have been variably shown to affect the risk of recurrence in pre-malignant breast lesions (Siziopikou 2013). Improved molecular characterisation of DCIS will offer additional, perhaps more conclusive, prognostic information and may provide the opportunity for personalised therapeutic options for patients with DCIS.

4.1.2 Gene expression profiling-derived classes and signatures

The developments of gene expression profiling (GEP) studies and related prognostic signature studies have dramatically unravelled the molecular heterogeneity and diversity in breast cancer (Sorlie et al., 2001). Moreover, they have contributed to the current understanding of breast cancer molecular complexity and led to the identification of distinct subtypes. Importantly, molecular subtyping and prognostic signatures that have arisen from GEP studies will have important impacts on future drug discoveries and improving clinical management (Weigelt and Reis-Filho 2010).

4.1.2.1 Molecular classes

4.1.2.1.1 History

A new classification system of biological breast cancer subtypes was adopted at the 12th St. Gallen International Breast Cancer Conference 2011 (Goldhirsch et al., 2011). The intrinsic molecular subtypes based on gene expression analyses (Perou et al., 2000, Sorlie et al., 2001) are, for practical purposes, approximated using clinicopathological criteria (Zhou et al., 2013). Systemic therapy recommendations for IBC according to the subtype classification were also proposed (Goldhirsch et al., 2013).

4.1.2.1.2 Importance of molecular classification

A clinically useful histopathological classification system for DCIS predicting prognosis, however, is still missing. Survival is excellent after a primary diagnosis of DCIS, but the risk of recurrence is considerably high (Early Breast Cancer Trialists' Collaborative Group (EBCTCG)) (Correa et al., 2010). Hence, identification of biomarkers to aid in individualised treatment decisions regarding surgery and radiotherapy is needed. Kerlikowske et al. (Kerlikowske et al., 2010) used IHC for biomarkers, including Ki67, showing that biomarkers were better than histopathological criteria for identifying risk groups for subsequent invasive cancer. In addition, Solin et al. (Solin et al., 2013) used a 21-gene array to identify risk groups after BCS without postoperative radiation.

4.1.2.1.3 Surrogate molecular subtypes for DCIS

The different subtypes are defined as follows:

1. Luminal A (ER and/or PR positive, HER2 negative and Ki67 <14%);
2. Luminal B/HER2- (ER and/or PR positive, HER2 negative and Ki67 ≥14%);
3. Luminal B/HER2+ (ER and/or PR positive, HER2 positive);
4. HER2+/ER- (non-luminal) (ER and PR negative and HER2 positive);
5. Triple Negative, (ER, PR and HER2 negative).

The IHC surrogate definitions are based on the 2011 St. Gallen guidelines (Goldhirsch et al., 2011).

4.1.2.2 Oestrogen receptor

Although there is a potentially very large pool of biomarkers on the horizon, only three are used in routine clinical practice worldwide: ER, PR and HER2 (Rakha et al., 2010). ER was one of the first biological markers to be studied in depth. It was identified by Ellwood Jensen in the late 1950s (Jensen and Jordan 2003), and ER status has been used since the mid-1970s, as one of the most valuable markers in breast cancer, both to predict responsiveness of the invasive tumour to endocrine therapy (Lari and Kuerer 2011) and as a prognostic factor for early recurrence and long-term outcome (Vuong et al., 2014).

The ER mean expression rate was reported to be 70% in a study that examined ER expression rate in DCIS (Lari and Kuerer 2011). Two studies have revealed ER expression to be inversely related to the nuclear grade of DCIS; for example, ER expression is higher in well-differentiated tumours than in poorly differentiated lesions (Meijnen et al., 2008, Altintas et al., 2009). Some studies have identified an association between ER-negative DCIS and the risk of local recurrence (Kerlikowske et al., 2010, Williams et al., 2015).

4.1.2.3 Progesterone receptor

PR is considered as an addition to ER in IBC where it is a weak prognostic factor contributing to recurrence-free survival and a predictor of response to endocrine therapy (Lari and Kuerer 2011). In previous studies, the mean PR expression rate was 60%. An inverse relationship was observed between PR expression and nuclear grade, similar to ER (Lebeau et al., 2003, Hanley et al., 2008); however, a direct positive

correlation was observed between PR and ER expression (Kulkarni et al., 2008). Few studies have shown a significant correlation between PR-negative DCIS and local recurrence (Ringberg et al., 2001, Provenzano et al., 2003).

4.1.2.4 HER2

4.1.2.4.1 HER2 protein expression

One of the interesting aspects of DCIS is that the expression of HER2 is related to development and progression of breast cancer (Solin et al., 2013) and its over-expression is frequently seen in high grade DCIS with or without necrosis (Roses et al., 2009). HER2 is a member of the type I receptor tyrosine kinase family, which consists of four related family members: HER2, EGFR (HER1), HER3, and HER4. The major role of HER2 is to act as a co-receptor in the dimerisation and activation of other HER members. The amplification of the HER2 gene results in a 50- to 100-fold increase in the number of surface HER2 receptors on cancer cells compared to normal mammary epithelial cells (Wilson et al., 2005). It was previously reported that the expression of HER2 in DCIS is associated with recurrence following surgical excision without RT (Holmes et al., 2011). However, the definitive role of HER2 as an independent risk factor for recurrence remains controversial (Borgquist et al., 2015).

IHC staining is the predominant method of determining HER2 status in breast cancer specimens due to its relative ease to perform, with rapid turnaround time and lower cost (Hanna et al., 1999). However,

commercially available antibodies have shown wide variation in sensitivity and specificity for FFPE tissue samples, in which tissue fixation and pre-treatment have a substantial effect on staining (Jimenez et al., 2000).

4.1.2.4.2 *HER2* gene amplification

HER2 gene amplification is primarily detected by FISH to detect the signals. This method is expensive and requires expertise with fluorescent microscopy using appropriate filters (Pothos et al., 2008). CISH offers an alternative method, and whilst it utilises *in situ* hybridisation technology of FISH, it takes advantage of a chromogenic signal detection that is assessed using light microscopy and is therefore a cheaper alternative. CISH is able to detect HER2 gene amplification and to minimise, if not eliminate, the borderline category with IHC (Penault-Llorca et al., 2009).

4.1.2.5 Proliferation marker – Ki67

Ki67 protein was first defined by Gerdes and colleagues, who developed a prototype monoclonal antibody Ki67 through immunising mice with the nuclei of the Hodgkin lymphoma cell line L428 (Gerdes et al., 1983). Cell cycle analysis revealed that the antigen is present in the nuclei of cells in all active proliferative phases (G1, S, and G2) as well as in M phase, while quiescent or resting cells in the G0 phase did not express it. Therefore, it has been used as a biomarker to assay the growth fraction of a given cell population (Colozza et al., 2005, Park et al., 2007). The nuclear antigen Ki67 is used to assess the proliferation

rate of breast cancer tumours, which is a key player in disease progression (de Azambuja et al., 2007). Variable mean Ki67 expression is observed, and attributable to factors such as tumour grade and HER2 status (Kuerer et al., 2011, Rakovitch et al., 2012). Furthermore, Ki67 is a predictor of local recurrence, either independently or in combination with other factors (Wilson et al., 2006, Kerlikowske et al., 2010).

4.2 Hypothesis and aims

It is hypothesised that classification of DCIS according to the molecular classification of breast cancer might help in patient risk stratification and that identification of biomarkers to aid in individualised treatment decisions regarding surgery and RT is needed. Therefore, implementing well-established biomarkers for IBC – ER, PR, HER2 and Ki67 – to classify DCIS tumours using IHC was an aim.

4.3 Patients and methods

4.3.1 Study cohort and TMA

This retrospective study was conducted on a consecutive series of primary pure DCIS and a control group of DCIS cases mixed with invasion (**Chapter 2**).

4.3.2 IHC and CISH

4.3.2.1 IHC

The IHC staining and evaluation of ER, PR, HER2 and Ki67 was performed using the TMA sections. Positive and negative controls were included in every assay, where internal control of breast tissue was used as a positive control for ER and PR, breast cancer tissue for

HER2 and tonsil tissue for Ki67. MIB1 antibody was used for evaluation of Ki67 expression. Nuclear staining was assessed for ER, PR and Ki67 and membrane staining for HER2 (**Chapter 2**) and (**Figure 4-1**). HER2 status was assessed using the HercepTest scoring method (Rakha et al., 2015). HER2 status was considered negative if the immunohistochemical score was 0 or 1+, equivocal if the score was 2+, and positive if the score was 3+.

4.3.2.2 CISH

CISH was performed according to the recommended method (**Chapter 2**). HER2 gene amplification was determined by CISH using the ZytoDot 2C SPEC ERBB2/CEN 17 Probe Kit (ZytoVision®, Germany). Interpretation of CISH was performed using a Nikon microscope equipped with a x63 objective lens. At least 20 malignant, non-overlapping cell nuclei were scored to assess the HER2 gene copy number. Chromosome 17 (represented by the red signals) was used as an internal control, particularly in the low HER2 gene copy number cases, as per the recommended protocol. HER2 gene amplification was defined as six or more signals per nucleus or when clusters (clumps of aggregated green signals) were identified in the cell nuclei in more than 50% of tumour cells (Wolff et al., 2013). If the average copy number was ≥4 to <6 per nucleus (equivocal), another 20 tumour cells were enumerated, and the final average copy number of the case was calculated from the total of 40 cells.

For ER and PR, a 1% cut-off value was used to dichotomise cases into positive and negative (Hammond et al., 2010b). For Ki67, the cut-off for differentiating low and high proliferative groups was 14% (Goldhirsch et al., 2013).

4.3.3 Statistical analysis

Association between final HER2 status and clinicopathological parameters using categorised data in the pure DCIS cohort was evaluated using the Chi-squared test. Survival rates were determined using the Kaplan-Meier method and compared by the log-rank test. Multivariate survival analyses using Cox's proportional hazard regression model was used to evaluate the associations between independent variables and local recurrence.

4.4 Results

The clinicopathological characteristics of pure DCIS cases included in the TMA are listed in **Table 4-1**.

4.4.1 Assessment of HER2 status

A total of 647 and 217 pure DCIS and DCIS mixed with invasion, respectively, were suitable for the analysis. In the pure DCIS group, 20% of cases were HER2 positive. Immunohistochemistry for HER2 was scored as 3+ in 9%, 2+ in 15% and 0 or 1+ in 76% (**Table 4-2 and Table 4-3**). All 3+ cases showed HER2 amplification with CISH; 72 (74%) cases of the 2+ group showed HER2 amplification. (**Figure 4-2**). Of the 493 cases with 0/1+ score using IHC, only one showed HER2

amplification. When IHC results were assessed with the exclusion of equivocal (2+) tumours, agreement between IHC and CISH in 0, 1+ and 3+ cases was 99.7% (κ-coefficient = 0.997). Interestingly, when the overall rate of positivity was analysed over the period of the study, the frequency of positivity reduced from 30% in the 1990s to 20% in 2012 (**Figure 4-3**).

In mixed DCIS and invasive carcinoma cases, the DCIS component was HER2 positive in 15% and the invasive component in 12% (p=0.051, **Table 4-4**). All eight discordant cases had extensive DCIS; six of them were of high grade.

4.4.2 Correlation with clinicopathological parameters and molecular biomarkers

In pure DCIS, HER2 positivity was associated with larger tumour size, high nuclear grade, comedo type DCIS, negative hormone receptor status and high Ki67 proliferation index (all p<0.0001). HER2 positive cases were more frequent in patients treated by mastectomy (p=0.001) (**Table 4-5**).

4.4.3 Characteristics of DCIS by surrogate molecular subtypes

577 pure DCIS cases could be classified into the surrogate molecular subtypes. Of the classified tumours, 348 were Luminal A (60%), 45 Luminal B/HER2- (7%), 46 Luminal B/HER2+ (8%), 66 HER2+ (12%) and 72 Triple Negative (13%) (**Table 4-6**).

The HER2+, Luminal B/HER2+ and Triple Negative subtypes were more often of larger tumour size (>40mm), higher tumour grade and more often aggressively treated with mastectomy compared to Luminal A and Luminal B/HER2- tumours. The Triple Negative subtype was also associated with presence of comedo necrosis.

4.4.4 Associations with the outcome

Nuclear grade, comedo necrosis and margin width were associated with the development of all ipsilateral recurrences (0.023, 0.013 and 0.029, respectively). When recurrences were stratified into *in situ* and invasive recurrences, HER2 was associated with the development of DCIS local recurrence (HR= 2.51, 95%CI (2.11-11.45), *p*=0.030), while tumour size was a predictor of invasive local recurrence (HR=2.63, 95%CI (1.13-6.09), *p*=0.024) (**Table 4-7**).

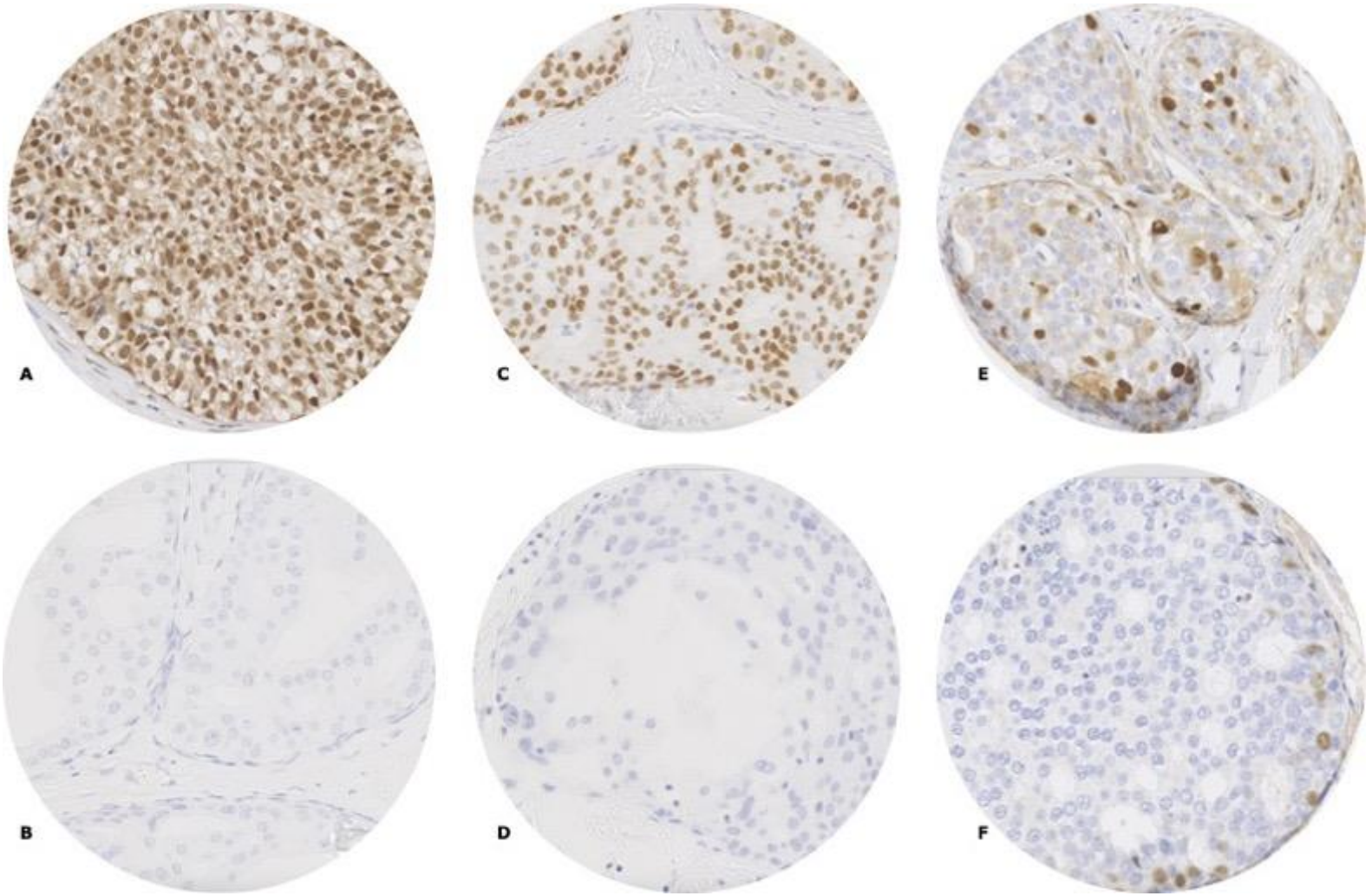


Figure 4-1: Immunohistochemistry of biomarkers used for characterisation of DCIS. (A) ER positive; (B) ER negative, (C) PR positive; (D) PR negative, (E) Ki67 high proliferative index; (F) Ki67 low proliferative index.

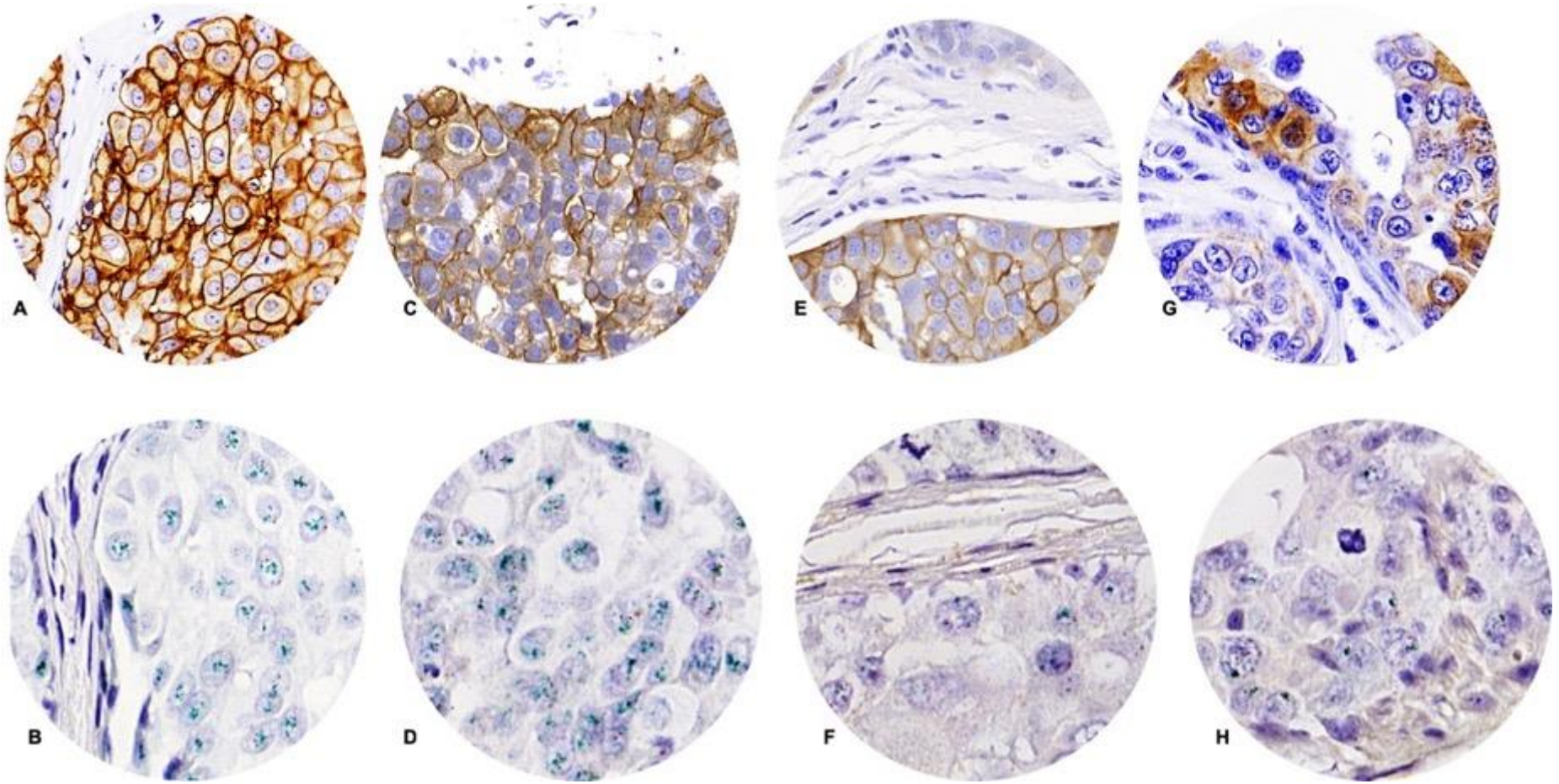


Figure 4-2: IHC of HER2 confirmed by CISH. (A and B) DCIS showing HER2 3+ (A) confirmed by CISH (B); (C and D) HER2 2+ (C) showing gene amplification (D); (E and F) HER2 2+ (E) without amplified gene (F); and (G and H) HER2 1+ (G) showing no gene amplification (H).

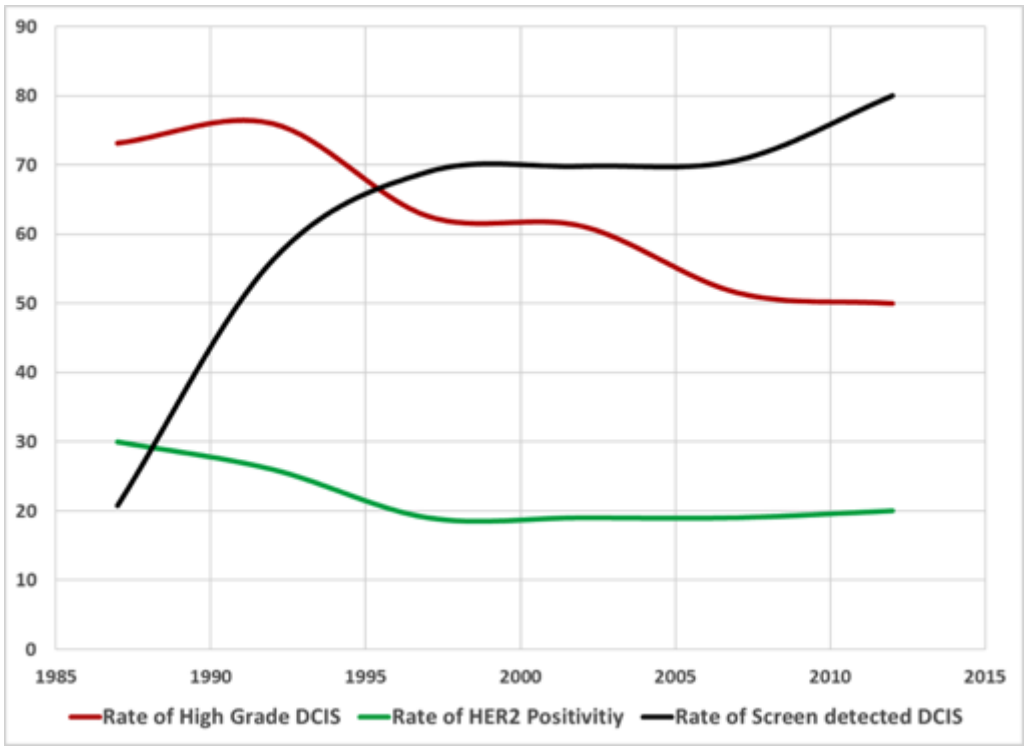


Figure 4-3: The rate of HER2 positivity, grade and screen-detected DCIS change over time. The graph shows that the rate of high-grade DCIS together with HER2 positivity declines over the years in contrast to the rate of screen-detected DCIS.

Table 4-1: Clinicopathological characteristics of patients with pure DCIS in the study

Parameter	Total (n=651) n (%)	BCS (n=308) n (%)	Mastectomy (n=343) n (%)	χ^2 (*p* value)
Age (years)*				
<40	22 (3.4)	5 (1.6)	17 (5)	5.86
40 to 60	379 (58.2)	179 (58.1)	200 (58.3)	(0.053)
>60	250 (38.4)	124 (40.3)	126 (36.7)	
Presentation				
Screening	337 (51.7)	187 (60.7)	150 (43.7)	18.74
Symptomatic	314 (48.3)	121 (39.3)	193 (56.3)	(**$1.5x10^{-5}$**)
Size*				
<16mm	224 (34.7)	170 (55.7)	54 (15.8)	161.11
16 to 40mm	247 (38.2)	114 (37.4)	133 (39.0)	(**$1.1x10^{-35}$**)
>40mm	175 (27.1)	21 (6.9)	154 (45.2)	
Grade				
Low	87 (13.4)	53 (17.2)	34 (9.9)	9.92
Intermediate	167 (25.7)	84 (27.3)	83 (24.2)	**(0.007)**
High	397 (60.9)	171 (55.5)	226 (65.9)	
Comedo necrosis				
Yes	416 (64.1)	179 (58.5)	237 (69.1)	7.89
No	233 (35.9)	127 (41.5)	106 (30.9)	**(0.005)**
ER Status				
Positive	428 (75.1)	211 (80.1)	217 (70.2)	8.53
Negative	142 (24.9)	50 (19.9)	92 (29.8)	**(0.004)**
PR Status				
Positive	302 (46.4)	159 (51.6)	143 (41.7)	6.44
Negative	349 (53.6)	149 (48.4)	200 (58.3)	**(0.011)**
Ki67 Index				
High (≥14%)	110 (23.4)	46 (21.4)	64 (25.0)	0.848
Low (<14%)	361 (76.6)	169 (78.6)	192 (75.0)	(0.357)

DCIS: Ductal carcinoma *in situ*; N: Number; X^2: Chi square, *p* value in **bold:** Significant.
ER: Oestrogen receptor; PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI).

Table 4-2: HER2 status in pure DCIS cases using IHC and CISH

HER2 CISH	HER2 IHC in pure DCIS			
	0/1+ (n=493) (%)	2+ (n=98) (%)	3+ (n=56) (%)	Total (n=647) (%)
Amplified	1 (0.2)	72 (73.5)	56 (100)	129 (20)
Non-amplified	492 (99.8)	26 (26.5)	0 (0)	518 (80)

Table 4-3: Comparison between HER2 status in pure DCIS cases using IHC and CISH

HER2 CISH	HER2 IHC in pure DCIS			
	0/1+ (n=493) (%)	2+ (n=98) (%)	3+ (n=56) (%)	Total (n=647) (%)
<4	489 (99)	26 (27)	0 (0)	515 (80)
4 to 6	3 (0.6)	3 (3)	1 (2)	7 (1)
>6	1 (0.4)	69 (70)	55 (98)	125 (19)

Table 4-4: HER2 status in DCIS cases mixed with invasion using IHC and CISH

CISH	HER2 IHC DCIS Component				HER2 IHC Invasive Component			
	1+	2+	3+	Total N. (%)	1+	2+	3+	Total N. (%)
Amplified	5	5	22	32 (14.7)	1	1	23	25 (11.5)
Non-amplified	180	5	0	185 (85.3)	191	1	0	192 (88.5)

Table 4-5: Association between final HER2 status and the clinicopathological parameters of pure DCIS cohort

Parameter	Total (n=646) n (%)	HER2 Status Positive (n=128) n (%)	HER2 Status Negative (n=518) n (%)	χ^2 (*p* value)
Age (years)*				
<40	22 (3.4)	7 (5.5)	15 (2.9)	2.12
40 to 60	376 (58.2)	74 (57.8)	302 (58.3)	(0.347)
>60	248 (38.4)	47 (36.7)	201 (38.8)	
Presentation				
Screening	336 (52.0)	69 (53.9)	267 (51.5)	0.23
Symptomatic	310 (48.0)	59 (46.1)	251 (48.5)	(0.632)
Size *				
<16mm	223 (34.8)	27 (21.4)	196 (38.1)	20.63
16 to 40mm	247 (38.5)	47 (37.3)	200 (38.8)	(**3.3×10^{-5}**)
>40mm	171 (26.7)	52 (41.3)	119 (23.1)	
Grade				
Low	87 (13.5)	0 (0.0)	87 (16.8)	58.58
Intermediate	166 (25.7)	13 (10.2)	153 (29.5)	**(1.9×10^{-13})**
High	393 (60.8)	115 (89.8)	278 (53.7)	
Comedo necrosis				
Yes	411 (63.8)	111 (86.7)	300 (58.1)	36.28
No	233 (36.2)	17 (13.3)	216 (41.9)	**(1.7×10^{-9})**
Management				
Mastectomy	339 (52.5)	84 (65.6)	255 (49.2)	11.06
BCS	307 (47.5)	44 (34.4)	263 (50.8)	(**0.001**)
Radiotherapy				
Yes	95 (14.7)	19 (14.8)	76 (14.7)	2.815
No	551 (85.3)	109 (85.2)	442 (85.3)	(0.093)
ER status				
Positive	426 (75.3)	45 (40.5)	381 (83.7)	88.86
Negative	140 (24.7)	66 (59.5)	74 (16.3)	**(5×10^{-20})**
PR status				
Positive	302 (46.7)	18 (14.1)	284 (54.8)	68.51
Negative	344 (53.3)	110 (85.9)	234 (45.2)	**(1.2×10^{-16})**
Ki67 Index				
High (≥14%)	108 (23.0)	46 (42.2)	62(17.2)	37.46
Low (<14%)	361 (77.0)	63 (57.8)	298 (82.8)	**(9.3×10^{-10})**

DCIS: Ductal carcinoma *in situ*; n: number; X^2: Chi square, *p* value in **bold:** significant.
BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI).

Table 4-6: Characteristics of DCIS by surrogate molecular subtypes

DCIS Characteristics	All (n=577) n (%)	Luminal A (n=348), %	Luminal B/HER- (n=45), %	Luminal B/HER+ (n=46), %	HER2+ (n=66), %	Triple negative (n=72), %	χ^2 (*p* value)
Age (years)*							
<40	21 (3.6)	10 (2.9)	2 (4.4)	3 (6.5)	3 (4.5)	3 (4.2)	3.146
40 to 60	333 (57.7)	201 (57.8)	25 (55.6)	29 (63.1)	38 (57.6)	40 (55.5)	(0.925)
>60	223 (38.6)	137 (39.3)	18 (40.0)	14 (30.4)	25 (37.9)	29 (40.3)	
Presentation							
Screening	297 (51.5)	171 (49.1)	25 (55.6)	23 (50.0)	38 (57.6)	40 (55.6)	2.564
Symptomatic	280 (48.5)	177 (50.9)	20 (44.4)	23 (50.0)	28 (42.4)	32 (44.4)	(0.633)
Size*							
<16	198 (34.4)	136 (39.1)	16 (35.6)	8 (17.8)	17 (26.2)	21 (29.2)	23.382
16 to 40	221 (38.4)	137 (39.3)	19 (42.2)	18 (40.0)	21 (32.3)	26 (36.1)	**(0.003)**
>40	156 (27.2)	75 (21.6)	10 (22.2)	19 (42.2)	27 (41.5)	25 (34.7)	
Grade							
Low	79 (13.7)	71 (20.4)	3 (6.7)	0 (0.0)	0 (0.0)	5 (6.9)	112.265
Intermediate	154 (26.7)	128 (36.8)	11 (24.4)	5 (10.9)	5 (7.6)	5 (6.9)	**(1.3×10^{-20})**
High	344 (59.6)	149 (42.8)	31 (68.9)	41 (89.1)	61 (92.4)	62 (86.2)	
Comedo necrosis							
Yes	119 (20.6)	50 (14.4)	4 (8.9)	14 (30.4)	21 (31.8)	30 (41.7)	39.337
No	458 (79.4)	298 (85.6)	41 (91.1)	32 (69.6)	45 (68.2)	42 (58.3)	**(5.9×10^{-8})**
Solid DCIS							
Yes	292 (50.6)	167 (48.0)	29 (64.4)	26 (56.5)	36 (54.5)	34 (47.2)	5.785
No	285 (49.4)	181 (52.0)	16 (35.6)	20 (43.5)	30 (45.5)	38 (52.8)	(0.216)

Cribriform DCIS							
Yes	265 (45.9)	182 (52.3)	18 (40.0)	19 (41.3)	22 (33.3)	24 (33.3)	15.535
No	312 (54.1)	166 (47.7)	27 (60.0)	27 (58.7)	44 (66.7)	48 (66.7)	**(0.004)**
Papillary DCIS							
Yes	75 (13.0)	60 (17.2)	7 (15.6)	1 (2.2)	2 (3.0)	5 (6.9)	18.699
No	502 (87.0)	288 (82.8)	38 (84.4)	45 (97.8)	64 (97.0)	67 (93.1)	**(0.001)**
Micropapillary DCIS							
Yes	158 (27.4)	100 (28.7)	13 (28.9)	10 (21.7)	19 (28.8)	16 (22.2)	2.138
No	419 (72.6)	248 (71.3)	32 (71.1)	36 (78.3)	47 (71.2)	56 (77.8)	(0.710)
Management							
Mastectomy	309 (53.6)	170 (48.9)	20 (44.4)	30 (65.2)	43 (65.2)	46 (63.9)	13.773
BCS	268 (46.4)	178 (51.1)	25 (55.6)	16 (34.8)	23 (34.8)	26 (36.1)	**(0.008)**

DCIS: Ductal carcinoma *in situ*; n: number; X^2: Chi square, *p* value in **bold**: significant
BCS: Breast Conserving Surgery
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI).

Table 4-7: Multivariate analysis for predictors of local recurrence in pure DCIS patients treated with breast conserving surgery

Outcome	HR (95% CI)	*p* value*
All local recurrence		
Tumour size	1.31 (0.36-2.55)	0.098
High tumour grade	3.43 (1.18-9.96)	**0.023**
Comedo necrosis	3.63 (1.44-7.82)	**0.013**
Margin width	0.63 (0.34-0.91)	**0.029**
Radiotherapy	0.95 (0.47-1.91)	0.890
ER status	1.09 (0.58-2.05)	0.777
HER2 status	0.89 (0.45-1.74)	0.734
Ki67 status	2.61 (0.33-5.01)	0.089
DCIS local recurrence		
Tumour size	1.01 (0.46-2.23)	0.423
High tumour grade	1.90 (0.21-4.72)	0.569
Comedo necrosis	1.24 (0.73-1.99)	0.372
Margin width	1.83 (0.15-11.96)	0.180
Radiotherapy	0.75 (0.35-1.59)	0.459
ER status	0.76 (0.29-1.92)	0.551
HER2 status	2.51 (2.11-11.45)	**0.030**
Ki67 status	2.44 (0.13-2.29)	0.067
Invasive local recurrence		
Tumour size	2.63 (1.13-6.09)	**0.024**
High tumour grade	1.15 (0.25-5.24)	0.871
Comedo necrosis	1.49 (0.814-2.76)	0.194
Margin width	0.69 (0.43-2.47)	0.578
Radiotherapy	1.17 (0.35-3.86)	0.780
ER status	1.01 (0.45-2.65)	0.833
HER2 status	0.49 (0.17-1.41)	0.184
Ki67 status	1.28 (0.41-3.88)	0.668

HR: Hazard ratio, CI: Confidence interval
ER: Oestrogen receptor, HER2: Human epidermal growth factor receptor 2.
* Bold **facing is significant.**

4.5 Discussion

DCIS is a heterogeneous disease, with molecular phenotypes seen in invasive cancer presenting in primary DCIS using IHC surrogate markers. These molecular phenotypes can be used to independently predict for both overall and, importantly, invasive recurrence (Williams et al., 2015). There is no clinically established classification of DCIS that supports the prognosis for an individual woman (Zhou et al., 2013). In invasive cancer, molecular subtyping predicts prognosis (Normanno et al., 2009, Su et al., 2011) but very little data has been published regarding DCIS (Zhou et al., 2010).

Despite the long-term follow-up, very sparse prognostic information using the intrinsic surrogate molecular subtype classification was determined. HER2 status is a known risk factor for recurrence in both invasive breast carcinoma and DCIS. Some publications have shown an increased risk of non-invasive recurrence in HER2+ tumours and have shown that the combination of HER2+ and high proliferation conferred an even higher risk of recurrence (Kerlikowske et al., 2010, Rakovitch et al., 2012). The data presented in this chapter confirms a higher risk for local recurrence in HER2+ tumours.

HER2 status can be detected by analysing the number of gene copies or measuring the amount of protein expression. The most widely used methods at present are IHC, FISH and CISH due to their propensity in evaluating HER2 in FFPE tissues in invasive carcinoma (Madrid and Lo 2004). Most HER2 studies have been performed by IHC, which is

widely accessible, easy to perform at a reasonable cost and is appropriate for initial HER2 assessment (Kakar et al., 2000). However, in cases with an IHC score of 2+, the inter-observer agreement is poor, and the predictive value is unsatisfactory for clinical use. This has led to the recommendation of additional testing measuring HER2 gene copy number status using an *in situ* hybridisation technique such as FISH or CISH, to avoid inaccurate prognostication and inappropriate treatment (Kakar et al., 2000, Thomson et al., 2001).

CISH is an alternative *in situ* hybridisation method to analyse gene amplification with a more feasible technique and assessment method (Tabarestani et al., 2015). Furthermore, several studies have demonstrated good correlation between CISH and FISH results (Gong et al., 2009, Garcia-Caballero et al., 2010).

In this study, similar to a previous study, but in invasive tumour (Madrid and Lo 2004), the concordance between 3+ IHC and CISH-amplified cases was 100%, denoting all HER2 over-expressing cases as having HER2 gene amplification. In contrast, the proportion of 2+ IHC that were HER2 amplified, as detected by CISH, was 73.5% (72/98), which is lower than previously reported (93%) (Arnould et al., 2003). The 26.5% (26/98) IHC-equivocal/HER2 non-amplified tumours in this study, all of which had 2+ IHC scores, is higher than the result obtained by other studies on invasive tumours (Tubbs et al., 2001, Zhao et al., 2002, Bartlett et al., 2008). The concordance between 0/1+ IHC and HER2 non-amplified tumours was 99.7%. A similar finding was reported by

Zhao and colleagues, who considered there to be a small undetermined percentage of amplified HER2 without protein over-expression (Zhao et al., 2002). The same study and other FISH studies on invasive carcinoma also report rare 3+ IHC tumours that are non-amplified (Tubbs et al., 2001, Zhao et al., 2002, Sapino et al., 2003, Bartlett et al., 2008); however, in this study, no such cases were detected.

The low-amplified HER2 category (≥ 4 to <6 CISH signals) was the most difficult to interpret, requiring an accurate enumeration of gene copy and the addition of more tumour cells to assess the final status. This step was particularly important because these cases could either resolve as amplified (4/7) or non-amplified (3/7). Signal clustering, more probably as a result of intra-chromosomal amplification of homogeneously staining regions, was immediately prominent in highly amplified cases and was easily assessed.

A lower HER2 expression was observed in pure DCIS compared to the previously published literature (Roses et al., 2009, Lari and Kuerer 2011). This might be related to the increasing detection of low- and intermediate-grade DCIS by screening mammography. In support of this, the HER2 positivity was higher (30%) in the earlier period of the study and declined over time to 20%.

The question of whether or not TMA cores are representative of whole tumours is frequently raised, as some tumours are heterogeneous and so a small sample of tissue may not always display the same biological

characteristics as a larger section (Malicka-Durczak et al., 2012). To increase the credibility of the TMA technique while reducing the probability of error associated with the difficulty of obtaining a representative sample, some studies use multiple cores from the same donor tissues, as it has been reported that only a few (up to four) cores are required to achieve a 100% agreement (Jourdan et al., 2003). However, even when as many as ten tissue cores are taken from a tumour, some disagreement may be noted (Camp et al., 2000, Mulrane et al., 2017). In the current study, 1mm diameter cores were used to construct the TMA of the pure DCIS tumours, increasing the probability of accurate sampling by taking large TMA cores. In cases with multiple cores, the initial HER2 status was assessed at the protein level followed by confirmation with *in situ* hybridisation. The high agreement rate between IHC and CISH results suggests that it is possible to apply TMAs to DCIS research, with the caveat that, for HER2, the CISH results may be more reliable, countering the discrepancy that can be observed in IHC for either technical or biological reasons.

The subsequent goal of this chapter was to classify DCIS tumours according to the molecular classification of breast cancer and to assess the role of surrogate molecular markers in progression of DCIS. HER2 positive DCIS was associated with predictors of local recurrence including larger tumour size, high nuclear grade, comedo type DCIS, negative hormone receptor status and high Ki67 proliferation index, which was consistent with other studies (Claus et al., 2001, DiGiovanna et al., 2002, Latta et al., 2002, Lebeau et al., 2003, Holmes et al., 2011,

Kuerer et al., 2011). In multivariate analysis, higher grade, comedo necrosis and margin status were associated with the development of local recurrence (invasive or DCIS). Similar to some studies, (Han et al., 2012, Rakovitch et al., 2012), HER2 positivity was a predictor of DCIS local recurrence; however, tumour size predicted invasive local recurrence.

DCIS patients with HER2 amplification had higher nuclear grade lesions and therefore are probably at risk of relapse more frequently than the HER2 unamplified group, but the prognostic role of HER2 over-expression in DCIS is still not fully clarified. Some studies suggest that patients with HER2-amplified DCIS are more frequently high nuclear grade and this aspect is related to an increased risk of relapse (Millis et al., 1996, Rohilla et al., 2016). In fact, some studies suggest that HER2 plays a major role in the transition from DCIS to IBC (Burkhardt et al., 2010, Williams et al., 2015), while others do not support this (Borgquist et al., 2015, Kim et al., 2016). In the current study, HER2 amplification was more frequent in the pure DCIS cohort (20%) than in the mixed DCIS (15%) despite the more prevalent higher-grade DCIS in the mixed cases. HER2 positivity alone was not predictive of recurrence as a whole or as invasive disease but was a predictor of DCIS recurrence. This result is consistent with the observation that HER2-positive DCIS tend to extend within the ductal system and even in the epidermis (Paget's disease) than in tumours associated with invasive disease. It is a personal observation that when HER2-positive DCIS is associated with early invasion, that invasive disease may show aggressive

features. Currently, HER2-positive invasive tumours are treated more aggressively regardless of their size (Joerger et al., 2011). This may suggest that HER2-positive DCIS tumour cells, although highly proliferative, are not highly invasive, whilst those that acquire invasive properties became aggressive and can metastasise.

In the present study, HER2 expression in the invasive component was seen less frequently as compared to the DCIS component adjacent to invasive carcinoma and pure DCIS, although this was of borderline statistical significance. Similarly, other studies (Park et al., 2006, Rohilla et al., 2016) have observed that HER2 positivity is often seen in patients with pure DCIS compared to those with microinvasive or invasive carcinoma. As it has been reported that patients with HER2+ invasive cancer have a poorer prognosis than those whose tumours do not express HER2 (Rom et al., 2013), the higher incidence of expression found in DCIS may therefore seem paradoxical. It was postulated that HER2 expression happens during the process of atypical hyperplasia in DCIS, and loss of HER2 expression occurs as DCIS develops into invasive disease. Another hypothesis is that HER2 overexpression in invasive carcinoma does not develop from DCIS but from the associated atypical hyperplasia (Hoque et al., 2002, Mylonas et al., 2005). Another theory explaining this phenomenon is that most invasive cancers develop from DCIS precursors, which have low expression of HER2 but are highly proliferating (Rohilla et al., 2016). Because such tumours are rapidly progressing, they are in the DCIS

stage for only a short period and would therefore be under-represented in population samples (Rohilla et al., 2016).

In summary, CISH is a promising, practical test that can be used in combination with IHC to determine HER2 status in DCIS. IHC is an easy technique to perform, moderately inexpensive, and able to detect a majority of breast cancer patients whose tumours have negative (0 or 1+) or positive (3+) HER2 status. This IHC/CISH test stratification not only confirms the status of the IHC borderline cases, but also keeps testing costs for HER2 status at a reasonable minimum. As HER2 status is not currently routinely measured in clinical practice, the aim was to show statistically significant correlations between the development of local recurrence as well as poor prognostic pathologic factors and HER2-positive DCIS, which could alert clinicians. The results might suggest considering routine assessment of HER2 in DCIS, similar to IBC.

Also confirmed in this study is the suitability of TMAs for assessment of HER2 gene amplification status in tumours arising from different patients, while maintaining a representative tissue sample in each case, with good reproducibility and credible results.

To conclude, the frequency of HER2 positivity, driven by gene amplification, in DCIS is comparable to IBC and is an independent predictor of recurrence. The results suggest the consideration of routine assessment of HER2 status for DCIS, as is commonly done for IBC.

5 Molecular Characterisation of DCIS

5.1 Introduction

5.1.1 DCIS progression to invasive carcinoma cascade

It is estimated that approximately half of all IBCs are associated with DCIS (Yeong et al., 2017, Doebar et al., 2018), and it is postulated that in a majority of these cases, the invasive component arises on top of the preceding DCIS, based on common genetic changes and shared copy number profiles (Damonte et al., 2008, Vincent-Salomon et al., 2008, Gao et al., 2009, Schnitt 2009, Johnson et al., 2012, Knudsen et al., 2012, Carraro et al., 2014, Doebar et al., 2016b). It has been further reported that numerous genomic changes shared by ADH, DCIS and IDC occur, strengthening the argument for the linear progression model (Gao et al., 2009).

However, the linear model hypothesis has been argued against as only 25% to 50% of untreated DCIS later progress to ipsilateral invasive carcinoma (Page et al., 1982, Page et al., 1995, Sanders et al., 2005). This leads to the demanding need to ask the question of when and whether DCIS will become invasive carcinoma. There is a variable percentage of IDCs associated with DCIS and that variation depends on the molecular subtype, with HER2-positive and luminal breast cancer more frequently associated with DCIS than basal-like cancers (Xu et al., 2016); however, neither molecular subtype nor histopathological characteristics are consistent or reproducible predictors of DCIS behaviour (Yeong et al., 2017). Two hypotheses have thus arisen on the progression of DCIS to IDC, that is, DCIS becomes invasive as a

result of intrinsic factors, namely genetic aberrations of the neoplastic cells or, alternatively, that extrinsic factors within the surrounding microenvironment drive DCIS progression, independent of or in conjunction with genetic changes within neoplastic cells (Cowell et al., 2013).

5.1.2 Tumour cell intrinsic factors involved in DCIS progression

Logically, significant cellular changes required for DCIS to overcome the physical barriers of the myoepithelium and BM entail *de novo* acquisition of equally profound genetic aberrations (Carraro et al., 2014). Accordingly, some research aimed to define predictive biomarkers and identify novel therapeutic targets by focusing on understanding the genetic differences between *in situ* and invasive phenotypes (Krings and Chen 2018). Recently, studies of the different compartments of DCIS and IDC have shed further light on the process of malignant progression and highlighted pathways for future research (Yeong et al., 2017). So far, only a broad concept of IBC precursors has been established today, and much remains unclear regarding the role of genomics of different components of tumour and stromal compartments in this process (Kitamura et al., 2018).

5.1.3 Molecular features of DCIS and its progression

5.1.3.1 Genomic changes

DCIS and invasive cancer are comparatively similar at the genomic level (Sonoda et al., 2013, Doebar et al., 2016b). Some studies suggested the likely existence of molecular abnormalities required for

the progression to an invasive phenotype at the premalignant stage, although it is still controversial (Petridis et al., 2016).

In contrast to that theory, other studies suggest that such abnormalities required for progression occur at any stage particularly from *in situ* to invasive stage (Hernandez et al., 2012, Heselmeyer-Haddad et al., 2012). According to this theory, distinct subclones with specific genetic changes are selected during the transition from DCIS to invasive disease. This leads to differences in the prevalence of specific mutations between neoplastic DCIS cells and their invasive counterparts (Turner and Reis-Filho 2012, Yap et al., 2012, Cowell et al., 2013).

Despite the absence of robust evidence supporting the mutation acquisition model of DCIS progression, there are some data suggesting the role of epigenetic changes such as DNA hypermethylation (Baylin et al., 2001). Aberrant hypermethylation of tumour suppressor genes takes place in both pure DCIS and the *in situ* component of DCIS to IDC, triggering carcinogenesis and perhaps also progression of pure DCIS (Pang et al., 2014). Downregulation of expression of epithelial cell-related genes also suggests that hypermethylation of gene promoters may play a role in this step of DCIS progression (Castro et al., 2008). In conclusion, these studies provide evidence of a common shared genetic susceptibility between DCIS and IDC, superimposed by a degree of alteration to DNA methylation patterns that might contribute to driving changes in gene expression that promote future progression (Doebar et al., 2018).

5.2 Tumour cell extrinsic factors involved in DCIS progression

Recent studies have investigated tumour cell extrinsic factors in the microenvironment that might drive the DCIS cells to acquire the invasive potential. DCIS, circumscribed by a dynamic layer of myoepithelium, exists within the context of a multicomponent stroma, accompanied by immune cells (Gorringe and Fox 2017) (**Figure 5-1**).

Studies have recently revealed alterations to the physical and biochemical properties of the tumour-associated matrix that promotes tumour progression and resistance to therapy. These changes disrupt the normal ECM architecture, which can adversely affect the functions of other stromal cell populations to corroborate tumour development (Shiga et al., 2015). Further studies on the earlier stages of breast cancer initiation have unravelled the importance of stromal factors in tumour evolution. Based on the radiological findings, it has previously been reported that high-density breast stroma is one of the strongest risk factors for invasive breast cancer, second to BRCA mutations (Hwang et al., 2007, Acerbi et al., 2015).

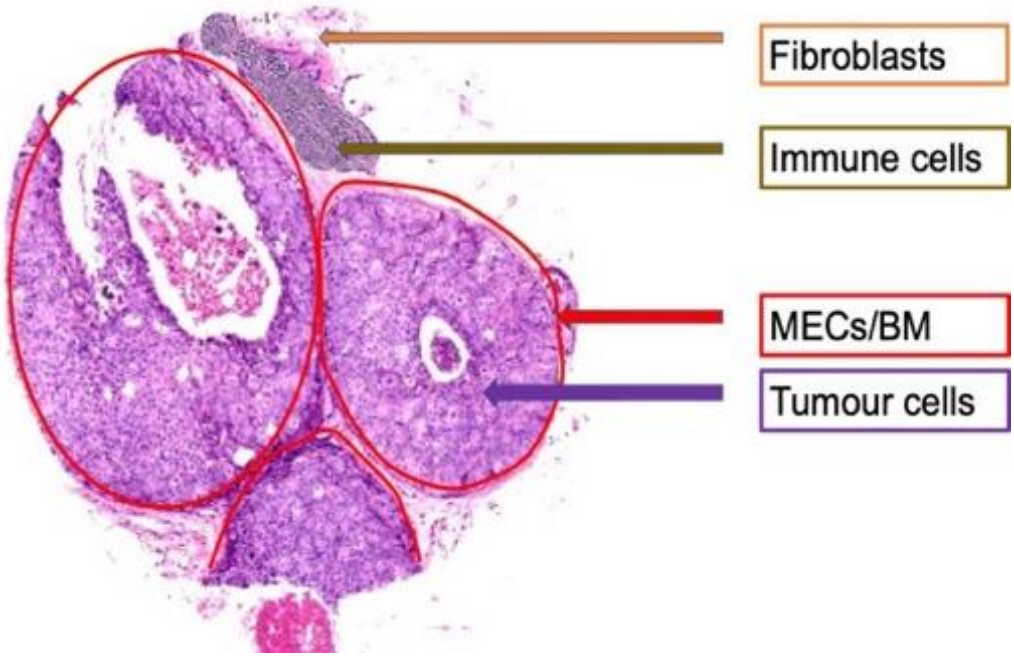


Figure 5-1: Factors contributing to the progression of ductal carcinoma in situ (DCIS) to invasive cancer. The diagram shows the different factors that are involved in the progression of DCIS to invasive breast carcinoma, including genetics of tumour cells, myoepithelial cells, BM, immune cells, fibroblasts and stroma.

5.2.1 Potential role of stromal matrix metalloproteinases (MMPs)

Although BM break is a hallmark of invasion and defines progression of malignancy, the intricacy of the underlying mechanisms remain incompletely understood (Wu et al., 2016). Using an indirect immunoperoxidase technique to detect type IV collagen, early studies established that the BM is intact in normal breast tissue, benign breast lesions and non-neoplastic mammary ducts adjacent to DCIS but shows small areas of disruption in pure DCIS (Rakha et al., 2018). Despite the importance of BM degradation during DCIS progression, little is known regarding the underlying mechanisms or identification of the triggers for MMP release by stromal cells. It is worthy to note the BM changes seen in DCIS are mediated either directly by tumour cells or indirectly through

changes to the stroma prior to or coincidental with emergence of the invasive phenotype (Rakha et al., 2018).

5.2.2 Myoepithelial cells (MECs) and DCIS progression

MECs both act as a physical barrier to the progression from *in situ* to invasive growth and have a crucial role in deterring tumour cell conversion to an invasive phenotype (Lo et al., 2017). Degradation and disruption of the BM appear to coincide with the loss of continuity of the MECs (Hsiao et al., 2011, Jiang et al., 2013), leading to the necessity to identify the key features of MEC biology, which are altered during the progression process (Pandey et al., 2010). Compared with MECs of normal tissue, DCIS MECs exhibit lower levels of gene expression associated with normal MECs' function, including thrombospondin, laminin and the oxytocin receptor, as well as higher expression of genes involved in cellular proliferation, migration, invasion and angiogenesis (Allinen et al., 2004). The effects of these changes *in vivo* have not been fully explored, and it is fundamental to define whether they represent some reactive changes to the emergent invasion or true changes that facilitate tumour cell invasion.

5.2.3 Immune cells in DCIS

Infiltration of immune cells into the stroma and tumour is one of the earliest events detected at the site of tumour progression. However, there is little known about the immune milieu of DCIS or the emergence of the anti-tumour immune response during progression to invasive carcinoma (Yeong et al., 2017). Simple H&E staining of tissue samples demonstrated increased inflammatory cell infiltration in DCIS compared

with IDC and control normal breast tissues (Sikandar et al., 2015, Miligy et al., 2017), indicating a potential link between the immune infiltrate with DCIS progression (Acerbi et al., 2015, Miligy et al., 2017).

5.2.4 Fibroblasts within the tumour microenvironment

Cancer-associated fibroblasts (CAFs) are one of the most critical components of the tumour microenvironment, modulating immune polarisation and stimulating cell growth via the production of growth factors and ECM proteins (Liao et al., 2009). Some studies have highlighted the need to understand the interplay between different cell types within the stroma in the case of CAFs, showing that the invasive potential of tumour epithelial cells can be concurrently corroborated by stromal cells, particularly fibroblasts, and inhibited by MECs (Gudjonsson et al., 2002), in an interaction leading to alterations in gene expression profiles of both cell types (Sotgia et al., 2012).

5.3 Identification of progression drivers

Although research has identified some genomic and transcriptomic changes within abnormal epithelial cells of DCIS together with altered microenvironment, so far, none of these alterations have proven to be reliable indicators of invasive progression or *in situ* recurrence (Dossus and Benusiglio 2015).

Since the genomic instability is one of the essential characteristics of cancer, it is responsible for its genetic and phenotypic heterogeneity (Schmitt et al., 2012). In the background of random aberrations due to genomic instability, there is a strong evidence for the selection of ’driver’

aberrations at the epidemiological, genetic and clinical levels (Wang et al., 2014). Conventionally, recurrent aberrations occurring at a rate higher than the background aberrations are believed to be drivers (Rane et al., 2015).

Somatic copy number alterations (SCNAs) are extremely common in cancer (Baudis 2007, Beroukhim et al., 2010). Therefore, proteins involved in tumour progression are anticipated to be highly diverse, integrated in complex interrelated networks of proteins and molecular pathways. Bearing in mind that some proteins may be key players in carcinogenesis and tumour progression makes these proteins ‘master regulatory‘ proteins. Therefore, a panel of molecular markers of their potential role in DCIS progression has been proposed and is subject to scrutiny (Rane et al., 2015).

It has been proposed that certain chromosomal abnormalities are common during progression from *in situ* to invasive disease, particularly 1q (Afghahi et al., 2015) and 20q (Kitamura et al., 2018). Among the genes located on each locus are thioredoxin interacting protein (TXNIP) located on 1q21.1 and aurora kinase A (AURKA), microtubule nucleation factor (TPX2) and non-receptor tyrosine kinase (SRC) located on 20q, which all show a role in the progression of cancer (Zou et al., 2009, Afghahi et al., 2015, Hou et al., 2016).

5.3.1 Thioredoxin interacting protein

Cancer cells are known for continuously experiencing oxidative stress, resulting from increased ROS generation (Lu and Holmgren 2012).

Enhanced oxidative stress has been implicated in the initiation and progression of cancer, promoting both cell survival and drug resistance (Yoshihara et al., 2014). However, unresolved high levels of oxidative stress beyond the capacity of the cancer cell to accommodate them causes critical damage to cellular DNA, proteins and lipids, ultimately leading to cell death (Zhou and Chng 2013). TXNIP, a negative regulator of the major anti-oxidant thioredoxin (TRX), has potent growth suppressive, metastasis inhibitory and pro-apoptotic functions (Kim et al., 2007). Loss of TXNIP expression is associated with the evolution of some solid tumours (Kim et al., 2007). In the breast, high expression of TXNIP is found in normal breast tissues (Li et al., 2017) with reduced immunointensity in invasive tumours (Butler et al., 2002, Miligy et al., 2018). Woolston and colleagues (Woolston et al., 2012) demonstrated that TXNIP is an independent predictor of outcome in a study of 98 locally advanced invasive breast cancer cases treated with anthracycline-based chemotherapy.

5.3.2 Aurora kinase A (AURKA)

Aurora kinase A (AURKA) belongs to the family of serine-threonine kinases that play an integral role in cell cycle regulation (Marumoto et al., 2005) by recruiting the cyclin B1/CDK1 complex and committing cells to mitosis (Portier et al., 2007). It has a key role in centrosome duplication and is a critical regulator of mitotic cell proliferation, particularly in mitotic entry, centrosome maturation, mitotic spindle assembly, and chromosome segregation processes (Wang et al., 2006). The AURKA gene is localised on the chromosome segment

20q13, which is commonly amplified in many human cancers (Eterno et al., 2016, Chen et al., 2017, Liao et al., 2018). Ectopic overexpression of the kinase induces chromosomal instability, centrosome anomalies, and tumourigenic transformation of human cells (Bischoff et al., 1998, Zhou et al., 1998). AURKA represents a unique proto-oncogenic mitotic kinase that is involved in the genetic pathways underlying the two most commonly observed phenotypic alterations in human cancer cells: aneuploidy and centrosome aberrations (Zhang et al., 2010). This property is critically relevant for breast cancer as a disease driven by chromosome copy number alterations (Ciriello et al., 2013).

AURKA is expressed at elevated levels in IBC (Liao et al., 2018, Lykkesfeldt et al., 2018), colorectal (Zhang et al., 2010), ovary (Gritsko et al., 2003) and gastric carcinomas (Zhou et al., 2018) and is associated with poor prognosis (Casorzo et al., 2015). It is one of the proliferation genes included within the Oncotype DX prognostic assay for DCIS (Solin et al., 2013) and IBC, which predicts the likelihood of ten-year recurrence in tamoxifen-treated, ER-positive, node-negative breast cancer patients (Paik et al., 2004, Ingoldsby et al., 2013, Ahmed et al., 2018). Moreover, AURKA is differentially expressed between normal breast epithelial cells and IBC (Moelans et al., 2011); however, the role of AURKA in DCIS has yet to be established.

5.3.3 Targeting protein for Xenopus kinesin-like protein 2 (TPX2)

TPX2, located on 20q11.21, is a prohibitin that belongs to the microtubule-associated protein family, is strictly regulated by cell cycle

and appears in the G1-S phase of the cell cycle and disappears after completion of mitosis (Cocchiola et al., 2014). It is involved in microtubule-associated proteins formed by spindle apparatus in mitosis (Zou et al., 2018) and was first described in 1997 (Heidebrecht et al., 1997). In the phase of mitosis (Neumayer et al., 2014), TPX2 is released through Ras-related nuclear protein GTPase pathway and activated by phosphorylation of AURKA; TPX2 and AURKA are mutually combined during the spindle apparatus assembly (van Gijn et al., 2018). Abnormality or structure destruction will result in errors in spindle apparatus assembly and abnormality of the chromosome will lead to tumour initiation (Yang et al., 2015). TPX2 is differentially expressed at mRNA level between breast DCIS and IBC and promotes the proliferation of breast cancer cells (Yang et al., 2015).

5.3.4 SRC non-receptor tyrosine kinase

SRC kinases are a family of non-receptor tyrosine kinases in which SRC is the prototypical member of this group of structurally related proteins expressed in mammalian cells (Frame 2002). SRC family kinases are regulatory proteins, playing key roles in cell differentiation, motility, invasion, proliferation and cell survival (Elsberger 2014). SRC is engaged in multiple cellular processes and takes a central role in cell-signalling-influencing processes, which are involved in oncogenesis (Wilson et al., 2006). Tumour progression from early disease to advanced or metastatic disease typically involves both tumour growth (cellular proliferation) and metastasis to distant sites (cellular migration) (Elsberger 2014).

Metastasis is a complex multistep process involving loss of cellular cohesion, hypermotility, intravasation, invasion into stroma, extravasation, colonisation to a distant site, and vasculogenesis (Chambers et al., 2002). Each of these processes is regulated to a great extent by SRC, suggesting that it plays a role in the advancement and metastasis of solid tumours (Browne et al., 2018). *In vitro* evidence for an SRC kinase role in breast cancer is convincing, but only supported by a few translational clinical studies (Yeatman 2004, Wilson et al., 2006).

5.4 Hypothesis

It is hypothesised that a powerful way to discover key genes playing causal roles in oncogenesis is to identify genomic regions that undergo frequent alteration in human cancers. To confirm the effect of a certain gene, detection of its protein expression is essential. Assessment of molecular markers can be used as surrogates of DCIS biology and behaviour and have the potential to predict patient outcome (Gorringe and Fox 2017).

Therefore, it was hypothesised that some genes that play a role as oncogenes (AURKA, TPX2 or SRC) or tumour suppressor genes (TXNIP) will have a role in progression from DCIS to invasive disease. This can be used as a tool to tailor treatment decisions of DCIS or have a therapeutic potential.

5.5 Patients and methods

5.5.1 Study Cohort

This retrospective study was conducted on a consecutive series of primary pure DCIS cases and a series of cases diagnosed as synchronous DCIS and invasive tumours (mixed DCIS and invasive breast cancer) as a validation set, prepared as TMA (**Chapter 2**). In addition, a set of whole tissue sections from ten cases containing DCIS and invasive tumours were assessed to evaluate heterogeneity and the pattern of TXNIP, AURKA, TPX2 and SRC expression in malignant breast lesions and adjacent stroma and normal tissue.

Patients' demographic information, histopathological parameters, management, radiotherapy and patient outcome data were collated, as previously described (**Chapter 3**).

Additionally, data on different molecular classes, p53 and Ki 67, was available for the cohort (Miligy et al., 2018). Ki67 high proliferation index was considered when more than or equal to 14% positively-stained tumour cell nuclei were detected (Ohara et al., 2016, Fulawka and Halon 2017). Aberrant p53 (clone DO7) staining was determined based on eyeballing the percentage and the staining pattern of p53 in tumour cell nuclei, which has been shown to correlate with mutation of the gene (Kobel et al., 2010, Kobel et al., 2016). Validation of the studied antibodies was performed as previously described (**Chapter 2**).

5.5.2 Analysis of the antibodies' mRNA expression in breast cancer

To confirm the prognostic and clinical significance of the targets in IBC, and given the deficiency of data on the transcriptomic profiles of DCIS, normalised gene expression (mRNA) data were analysed using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset that comprises 1980 tumours with long-term follow-up (Curtis et al., 2012). mRNA expression levels of the target biomarkers were analysed against the clinicopathological and outcome data of the METABRIC cohort.

5.6 Results

5.6.1 Specificity of the antibodies used in the study

Western blot revealed that all the antibodies used in this study showed their specific band(s) at the predicted molecular weight(s), as listed by the supplying company. AURKA and SRC had two epitopes shown as two bands (**Figure 5-2**).

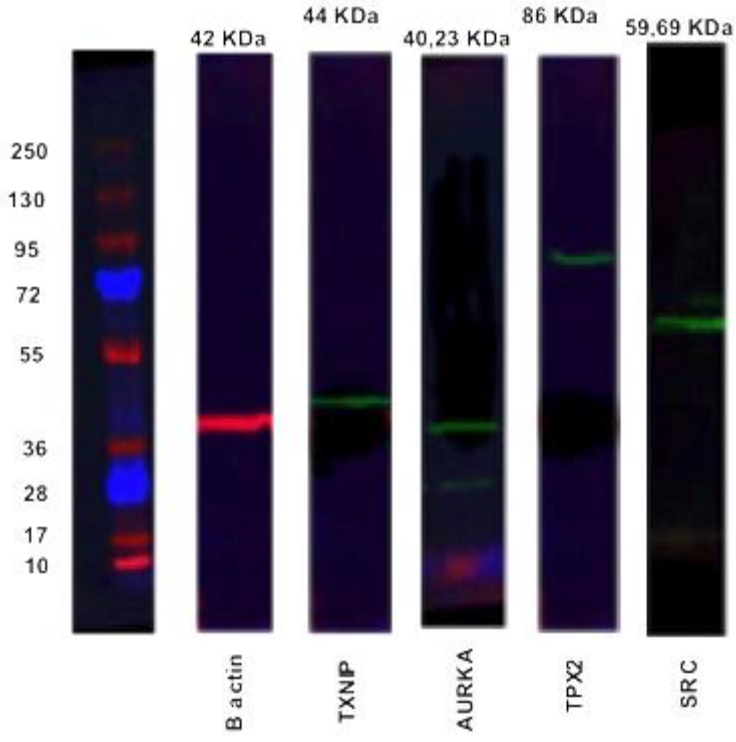


Figure 5-2: Western Blot of the antibodies used in the study.

5.6.2 Frequency and localisation pattern of the studied antibodies

Assessment of whole tissue sections of the studied biomarkers revealed a homogenous distribution pattern and that confirmed the validity of using TMAs to assess their expression. Assessment of 50% of the tissue cores was done for a second time by the author to evaluate the intra-observer concordance that was ranged between 0.88 and 0.97.

5.6.2.1 TXNIP

TXNIP showed cytoplasmic expression with occasional nuclear staining in normal luminal and myoepithelial cells. Variable staining intensities and localisation were detected in the different morphological components, with occasional strong nuclear staining in normal breast epithelial cells, strong to moderate cytoplasmic staining in DCIS (with occasional membrane accentuation), and mild to negative cytoplasmic staining in invasive tumours (with no nuclear staining). TXNIP-positive pure DCIS tumour cells showed distinct cytoplasmic staining with occasional peripheral/perimembrane accentuation and infrequent nuclear staining in some low-grade tumours (**Figure 5-3A**). After exclusion of uninformative cores (i.e. lost cores, folded tissue during processing and cores containing less than 15% tumour cells), a total of 608 pure DCIS and 231 tumours with mixed DCIS and invasive

components were included in the analysis. TXNIP expression exhibited a unimodal distribution with a median H-score of 185 (range 0–300) in the pure DCIS.

When pure DCIS was compared to DCIS mixed with IBC, the TXNIP cytoplasmic mean H-score was higher in pure *in situ* tumours compared with the DCIS component of the mixed cases ($\bar{X} \pm$SD=185 $\pm$ 76 for pure DCIS, $\bar{X} \pm$SD= 112 $\pm$ 88 for DCIS component of the mixed cases; t-test= 15.8 and p<0.0001). In the mixed cases, TXNIP cytoplasmic mean H-score was higher in the DCIS component when compared with the adjacent invasive component ($\bar{X} \pm$SD= 112 $\pm$ 88, $\bar{X} \pm$SD= 83 $\pm$ 78, respectively; Z=16.6 and p<0.0001) (**Figure 5-4A**).

5.6.2.2 AURKA and TPX2

Assessment of ten whole-tissue sections from IBC mixed with DCIS revealed nuclear expression of AURKA and TPX2. Adjacent normal breast terminal duct-lobular units showed negative or very weak cytoplasmic staining (**Figure 5-3B and Figure 5-3C**).

After unbiased exclusion of uninformative cores, a total of 632 and 631 pure DCIS (for AURKA and TPX2, respectively) and 217 tumours with mixed DCIS and invasive components were included in the analysis. AURKA and TPX2 expression showed a unimodal distribution with a median percentage of 30% AURKA and 25% TPX2 positive nuclei in pure DCIS, 48% AURKA and 30% TPX2 in the DCIS component of

mixed cases and 65% AURKA and 45% TPX2 in invasive component of the latter cohort.

Within the pure DCIS series, high AURKA expression (>30% of nuclei positive) was observed in 15% of cases and this was a statistically significantly lower proportion than in the DCIS component of the mixed cases (29%, X^2=9.8, p=0.004 with high expression) and the invasive component (55%, X^2=33.1, p<0.0001 with high expression). Similar results were observed when analysis was performed on a continuous scale (p=0.030). A statistically significant difference in AURKA expression was also observed between tumour cells of the components of the mixed DCIS/IBC cases (p<0.0001) (**Figure 5-4B**).

Regarding TPX2, within the pure DCIS series, high TPX2 expression (>25% of nuclei positive) was observed in 15% of cases and this was a statistically significantly lower proportion than in the DCIS component of the mixed cases (30%, X^2=7.2, p=0.005 with high expression) and the invasive component (45%, X^2=22.6, p<0.0001 with high expression). Similar results were observed when analysis was performed on a continuous scale (p=0.012). A statistically significant difference in TPX2 expression was also observed between tumour cells of the components of the mixed DCIS/IBC cases (p<0.0001) (**Figure 5-4C**).

5.6.2.3 SRC

Assessment of whole-tissue sections from IBC with DCIS revealed cytoplasmic expression of SRC. Adjacent normal breast terminal duct-

lobular units showed negative or very weak cytoplasmic staining of SRC (**Figure 5-3D**).

A total of 506 pure DCIS and 217 tumours with mixed DCIS and invasive components were included in the analysis. SRC expression showed a unimodal distribution with a median H score of 100 (range 0–200). Within the pure DCIS series, high SRC expression was observed in 54% of cases and this was a significantly lower than in the DCIS component of the mixed cases (H score=125, X^2=6.5, *p*=0.010 with high expression) and the invasive component (H score=165, X^2=15.5, *p*<0.0001 with high expression). Similar results were observed when analysis was performed on a continuous scale (*p*=0.033). A statistically significant difference in SRC expression was also observed between tumour cells of the components of the mixed DCIS/IBC cases (*p*<0.0001) (**Figure 5-4D**).

5.6.3 Association of the studied biomarkers and patients' clinicopathological parameters

5.6.3.1 TXNIP

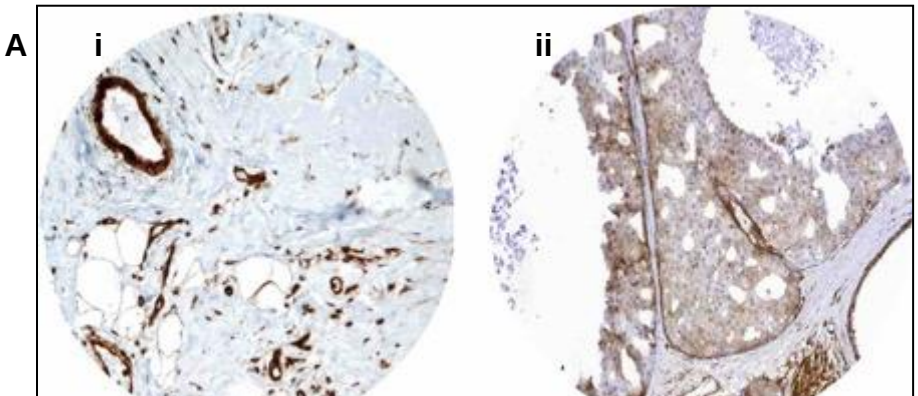
High/positive TXNIP cytoplasmic expression was significantly associated with good prognostic factors including small tumour size (*p*=0.008), low tumour grade (*p*<0.0001), no radiotherapy status (0.047), positive ER and PR status (*p*<0.0001), negative HER2 status (*p*=0.003), low Ki67 (*p*=0.001) and luminal (ER+/HER2-) phenotype (*p*<0.0001) (**Table 5-1**). Analysis of the continuous data revealed approximately consistent results (**Table 5-2**).

Analysis of TXNIP mRNA levels in IBC of the METABRIC cohort revealed significant association between high TXNIP expression and small tumour size (p<0.0001), low histological grade (p<0.0001) and with ER, PR positive and HER2 negative phenotype (p<0.0001, p=0.011 and p=0.004, respectively) and Luminal A intrinsic subtypes according to the PAM50 classification (p=0.021) (**Table 5-3)**.

5.6.3.2 AURKA, TPX2 and SRC

High expression of AURKA, TPX2 and SRC was significantly associated with poor prognostic factors such as large tumour size, high nuclear grade and negative hormone receptor expression (**Table 5-4, Table 5-7 and Table 5-10**). Analysis of the continuous data for the biomarkers' expression showed approximately similar results (**Table 5-5, Table 5-8 and Table 5-11**).

To validate the prognostic importance of AURKA, TPX2 and SRC in IBC, the METABRIC cohort was used (Curtis et al., 2012) to assess the correlation between the biomarkers' mRNA levels and clinicopathological variables and outcome. High AURKA, TPX2 and SRC mRNA level was associated with factors of poor prognosis such as young patient age, high tumour grade, negative hormone receptor expression and positive HER2 status (**Table 5-6, Table 5-9 and Table 5-12**). All statistical analyses were performed by the author.



C

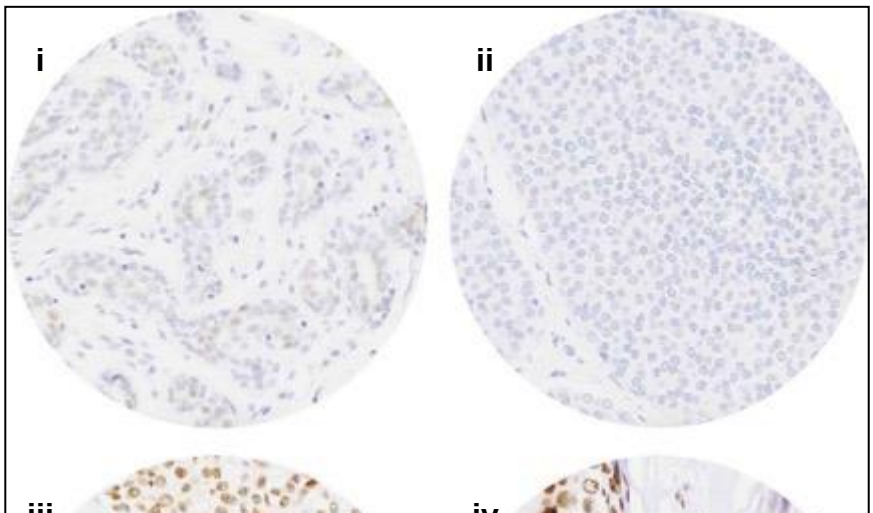


Figure 5-3: Different intensities of expression of the biomarkers used in the study; (A) TXNIP (cytoplasmic), (B) AURKA (nuclear), (C) TPX2 (nuclear) and (D) SRC (cytoplasmic), from the left to the right: normal tissue, negative to mild, moderate to strong and expression in the mixed cases. All pictures were taken using digital pathology system at 20x magnification.

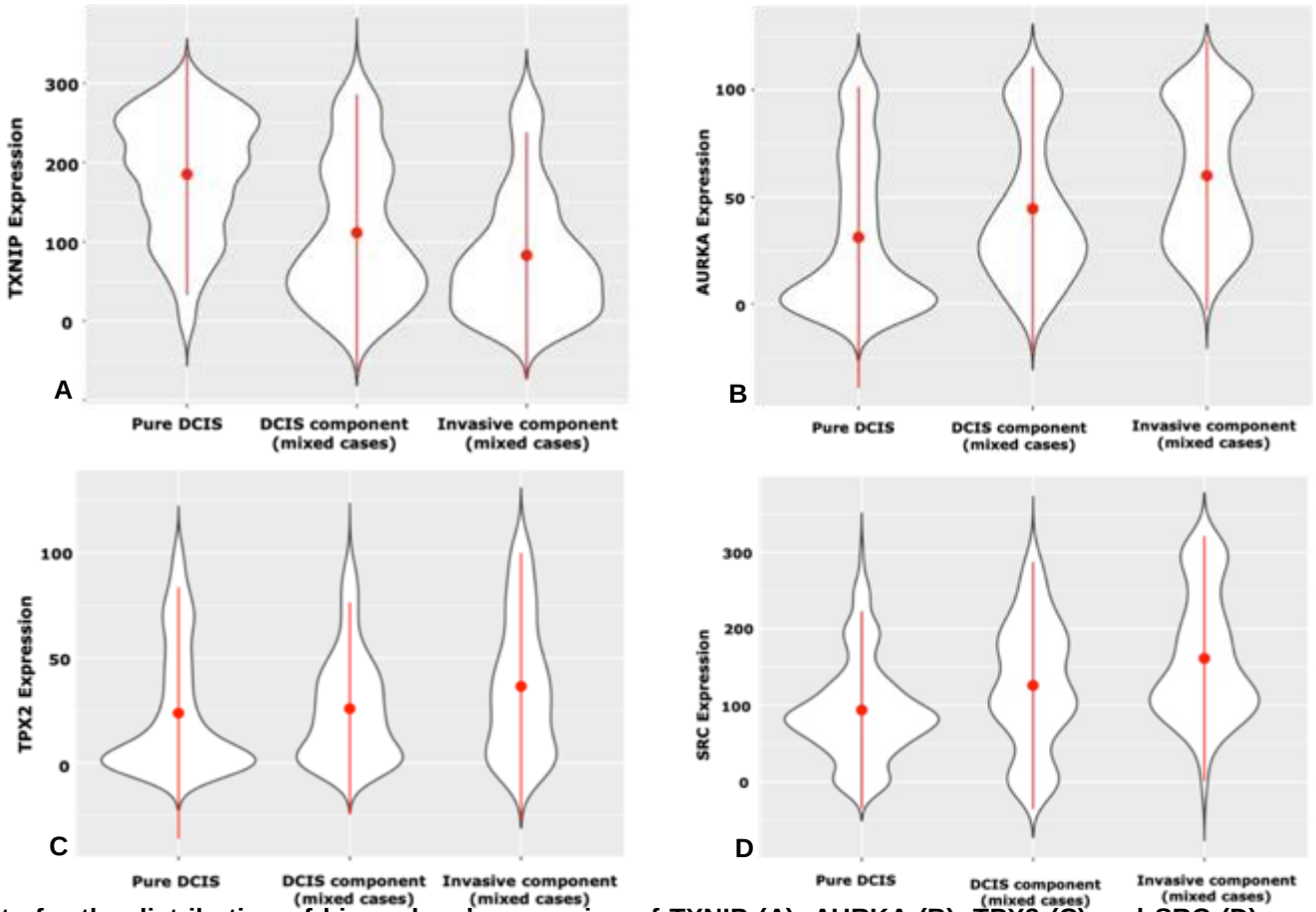


Figure 5-4: Violin plots for the distribution of biomarkers' expression of TXNIP (A), AURKA (B), TPX2 (C) and SRC (D) expression in pure DCIS, DCIS component and invasive component of the mixed cohort. The central line represents the interquartile range and the red dots refer to the median. Graphs are produced by R studio software (v 1.1.463).

Table 5-1: Correlation between TXNIP expression and the clinicopathological variables of pure DCIS cases

		TXNIP Expression		
Parameter	**Total (n=608) n (%)**	**Low (n=266) n (%)**	**High (n=342) n (%)**	**χ^2 (*p* value)**
Age (years)*				
<40	22 (3.6)	12 (4.5)	10 (2.9)	1.118
40 to 60	355 (58.4)	155 (58.3)	200 (58.5)	(0.572)
>60	231 (38.0)	99 (37.2)	132 (38.6)	
Presentation				
Screening	316 (52.0)	139 (52.3)	177 (51.8)	0.015
Symptomatic	292 (48.0)	127 (47.7)	165 (48.2)	(0.902)
Size*				
<16mm	203 (33.7)	74 (27.8)	129 (38.3)	9.781
16 to 40mm	236 (39.1)	121 (45.5)	115 (34.1)	(**0.008**)
>40mm	164 (27.2)	71 (26.7)	93 (27.6)	
Grade				
Low	85 (14)	20 (7.5)	65 (19.0)	25.194
Intermediate	156 (25.6)	58 (21.8)	98 (28.7)	**(1.6×10^{-6})**
High	367 (60.4)	188 (70.7)	179 (52.3)	
DCIS histologic type				
Single	300 (49.3)	129 (48.5)	171 (50.0)	0.135
Mixture**	308 (50.7)	137 (51.5)	171 (50.0)	(0.713)
Comedo necrosis				
Yes	124 (20.4)	56 (21.1)	68 (19.9)	0.126
No	484 (79.6)	210 (78.9)	274 (80.1)	(0.723)
Associated LCIS				
Yes	55 (9.0)	20 (7.5)	35 (10.2)	1.341
No	553 (91.0)	246 (92.5)	307 (89.8)	(0.247)
Associated Paget's				
Yes	25 (10.0)	15 (10.5)	10 (5.6)	2.808
No	295 (90.0)	128 (89.5)	167 (94.4)	(0.246)
Management+				
Mastectomy	320 (52.6)	142 (53.4)	178 (52.0)	0.107
BCS	288 (47.4)	124 (46.6)	164 (48.0)	(0.743)
Radiotherapy***				
Yes	87 (21.6)	42 (33.9)	45 (27.4)	3.930
No	201 (78.4)	82 (66.1)	119 (72.6)	**(0.047)**
ER Status				
Positive	412 (75.3)	157 (64.1)	255 (84.4)	30.150
Negative	135 (24.7)	88 (35.9)	47 (15.6)	**(3.9×10^{-8})**
PR Status				
Positive	296 (48.7)	105 (39.5)	191 (55.8)	16.058
Negative	312 (51.3)	161 (60.5)	151 (44.2)	**(6.1×10^{-5})**
HER2 Status****				
Negative	484 (80.1)	197 (74.6)	287 (84.4)	8.948
Positive	120 (19.9)	67 (25.4)	53 (15.6)	**(0.003)**
Ki67 Index				
Low (<14%)	350 (76.6)	150 (69.4)	200 (83.0)	11.651
High (≥14%)	107 (23.4)	66 (30.6)	41 (17.0)	**(0.001)**
P53 Mutation				
Inactivating (0)	105 (24.5)	53 (27.3)	52 (22.2)	3.613
Normal (>0-50%)	255 (59.6)	106 (54.6)	149 (63.7)	(0.164)
Missense (>50%)	68 (15.9)	35 (18.1)	33 (14.1)	

Molecular Classes				
Luminal A	334 (60.2)	116 (47.4)	218 (70.3)	40.356
Luminal B (HER2-)	44 (7.9)	26 (10.6)	18 (5.8)	**($3.6x10^{-8}$)**
Luminal B (HER2+)	45 (8.1)	17 (6.9)	28 (9.1)	
HER2+	63 (11.4)	41 (16.7)	22 (7.1)	
Triple negative	69 (12.4)	45 (18.4)	24 (7.7)	

DCIS: Ductal carcinoma *in situ*, N: Number, X^2: Chi square, ***p* value in bold: significant**.
TXNIP: Thioredoxin interacting protein, LCIS: Lobular carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation
***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic *in situ* hybridisation (CISH).

Table 5-2: Correlation between TXNIP expression and clinicopathological parameters in the pure DCIS cohort using continuous data

Clinicopathological Parameters	Number of cases (n=608)	TXNIP expression	
		Mean Rank	*p*-value
Age (years)*			
<40	22	280.64	0.765
40 to 60	355	303.43	
>60	231	308.42	
Presentation			
Screening	316	299.81	0.493
Symptomatic	292	309.57	
Size*			
<16mm	203	319.21	0.213
16-40mm	236	291.07	
>40mm	164	296.42	
Grade			
Low	85	398.14	**<0.0001**
Moderate	156	326.56	
High	367	273.44	
DCIS histologic type			
Single	300	307.64	0.663
Mixture**	308	301.44	
Comedo necrosis			
Yes	124	300.26	0.763
No	484	305.59	
Associated LCIS			
Yes	55	321.38	0.454
No	553	302.82	
Associated Paget's			
Yes	25	130.13	0.098
No	295	162.43	
Management[+]			
Mastectomy	320	299.07	0.421
BCS	288	310.53	
Radiotherapy***			
Yes	87	135.24	**0.049**
No	201	148.51	
ER Status			
Positive	412	296.07	**<0.0001**
Negative	135	206.63	
PR Status			
Positive	296	337.31	**<0.0001**
Negative	312	273.37	
HER2 status******			
Negative	484	311.83	**0.008**
Positive	120	264.88	
Ki67 Index			
Low (<14%)	350	241.63	**<0.0001**

High (≥14%)	107	187.70	
P53 mutation			
Inactivating (0%)	105	194.31	**0.019**
Normal (1-50%)	255	228.31	
Missense (>50%)	68	193.89	
Molecular classes			
Luminal A	334	308.84	**<0.0001**
Luminal B (HER2-)	44	236.85	
Luminal B (HER2+)	45	291.41	
HER2+	63	217.94	
Triple negative	69	201.03	

Mean Rank: Mann-Whitney or Kruskal-Wallis tests
DCIS: Ductal carcinoma *in situ*, N: Number, ***p* value in bold: significant**.
TXNIP: Thioredoxin interacting protein, LCIS: Lobular carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation, ***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic *in situ* hybridisation (CISH).

Table 5-3: Correlation between TXNIP mRNA and the clinicopathological parameters in the METABRIC series of invasive breast cancers

Clinicopathological parameters	Number of cases	Mean TXNIP mRNA level	*p*-value
Patient Age (years)			
<50	383	10.0	0.957
≥50	1556	9.9	
Tumour Size (mm)			
≤20	858	10.1	**<0.0001**
>20	1101	9.0	
Histologic Grade			
1	170	10.2	
2	770	10.0	**<0.0001**
3	952	9.8	
Lymph node metastasis			
Negative	1035	10.0	0.091
Positive	938	9.7	
Oestrogen Receptor Status			
Positive	1506	10.2	**<0.0001**
Negative	474	9.7	
Progesterone Receptor Status			**0.011**
Positive	1044	10.1	
Negative	940	9.8	
HER2 Status			
Negative	1733	10.0	**0.004**
Positive	247	9.8	
PAM50 molecular classes			
Luminal A	718	10.1	
Luminal B	488	9.7	**0.021**
Basal-like	329	9.8	
HER2 enriched	240	9.9	
Normal like	199	10.5	

Significant *p* values are represented in **bold**.
METABRIC: Molecular Taxonomy of Breast Cancer International Consortium,
TXNIP: Thioredoxin interacting protein.
PAM50: Prediction analysis of microarray 50

Table 5-4: Correlation between AURKA expression and the clinicopathological variables of pure DCIS cases

Parameter	Total (n=632) n (%)	AURKA Expression Low (n=533) n (%)	AURKA Expression High (n=99) n (%)	χ^2 (*p* value)
Age (years)*				
<40	20 (3.2)	15 (2.8)	5 (5.1)	1.606
40 to 60	370 (58.4)	311 (58.3)	59 (59.6)	(0.448)
>60	242 (38.3)	207 (38.8)	35 (35.3)	
Presentation				
Screening	330 (52.2)	285 (53.5)	45 (45.5)	2.150
Symptomatic	302 (47.8)	248 (46.5)	54 (54.5)	(0.143)
Size*				
<16mm	221 (35.2)	200 (37.8)	21 (21.4)	10.459
16 to 40mm	243 (38.8)	200 (37.8)	43 (43.9)	**(0.005)**
>40mm	163 (26.0)	129 (24.4)	34 (34.7)	
Grade				
Low	85 (13.4)	77 (14.5)	8 (8.1)	13.074
Intermediate	158 (25.0)	144 (27.0)	14 (14.1)	**(0.001)**
High	389 (61.6)	312 (58.5)	77 (77.8)	
DCIS histologic type				
Single	315 (49.8)	267 (50.1)	48 (48.5)	0.086
Mixture**	317 (50.2)	266 (49.9)	51 (51.5)	(0.769)
Comedo necrosis				
Yes	140 (22.2)	113 (21.2)	27 (27.3)	1.785
No	492 (77.8)	420 (78.8)	72 (72.7)	(0.182)
Associated LCIS				
Yes	55 (8.7)	48 (9.0)	7 (7.1)	0.393
No	577 (91.3)	485 (91.0)	92 (92.9)	(0.531)
Associated Paget's				
Yes	27 (8.2)	24 (8.6)	3 (6.3)	0.292
No	301 (91.8)	256 (91.4)	45 (93.7)	(0.589)
Management+				
Mastectomy	328 (51.9)	280 (52.5)	48 (48.5)	0.548
BCS	304 (48.1)	253 (47.5)	51 (51.5)	(0.459)
Radiotherapy***				
Yes	90 (29.6)	78 (30.8)	12 (23.5)	1.085
No	214 (70.4)	175 (69.2)	39 (76.5)	(0.297)
ER Status				
Positive	414 (75.1)	368 (81.2)	46 (46.9)	50.732
Negative	137 (24.9)	85 (18.8)	52 (53.1)	**(1.1×10^{-12})**
PR Status				
Positive	289 (45.7)	259 (48.6)	30 (30.3)	11.254
Negative	343 (54.3)	274 (51.4)	69 (69.7)	**(0.001)**
HER2 Status****				
Negative	503 (80.2)	448 (84.7)	55 (56.1)	42.525
Positive	124 (19.8)	81 (15.3)	43 (43.9)	**(6.9×10^{-11})**
Ki67 Index				
Low (<14%)	350 (77.1)	294 (80.5)	56 (62.9)	12.589
High (≥14%)	104 (22.9)	71 (19.5)	33 (37.1)	**(3.8×10^{-4})**

p53 Mutation				
Inactivating (0%)	108 (25.2)	91 (26.1)	17 (21.0)	11.227
Normal (>0-50%)	252 (58.7)	211 (60.7)	41 (50.6)	**(0.004)**
Missense (>50%)	69 (16.1)	46 (13.2)	23 (28.4)	
Molecular Classes				
Luminal A	338 (60.6)	310 (67.4)	28 (28.6)	73.064
Luminal B (HER2-)	43 (7.7)	35 (7.6)	8 (8.2)	**($5.1x10^{-15}$)**
Luminal B (HER2+)	44 (7.9)	33 (7.2)	11 (11.2)	
HER2+	64 (11.5)	32 (7.0)	32 (32.6)	
Triple negative	69 (12.3)	50 (10.8)	19 (19.4)	

DCIS: Ductal carcinoma *in situ*, N: Number, X^2: Chi square, ***p* value in bold: significant**.
AURKA: Aurora kinase A, LCIS: Lobular carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation
***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic in situ hybridisation (CISH).

Table 5-5: Correlation between AURKA expression as a continuous parameter (% of positive nuclei) and clinicopathological parameters in pure DCIS

Clinicopathological Parameters	Number of cases (N=632)	AURKA expression	
		Mean Rank	*p*-value
Age (years)*			
<40	20	370.60	0.364
40 to 60	370	313.97	
>60	242	315.90	
Presentation			
Screening	330	298.78	0.070
Symptomatic	302	335.86	
Size*			
<16mm	221	282.68	**0.002**
16-40mm	243	337.49	
>40mm	163	321.45	
Grade			
Low	85	307.24	**0.001**
Moderate	158	302.69	
High	389	324.13	
DCIS histologic types			
Single	315	319.86	0.628
Mixture**	317	313.16	
Comedo necrosis			
Yes	140	338.21	0.094
No	492	310.32	
Associated LCIS			
Yes	55	333.82	0.439
No	577	314.85	
Associated Paget's			
Yes	27	131.85	0.051
No	301	167.43	
Management+			
Mastectomy	328	321.30	0.471
BCS	304	311.32	
Radiotherapy***			
Yes	90	314.55	0.907
No	214	316.83	
ER Status			
Positive	414	258.75	**<0.0001**
Negative	137	328.13	
PR Status			
Positive	289	318.51	**0.042**
Negative	343	314.81	
HER2 status******			
Negative	503	296.38	**<0.0001**
Positive	124	385.48	

Ki67 Index			
Low (<14%)	350	221.47	**0.049**
High (≥14%)	104	247.79	
P53 mutation			
Inactivating (0)	108	191.45	**0.041**
Normal (1-50%)	252	221.61	
Missense (>50%)	69	227.72	
Molecular classes			
Luminal A	338	251.06	**<0.0001**
Luminal B (HER2-)	43	295.09	
Luminal B (HER2+)	44	316.08	
HER2+	64	372.37	
Triple negative	69	299.63	

Mean Rank: Mann-Whitney or Kruskal-Wallis tests
DCIS: Ductal carcinoma *in situ*, N: Number, X^2: Chi square, ***p* value in bold: significant**.
AURKA: Aurora kinase A, LCIS: Lobular carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation
***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic in situ hybridisation (CISH).

Table 5-6: Correlation between AURKA mRNA level and the clinicopathological parameters in the METABRIC series of invasive breast cancers

Clinicopathological parameters	Number of cases	Mean AURKA mRNA level	*p*-value
Patient Age (years)			
<50	421	7.7	**0.001**
≥50	1547	7.5	
Tumour Size (mm)			
≤20	855	7.4	0.740
>20	1093	7.6	
Histologic Grade			
1	170	8.2	
2	770	8.2	**0.034**
3	952	8.3	
Lymph node metastasis			
Negative	1028	7.4	0.073
Positive	933	7.6	
Oestrogen Receptor Status			
Positive	1496	7.3	**<0.0001**
Negative	472	8.1	
Progesterone Receptor Status			**<0.0001**
Positive	1033	7.2	
Negative	935	7.8	
HER2 Status			
Negative	1724	7.4	**<0.0001**
Positive	244	8.0	
PAM50 molecular classes			
Luminal A	712	6.9	
Luminal B	486	7.9	**<0.0001**
Basal-like	329	8.0	
HER2 enriched	239	8.2	
Normal like	201	6.8	

Significant p values are in bold
METABRIC; Molecular Taxonomy of Breast Cancer International Consortium,
AURKA: Aurora Kinase A
PAM50: Prediction analysis of microarray 50

Table 5-7: Correlation between TPX2 expression and the clinicopathological variables of pure DCIS cases

		TPX2 Expression		
Parameter	**Total (n=631) n (%)**	**Low (n=320) n (%)**	**High (n=311) n (%)**	**χ^2 (*p* value)**
Age (years)*				
<40	20 (3.2)	11 (3.4)	9 (2.9)	1.450
40 to 60	368 (58.3)	193 (60.3)	175 (56.3)	(0.484)
>60	243 (38.5)	116 (36.3)	127 (40.8)	
Presentation				
Screening	331 (52.5)	165 (51.6)	166 (53.4)	0.208
Symptomatic	300 (47.5)	155 (48.4)	145 (46.6)	(0.648)
Size*				
<16mm	222 (35.5)	132 (41.6)	90 (29.1)	15.765
16 to 40mm	240 (38.3)	121 (38.2)	119 (38.5)	**(3.7×10^{-4})**
>40mm	164 (26.2)	64 (20.2)	100 (32.4)	
Grade				
Low	85 (13.5)	50 (15.6)	35 (11.3)	4.878
Intermediate	158 (25.0)	81 (25.3)	77 (24.7)	**(0.030)**
High	388 (61.5)	189 (59.1)	199 (64.0)	
DCIS histologic type				
Single	315 (49.9)	161 (51.4)	154 (48.4)	0.572
Mixture**	316 (50.1)	152 (48.6)	164 (51.6)	(0.450)
Comedo necrosis				
Yes	140 (22.2)	78 (24.4)	62 (19.9)	1.800
No	491 (77.8)	242 (75.6)	249 (80.1)	(0.180)
Associated LCIS				
Yes	55 (8.7)	32 (10.0)	23 (7.4)	1.345
No	576 (91.3)	288 (90.0)	288 (92.6)	(0.246)
Associated Paget's				
Yes	27 (8.3)	14 (9.6)	13 (7.3)	2.808
No	299 (91.7)	132 (90.4)	166 (92.7)	(0.246)
Management+				
Mastectomy	326 (51.7)	151 (47.2)	175 (56.3)	5.210
BCS	305 (48.3)	169 (52.8)	136 (43.7)	**(0.022)**
Radiotherapy***				
Yes	91 (29.8)	39 (23.4)	52 (37.7)	7.410
No	214 (70.2)	128 (76.6)	86 (62.3)	**(0.006)**
ER Status				
Positive	413 (75.1)	216 (76.9)	197 (73.2)	2.980
Negative	137 (24.9)	65 (23.1)	72 (26.8)	(0.060)
PR Status				
Positive	291 (53.9)	157 (49.1)	134 (43.1)	2.266
Negative	340 (46.1)	163 (50.9)	177 (56.9)	**(0.013)**
HER2 Status****				
Negative	503 (80.4)	258 (81.1)	245 (79.5)	0.249
Positive	123 (19.6)	60 (18.9)	63 (20.5)	(0.617)
Ki67 Index				
Low (<14%)	349 (77.0)	175 (76.1)	174 (78.0)	0.241
High (≥14%)	104 (23.0)	55 (23.9)	49 (22.0)	(0.624)
P53 mutation				
Inactivating (0)	109 (25.5)	46 (21.5)	63 (29.4)	4.149
Normal (>0-50%)	254 (59.3)	131 (61.2)	123 (57.5)	(0.126)
Missense (>50%)	65 (15.2)	28 (17.3)	37 (13.1)	

Molecular Classes				
Luminal A	338 (60.7)	174 (51.5)	164 (59.9)	1.020
Luminal B (HER2-)	43 (7.7)	19 (6.7)	24 (8.7)	(0.907)
Luminal B (HER2+)	43 (7.7)	22 (7.8)	21 (7.7)	
HER2+	64 (11.5)	34 (12.0)	30 (10.9)	
Triple negative	69 (12.4)	34 (12.0)	35 (12.8)	

DCIS: Ductal carcinoma *in situ*, N: Number, X^2: Chi square, ***p*** **value in bold: significant**.
TPX2: Targeting protein for Xenopus kinesin-like protein 2, LCIS: Lobular carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation
***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic in situ hybridisation (CISH).

Table 5-8: Correlation between TPX2 expression and clinicopathological parameters in the pure DCIS cohort using continuous data

Clinicopathological Parameters	Number of cases (N=631)	TPX2 expression	
		Mean Rank	*p*-value
Age (years)*			
<40	20	320.68	0.637
40 to 60	368	321.46	
>60	243	307.34	
Presentation			
Screening	331	310.83	0.452
Symptomatic	300	321.70	
Size*			
<16mm	222	279.77	**0.004**
16-40mm	240	311.44	
>40mm	164	340.65	
Grade			
Low	85	305.50	**0.001**
Moderate	158	321.29	
High	388	354.11	
DCIS histologic types			
Single	315	313.47	0.727
Mixture**	316	318.52	
Comedo necrosis			
Yes	140	325.55	0.480
No	491	313.28	
Associated LCIS			
Yes	55	327.83	0.613
No	576	314.87	
Associated Paget's			
Yes	27	149.22	0.424
No	299	164.25	
Management[+]			
Mastectomy	326	334.33	**0.014**
BCS	305	298.85	
Radiotherapy***			
Yes	91	131.34	**0.005**
No	214	162.21	
ER Status			
Positive	413	259.53	**0.045**
Negative	137	280.80	
PR Status			
Positive	291	296.33	**0.003**
Negative	340	338.99	
HER2 status****			
Negative	503	314.88	0.697
Positive	123	307.84	
K67 Index			
Low (<14%)	349	225.93	0.748

High (≥14%)	104	230.60	
P53 expression			
Inactivating (0%)	109	197.81	0.126
Normal (1-50%)	254	217.87	
Missense (>50%)	65	229.32	
Molecular classes			
Luminal A	338	282.28	0.739
Luminal B (HER2-)	43	275.07	
Luminal B (HER2+)	43	295.65	
HER2+	64	277.23	
Triple negative	69	256.66	

Mean Rank: Mann-Whitney or Kruskal-Wallis tests
DCIS: Ductal carcinoma *in situ*, N: Number, X^2: Chi square, ***p* value in bold: significant**.
TPX2: Targeting protein for Xenopus kinesin-like protein 2, LCIS: Lobular carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation
***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic in situ hybridisation (CISH).

Table 5-9: Correlation between TPX2 mRNA level and the clinicopathological parameters in the METABRIC series of invasive breast cancers

Clinicopathological parameters	Number of cases	Mean TPX2 mRNA level	*p*-value
Patient Age (years)			
<50	383	7.1	**<0.0001**
≥50	1556	6.8	
Tumour Size (mm)			
≤20	858	6.7	**<0.0001**
>20	1101	6.9	
Histologic Grade			
1	170	6.2	
2	770	6.6	**<0.0001**
3	952	7.3	
Lymph node metastasis			
Negative	1035	6.8	0.089
Positive	938	6.9	
Oestrogen Receptor Status			
Positive	1506	6.7	**<0.0001**
Negative	474	7.4	
Progesterone Receptor Status			**<0.0001**
Positive	1040	6.7	
Negative	940	7.1	
HER2 Status			
Negative	1733	6.8	**<0.0001**
Positive	247	7.1	
PAM50 molecular classes			
Luminal A	718	6.4	
Luminal B	488	7.2	**<0.0001**
Basal-like	329	7.7	
HER2 enriched	240	7.2	
Normal like	199	6.2	

Significant p values are in bold

METABRIC; Molecular Taxonomy of Breast Cancer International Consortium, TPX2: Targeting protein for Xenopus kinesin-like protein 2.

PAM50: Prediction analysis of microarray 50.

Table 5-10: Correlation between SRC expression and the clinicopathological variables of pure DCIS

	SRC Expression			
Parameter	**Total (n=506) n (%)**	**Low (n=234) n (%)**	**High (n=272) n (%)**	**χ^2 (*p* value)**
Age (years)*				
<40	20 (4.0)	7 (3.0)	13 (4.8)	1.128
40 to 60	293 (57.9)	139 (59.4)	154 (56.6)	(0.544)
>60	193 (38.1)	88 (37.6)	105 (38.6)	
Presentation				
Screening	259 (51.2)	127 (54.3)	132 (48.5)	1.661
Symptomatic	247 (48.8)	107 (45.7)	140 (51.5)	(0.197)
Size*				
<16mm	165 (32.8)	84 (35.9)	81 (30.1)	3.290
16 to 40mm	193 (38.4)	91 (38.9)	102 (37.9)	(0.193)
>40mm	145 (28.8)	59 (25.2)	86 (32.0)	
Grade				
Low	72 (14.2)	45 (19.2)	27 (9.9)	24.643
Intermediate	129 (25.5)	75 (32.1)	54 (19.9)	**(4×10^{-6})**
High	305 (60.3)	114 (48.7)	191 (70.2)	
DCIS histologic type				
Single	246 (48.6)	110 (47.0)	136 (50.0)	0.451
Mixture**	260 (51.4)	124 (53.0)	136 (50.0)	(0.502)
Comedo necrosis				
Yes	104 (20.6)	35 (15.0)	69 (25.4)	8.349
No	402 (79.4)	199 (85.0)	203 (74.6)	**(0.004)**
Associated LCIS				
Yes	46 (9.1)	28 (12.0)	18 (6.6)	4.353
No	460 (90.9)	206 (88.0)	254 (93.4)	(0.051)
Associated Paget's				
Yes	17 (6.8)	3 (2.7)	14 (9.7)	5.108
No	249 (93.2)	109 (97.3)	140 (90.3)	(0.078)
Management+				
Mastectomy	266 (52.6)	111 (47.4)	155 (57.0)	4.600
BCS	240 (47.4)	123 (52.6)	117 (43.0)	**(0.032)**
Radiotherapy				
Yes	76 (31.7)	40 (32.5)	36 (30.8)	0.085
No	164 (68.3)	83 (67.5)	81 (69.2)	(0.771)
ER Status				
Positive	369 (75.5)	193 (84.6)	176 (67.4)	19.478
Negative	120 (24.5)	35 (15.4)	85 (32.6)	**(1.1×10^{-5})**
PR Status				
Positive	271 (53.6)	147 (62.8)	124 (45.6)	15.017
Negative	235 (46.4)	87 (37.2)	148 (54.4)	**(0.0001)**
HER2 Status***				
Negative	395 (78.5)	199 (85.8)	196 (72.3)	13.413
Positive	108 (21.5)	33 (14.2)	75 (27.7)	**(0.0002)**
Ki67 Index				
Low (<14%)	328 (77.4)	146 (77.7)	182 (77.1)	0.017
High (≥14%)	96 (22.6)	42 (22.3)	54 (22.9)	(0.895)
P53 mutation				
Inactivating (0%)	100 (24.9)	27 (14.7)	73 (33.5)	20.369
Normal (1-50%)	242 (60.2)	130 (70.7)	112 (51.4)	**(3.8×10^{-5})**
Missense (>50%)	60 (14.9)	27 (14.7)	33 (15.1)	

Molecular Classification	289 (59.0)	152 (66.9)	137 (52.1)	23.850
Luminal A	42 (8.6)	24 (10.6)	18 (6.9)	**(8.6×10^{-5})**
Luminal B (HER2-)	41 (8.4)	17 (7.5)	24 (9.1)	
Luminal B (HER2+)	63 (12.8)	14 (6.2)	49 (18.6)	
HER2+	55 (11.2)	20 (8.8)	35 (13.3)	
Triple negative				

DCIS: Ductal carcinoma *in situ*, N: Number, X^2: Chi square, ***p* value in bold: significant**.
SRC: non-receptor tyrosine kinase, LCIS: Lobular carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation
***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic in situ hybridisation (CISH).

Table 5-11: Correlation between SRC expression and clinicopathological parameters in the pure DCIS cohort using continuous data

Clinicopathological Parameters	Number of cases (N=506)	SRC expression	
		Mean Rank	*p*-value
Age (years)*			
<40	20	225.05	0.544
40 to 60	293	356.52	
>60	193	251.86	
Presentation			
Screening	259	246.29	0.276
Symptomatic	247	260.38	
Size*			
<16mm	165	263.04	0.194
16-40mm	193	253.58	
>40mm	145	237.33	
Grade			
Low	72	231.06	**<0.0001**
Moderate	129	383.59	
High	305	294.63	
DCIS histologic types**			
Single	246	258.80	0.400
Mixture	260	247.90	
Comedo necrosis			
Yes	104	267.44	**<0.0001**
No	402	199.61	
Associated LCIS			
Yes	46	248.21	0.110
No	460	306.37	
Associated Paget's			
Yes	17	137.99	0.251
No	249	116.66	
Management[+]**			
Mastectomy	266	217.66	0.008
BCS	240	237.12	
Radiotherapy***			
Yes	76	251.50	0.466
No	164	264.64	
ER Status			
Positive	369	187.45	**<0.0001**
Negative	120	263.72	
PR Status			
Positive	271	219.43	**<0.0001**
Negative	235	283.04	
HER2 status******			
Negative	395	201.21	**<0.0001**
Positive	108	265.89	

Ki67 Index			
Low (<14%)	328	213.14	0.843
High (≥14%)	96	210.33	
P53 mutation			
Inactivating (0%)	100	148.56	**<0.0001**
Normal (1-50%)	242	193.23	
Missense (>50%)	60	225.43	
Molecular classes			
Luminal A	289	186.44	**<0.0001**
Luminal B (HER2-)	42	221.09	
Luminal B (HER2+)	41	233.59	
HER2+	63	272.00	
Triple negative	55	260.86	

Mean Rank: Mann-Whitney or Kruskal-Wallis tests
DCIS: Ductal carcinoma *in situ*; n: number
***p* value in bold: significant**, SRC: non-receptor tyrosine kinase, LCIS: Lobular Carcinoma *in situ*, BCS: Breast Conserving Surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation, ***Radiotherapy status is for cases treated with BCS, ****HER2 final status is achieved using combination of IHC and chromogenic *in situ* hybridisation (CISH).

Table 5-12: Correlation between SRC mRNA level and the clinicopathological parameters in the METABRIC series of invasive breast cancers

Clinicopathological parameters	Number of cases	Mean SRC mRNA level	*p*-value
Patient Age (years)			
<50	421	7.7	**0.001**
≥50	1547	7.5	
Tumour Size (mm)			
≤20	855	7.4	0.740
>20	1093	7.6	
Histologic Grade			
1	170	8.2	
2	770	8.2	**0.034**
3	952	8.3	
Lymph node metastasis			
Negative	1028	7.4	0.073
Positive	933	7.6	
Oestrogen Receptor Status			
Positive	1496	7.3	**<0.0001**
Negative	472	8.1	
Progesterone Receptor Status			**<0.0001**
Positive	1033	7.2	
Negative	935	7.8	
HER2 Status			
Negative	1724	7.4	**<0.0001**
Positive	244	8.0	
PAM50 molecular classes			
Luminal A	712	6.9	
Luminal B	486	7.9	**<0.0001**
Basal-like	329	8.0	
HER2 enriched	239	8.2	
Normal like	201	6.8	

Significant p values are in bold

METABRIC; Molecular Taxonomy of Breast Cancer International Consortium, SRC: non-receptor tyrosine kinase

PAM50: Prediction analysis of microarray 50

5.6.4 Association of the studied biomarkers and patient outcome

5.6.4.1 TXNIP

In IBC, positive/high *TXNIP* mRNA level was associated with better outcome in terms of longer breast cancer specific survival (p=0.002) (**Figure 5-5**). These findings confirmed the tumour suppressor function of TXNIP in invasive breast cancer and its potential prognostic value and supported investigating its role in DCIS.

Univariate analysis in pure DCIS showed that patients with positive TXNIP had a significantly longer local recurrence free survival (LRFS) (log rank (LR)=7.99, 95%CI:0.34-0.83 and p=0.009). When the analysis was confined to patients who underwent BCS, to avoid the biased impact of surgical management on outcome prediction, TXNIP expression retained its significant association with improved outcome (LR=6.67, 95%CI:0.39-0.88 and p=0.001). It was also associated with improved outcome in the group of patients treated with BCS without post-operative radiotherapy (p=0.02) (**Figure 5-6**). No significant association was detected between TXNIP expression and the type of local recurrence.

The multivariate Cox regression model showed that high TXNIP expression was an independent predictor of good outcome in pure DCIS (p<0.0001, HR 0.51, 95% CI 0.32-0.81) (**Table 5-13** and **Figure 5-7**).

5.6.4.2 AURKA, TPX2 and SRC

In IBC, high AURKA, TPX2 and SRC mRNA was associated with worse outcome in terms of shorter breast-cancer-specific survival (HR=1.4, 95% CI=1.1-1.6, p<0.0001, HR=1.9, 95% CI=1.3-1.9, p<0.0001 and HR=1.4, 95% CI=1.2-1.8, p<0.0001, respectively) (**Figure 5-8A, 5-8B and 5-8C)**.

In univariate analysis of the pure DCIS cohort (**Figure 5-9, 5-10 and 5-11**), patients with high AURKA, TPX2 and SRC expression had a significantly shorter LRFS (all recurrences either as *in situ* or invasive disease) in pure DCIS (HR=2.9, 95% CI=1.7-4.9 and p<0.0001, HR=2.9, 95% CI=1.7-4.9 and p=0.026 and HR=2.2, 95% CI=1.6-3.9 and p=0.007, respectively). Association with a shorter local recurrence-free interval was observed in patients treated with BCS without postoperative radiotherapy (HR=2.7, 95% CI=1.6-4.6 and p<0.0001, HR=1.8, 95% CI=1.1-3.2 and p=0.01 and HR=1.5, 95% CI=1.1-2.9 and p=0.001, respectively). However, the significant association with poor outcome was not maintained in patients treated with either mastectomy or BCS followed by radiotherapy. High AURKA, TPX2 and SRC expression was associated with recurrence as invasive disease (HR=2.9, 95% CI=1.5-5.8 and p=0.002, HR=1.9, 95% CI=1.5-4.8 and p=0.040 and HR=1.4, 95% CI=1.1-3.8 and p=0.017, respectively), particularly in patients treated with BCS without adjuvant radiotherapy (HR=2.7, 95% CI=1.4-5.2, p=0.002, HR=1.3, 95% CI=1.1-3.2, p=0.045 and HR=1.3, 95% CI=1.1-3.1, p=0.002, respectively).

Multivariate survival analysis showed that high AURKA, TPX2 and SRC expression was a poor prognostic factor for local recurrence in patients treated with BCS independent of other factors associated with an increased risk of local recurrence including patient age, tumour size, tumour grade, comedo necrosis, margin status, molecular classes and radiotherapy, either for all recurrences (*in situ* or invasive) (HR=6.9, 95% CI=2.7-7.0, $p<0.0001$, HR=1.8, 95% CI=1.1-3.1, $p=0.024$ and HR=5.5, 95% CI=1.7-6.9, $p=0.009$, respectively) or when restricting the analysis to invasive recurrence (HR=3.9, 95% CI=1.7-7.1, $p=0.001$, HR=2.1, 95% CI=1.4-4.8, $p=0.049$ and HR=2.9, 95% CI=1.7-5.4, $p=0.021$, respectively) (**Table 5-14, 5-15 and 5-16** and **Figure 5-12, 5-13 and 5-14**).

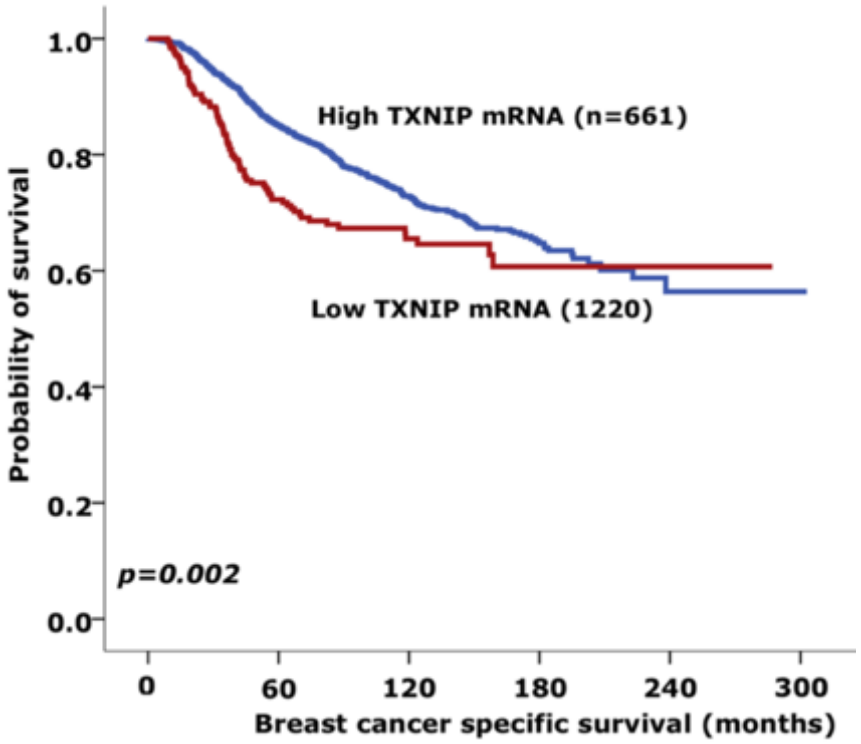


Figure 5-5: Association between TXNIP mRNA level and breast-cancer-specific survival in invasive breast cancer of the METABRIC series (n=1980). The cohort was dichotomised into high and low based on the median level of TXNIP mRNA (6.7).

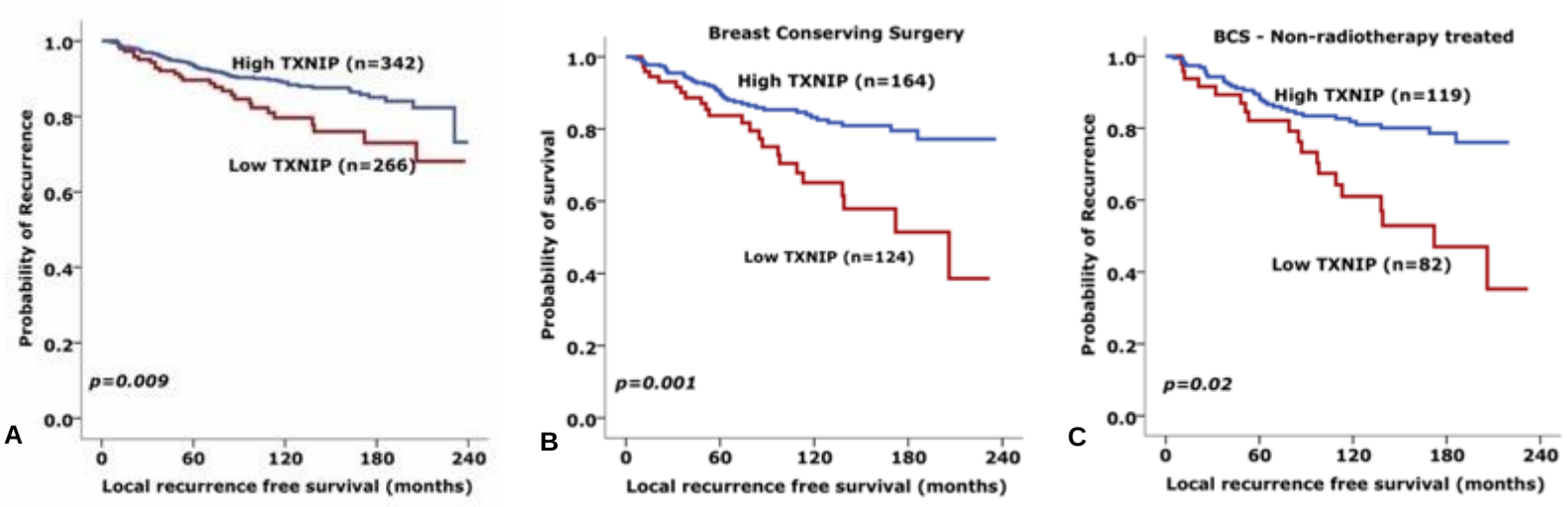


Figure 5-6: Kaplan-Meier curves show that high TXNIP expression is associated with: (A) longer LRFS in the whole pure DCIS series (p=0.009); (B) improved survival in patients treated with BCS (p=0.001); and (C) improved outcome in non-radiotherapy treated patients (p=0.02). Dichotomisation of the cohort was baed on the median TXNIP H score of 185.

Table 5-13: Multivariate Cox proportional hazards including TXNIP for predictors of local recurrence in pure DCIS patients

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
TXNIP expression	0.5	0.3	0.8	**<0.0001**
Patient Age	0.6	0.2	1.1	0.542
DCIS size	1.5	2.1	4.3	**<0.0001**
DCIS nuclear Grade	1.8	1.2	2.7	**<0.0001**
Comedo necrosis	0.9	0.5	1.7	0.911
Molecular classes	0.9	0.5	1.2	0.342
Radiotherapy	0.4	0.2	0.9	**0.040**
Margin status	0.8	0.4	1.3	0.752

HR: Hazard ratio, CI: Confidence interval, DCIS: Ductal carcinoma *in situ*, TXNIP: Thioredoxin interacting protein
Significant *p* values are in **bold**

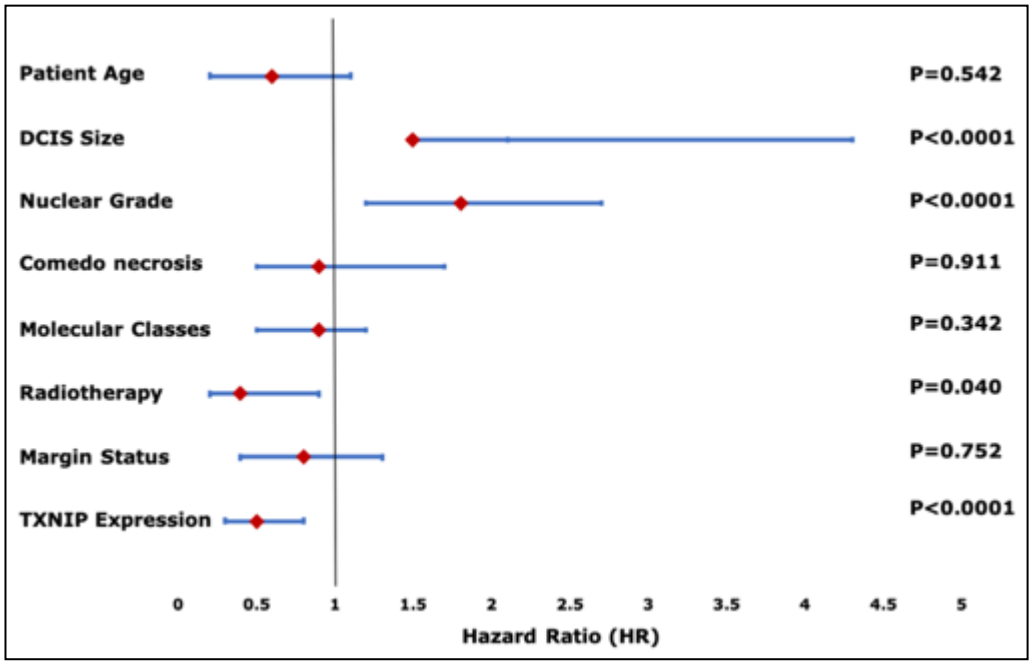


Figure 5-7: Forest plots (blobbogram) showing the hazard ratio of the different clinicopathological parameters and TXNIP, and ipsilateral tumour recurrence for patients treated with breast conserving surgery in pure DCIS cohort based on the multivariate analysis results for all recurrences, whether DCIS or invasive disease.

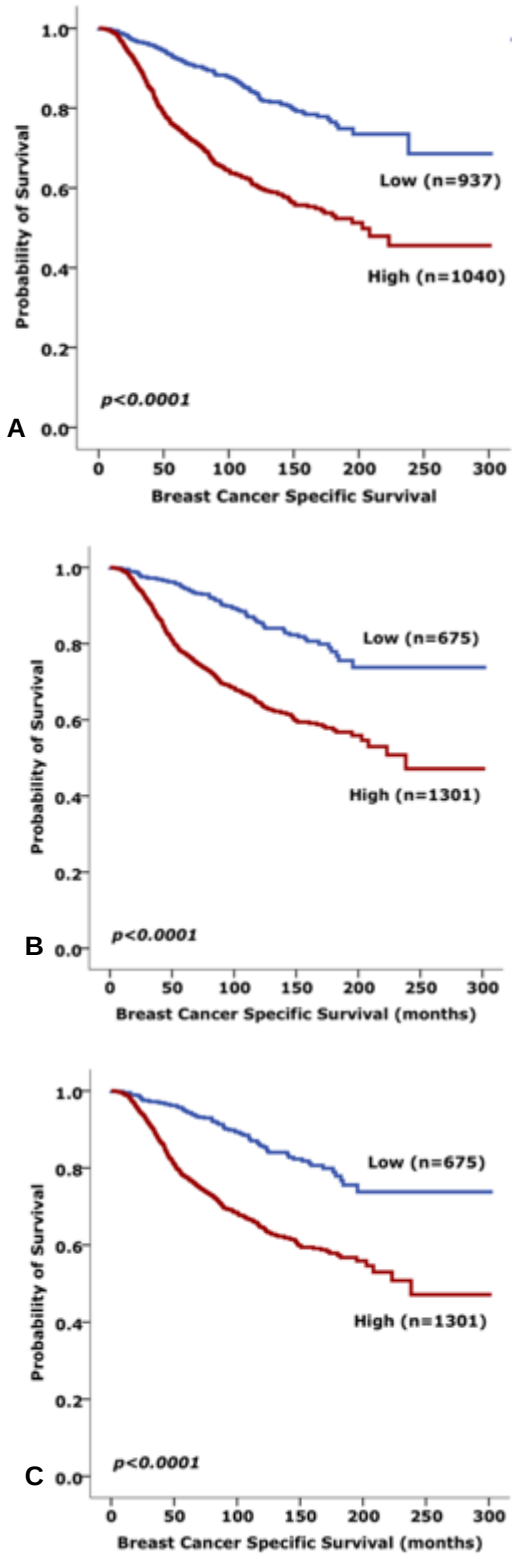


Figure 5-8: Association between AURKA (A), TPX2 (B) and SRC (C) mRNA level and outcome in terms of breast-cancer-specific survival (BCSS) in invasive breast cancer of the METABRIC series. The cohort was split into high and low mRNA expression based on the median (7.35, 6.49 and 8.59, respectively).

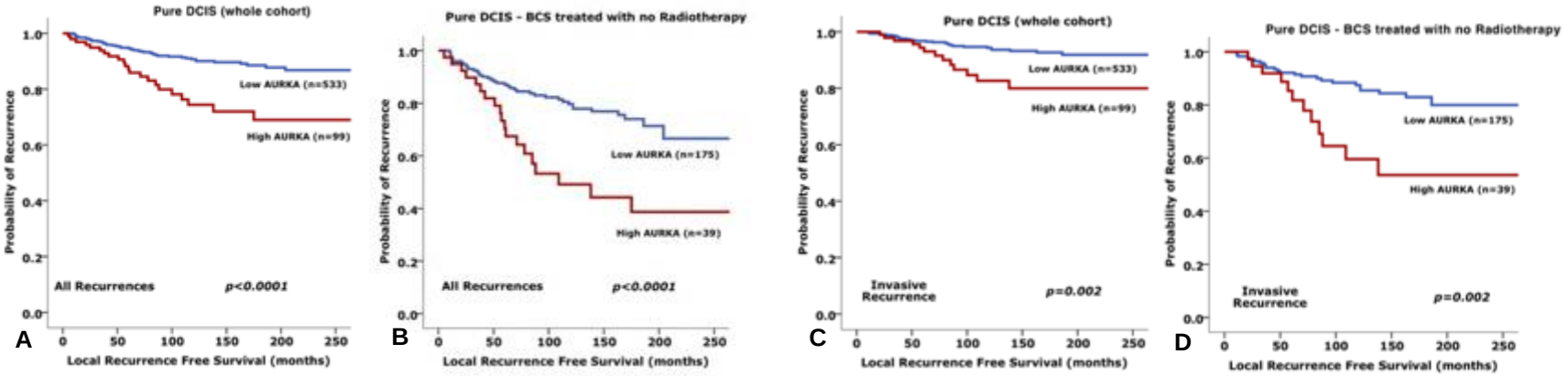


Figure 5-9: Kaplan-Meier curves show that high expression of AURKA is associated with shorter local recurrence-free interval in the whole pure DCIS series (A), and in breast conserving surgery (BCS) without adjuvant radiotherapy (B). High expression also showed an association with shorter local recurrence-free interval as invasive disease in the whole series (C) and in patients treated with breast conserving surgery without adjuvant radiotherapy (D). Dichotomisation of the cases was according to the median AURKA percentage of 30%.

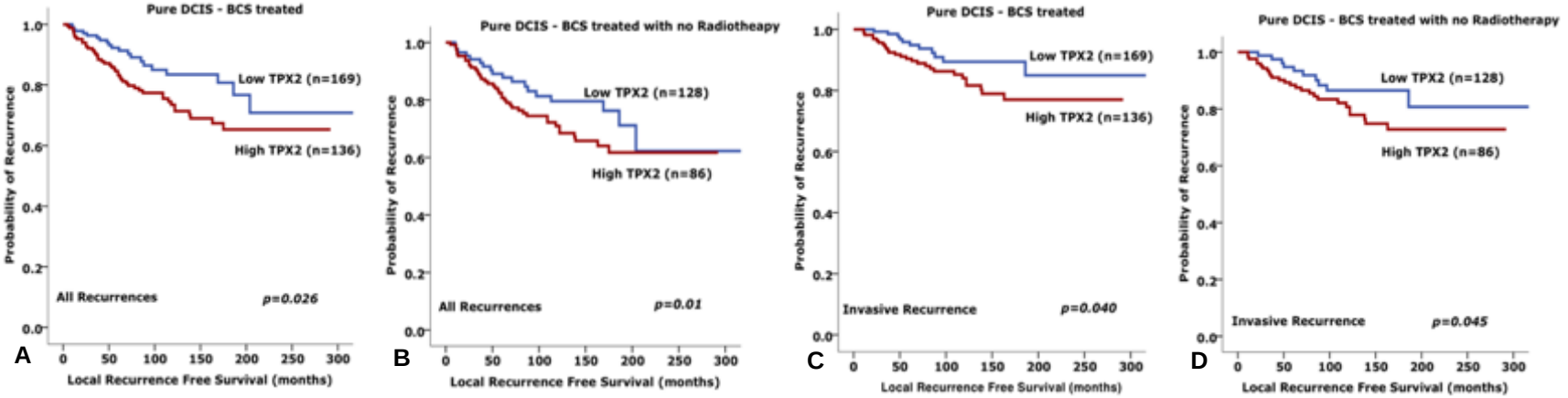


Figure 5-10: Kaplan-Meier curves show that high expression of TPX2 is associated with shorter local recurrence-free interval in breast conservative surgery (BCS)-treated pure DCIS series (A), and in BCS without adjuvant radiotherapy (B). High TPX2 expression also showed an association with shorter local recurrence-free interval as invasive disease in BCS-treated patients in the same series (C) and in patients treated with BCS without adjuvant radiotherapy (D). Dichotomisation of the cases was according to the median TPX2 percentage of 25%.

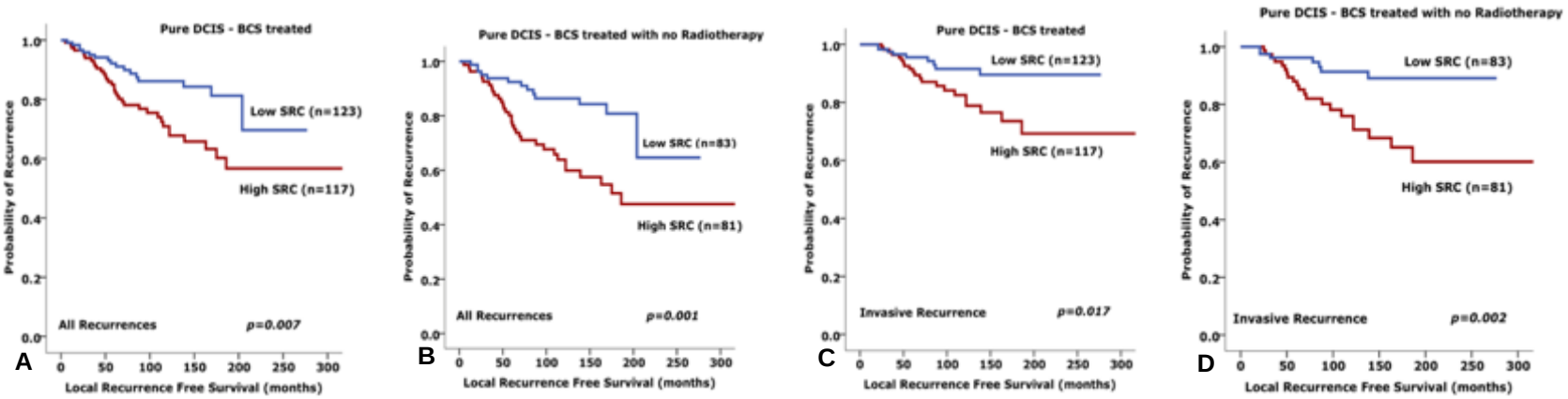


Figure 5-11: Kaplan-Meier curves show that high expression of SRC is associated with shorter local recurrence-free interval in breast conserving surgery (BCS)-treated pure DCIS series (A), and in BCS without adjuvant radiotherapy (B). High expression also showed an association with shorter local recurrence-free interval as invasive disease in BCS-treated patients in the same series (C) and in patients treated with breast conserving surgery without adjuvant radiotherapy (D). Dichotomisation of the cases was according to the median SRC H score of 100.

Table 5-14: Multivariate survival analysis (Cox regression model) of variables predicting outcome in terms of ipsilateral local recurrence in patients treated by breast conserving surgery in pure DCIS cohort, including AURKA

A) All recurrences

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
High AURKA expression	6.9	2.7	7.0	**$4.5x10^{-5}$**
Patient Age	0.6	0.2	2.2	0.631
DCIS size	0.8	0.4	1.5	0.531
DCIS nuclear Grade	2.8	0.9	4.5	0.057
Comedo necrosis	1.8	0.8	3.7	0.096
Molecular classes	0.8	0.6	1.2	0.260
Radiotherapy	0.3	0.1	0.9	**0.045**
Margin status	0.8	0.4	1.3	0.752

B) Invasive recurrence

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
High AURKA expression	3.9	1.7	7.1	**0.001**
Patient Age	0.5	0.1	4.5	0.577
DCIS size	3.2	1.1	6.5	**0.045**
DCIS nuclear Grade	4.7	1.1	6.0	**0.040**
Comedo necrosis	1.3	0.5	3.7	0.509
Molecular classes	0.1	0.1	7.1	0.055
Radiotherapy	0.2	0.1	4.5	**0.025**
Margin Status	0.8	0.3	6.5	0.588

HR: Hazard ratio, CI: Confidence interval, DCIS: Ductal carcinoma *in situ*, AURKA: Aurora kinase A
Significant *p* values are represented in **bold**

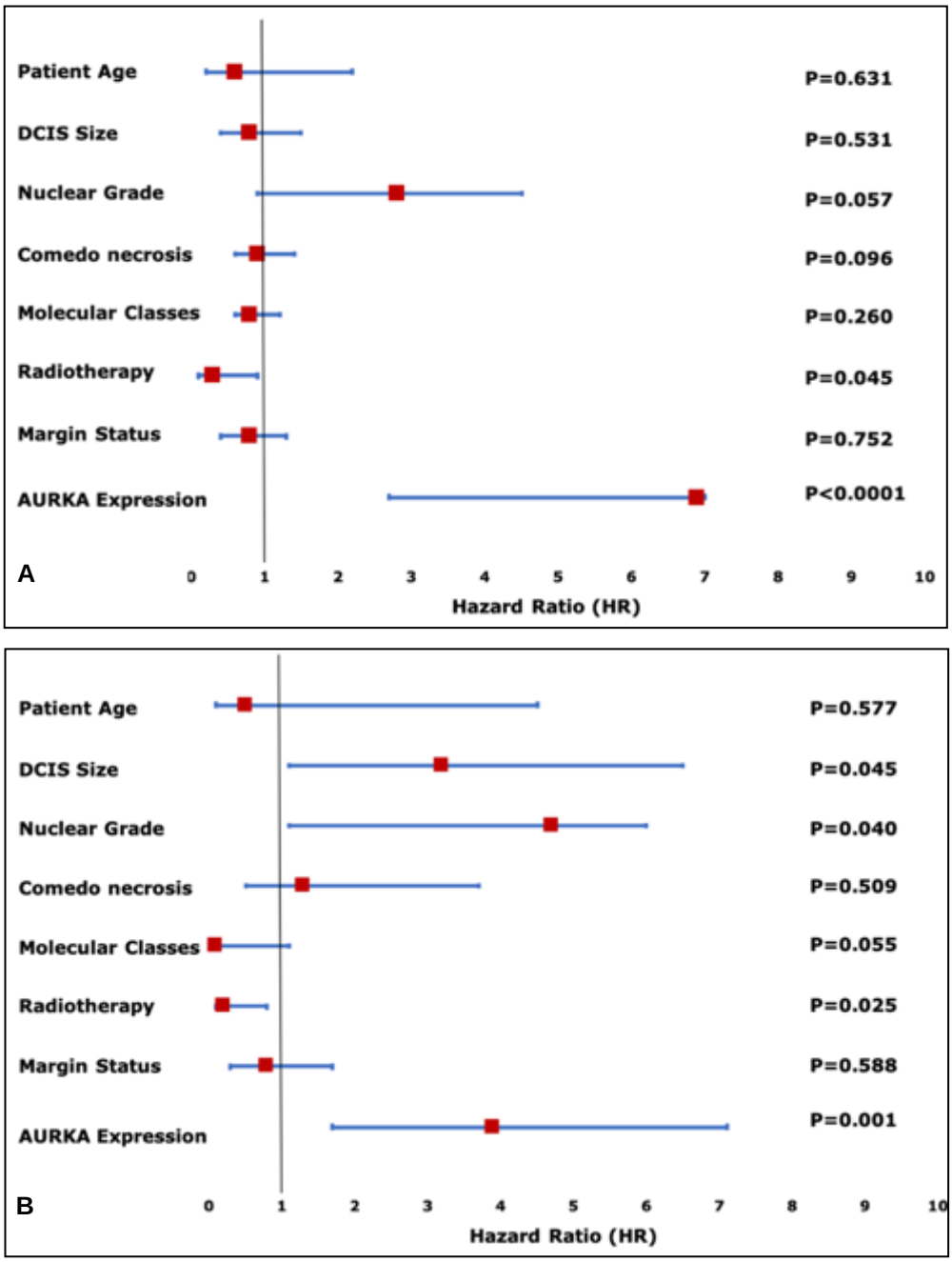


Figure 5-12: Forest plots (blobbogram) showing the hazard ratio of the different clinicopathological parameters and AURKA, and ipsilateral tumour recurrence for patients treated with breast conserving surgery in pure DCIS cohort based on the multivariate analysis results for: A) all recurrences whether DCIS or invasive disease; and B) for invasive recurrence only.

Table 5-15 : Multivariate survival analysis (Cox regression model) of variables predicting outcome in terms of ipsilateral local recurrence in patients treated by breast conserving surgery in pure DCIS cohort, including TPX2

A) All recurrences

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
TPX2 expression	1.8	1.1	3.1	**0.024**
Patient Age	0.4	0.2	0.8	**0.009**
DCIS size	3.7	1.2	6.0	**0.020**
DCIS nuclear Grade	3.8	1.3	6.5	**0.017**
Comedo necrosis	1.6	0.9	3.1	0.094
Molecular classes	1.7	0.7	4.3	0.198
Radiotherapy	0.3	0.1	0.7	**0.005**
Margin status	1.3	0.7	2.2	0.354

B) Invasive recurrence

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
TPX2 expression	2.1	1.4	4.8	**0.049**
Patient Age	0.7	0.2	2.3	0.588
DCIS size	7.3	2.2	6.0	**0.001**
DCIS nuclear Grade	4.9	1.1	6.6	**0.036**
Comedo necrosis	1.5	0.6	3.9	0.423
Molecular classes	0.5	0.1	2.2	0.343
Radiotherapy	0.2	0.1	0.7	**0.015**
Margin Status	0.5	0.4	1.7	0.856

HR: Hazard ratio, CI: Confidence interval, DCIS: Ductal carcinoma *in situ*, TPX2: Targeting protein for Xenopus kinesin-like protein 2
Significant *p* values are represented in **bold**

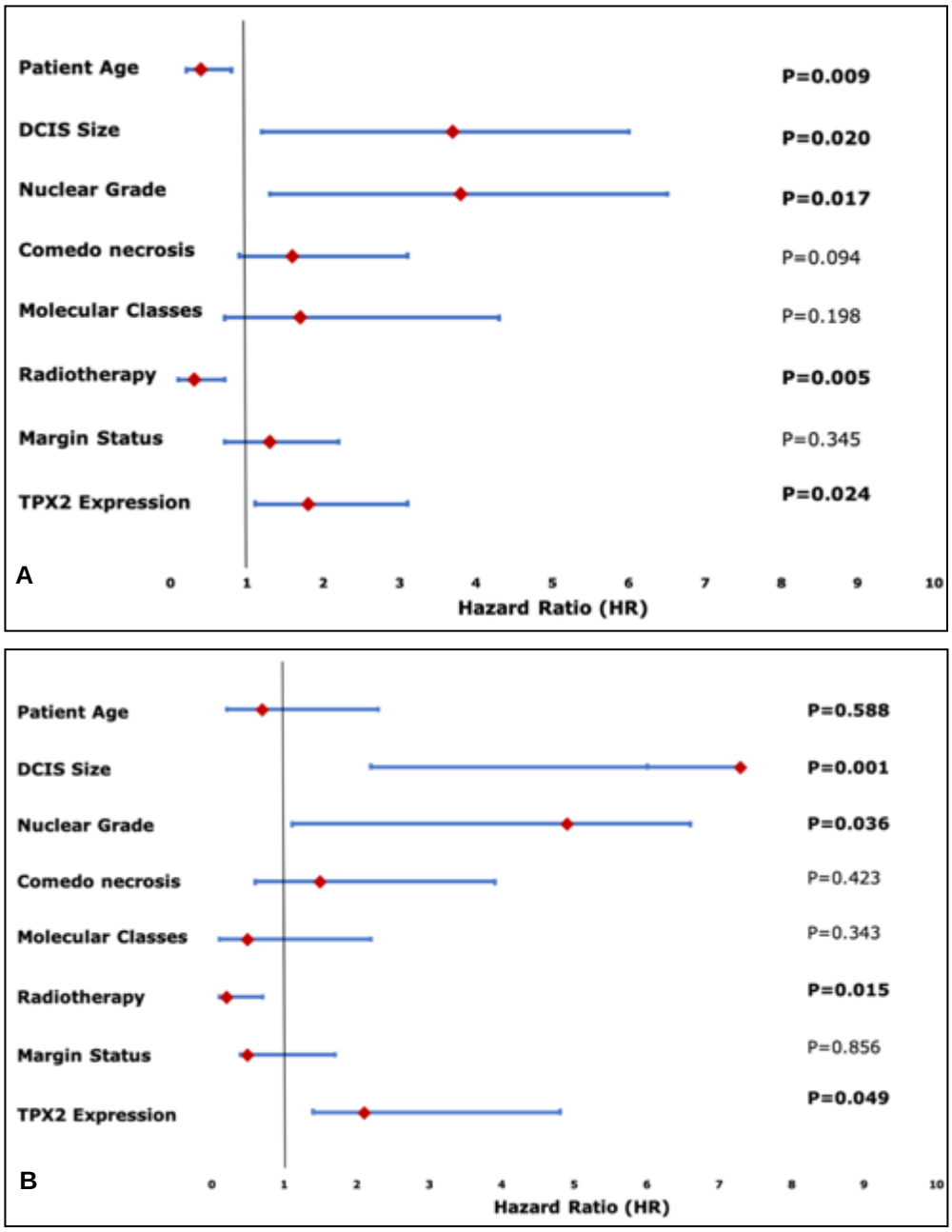


Figure 5-13: Forest plots (blobbogram) showing the hazard ratio of the different clinicopathological parameters and TPX2, and ipsilateral tumour recurrence for patients treated with breast conserving surgery in pure DCIS cohort based on the multivariate analysis results for: A) all recurrences whether DCIS or invasive disease; and B) for invasive recurrence only.

Table 5-16: Multivariate survival analysis (Cox regression model) of variables predicting outcome in terms of ipsilateral local recurrence in patients treated by breast conserving surgery in pure DCIS cohort, including SRC

A) All recurrences

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
SRC expression	5.5	1.7	6.9	**0.009**
Patient Age	0.4	0.2	0.7	**0.003**
DCIS size	1.1	0.6	1.8	0.888
DCIS nuclear Grade	3.2	1.1	5.6	**0.036**
Comedo necrosis	1.7	0.8	3.4	0.130
Molecular classes	1.9	0.8	4.5	0.127
Radiotherapy	0.3	0.1	0.7	**0.007**
Margin status	1.3	0.7	2.2	0.354

B) Invasive recurrence

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
SRC expression	2.9	1.7	5.4	**0.021**
Patient Age	0.7	0.2	2.5	0.704
DCIS size	2.2	1.3	5.6	**0.017**
DCIS nuclear Grade	2.0	1.1	5.9	**0.046**
Comedo necrosis	1.4	0.5	3.7	0.463
Molecular classes	0.5	0.1	2.2	0.343
Radiotherapy	0.2	0.1	0.8	**0.020**
Margin Status	0.9	0.4	1.9	0.801

HR: Hazard ratio, CI: Confidence interval, DCIS: Ductal carcinoma *in situ*, SRC: non-receptor tyrosine kinase
Significant *p* values are represented in **bold**

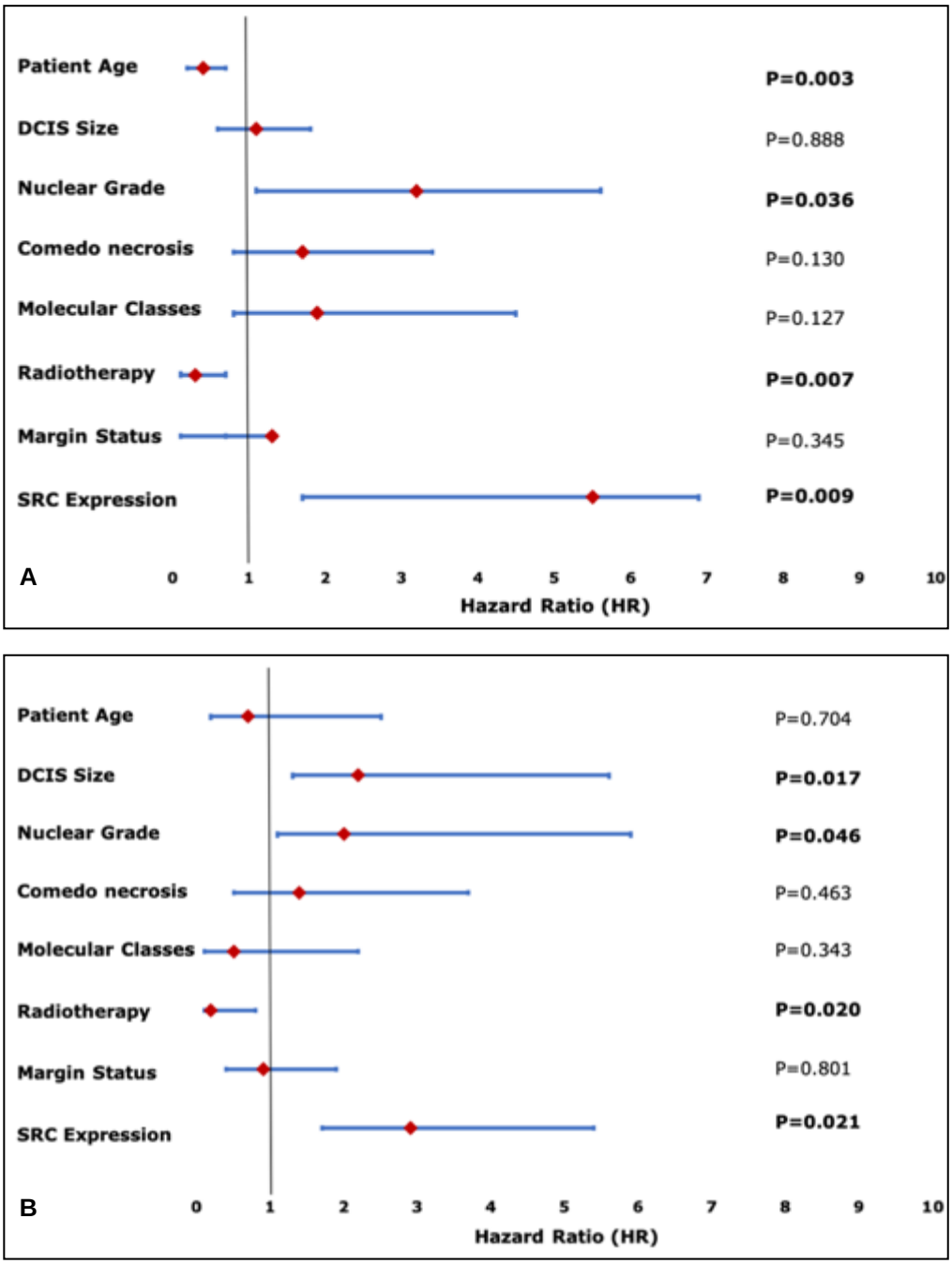


Figure 5-14: Forest plots (blobbogram) showing the hazard ratio of the different clinicopathological parameters and SRC, and ipsilateral tumour recurrence for patients treated with breast conserving surgery in pure DCIS cohort based on the multivariate analysis results for: A) all recurrences, whether DCIS or invasive disease; and B) for invasive recurrence only.

5.7 Discussion

The treatment of DCIS remains a challenge as the clinicopathological features of the disease do not reliably stratify patients into distinct risk groups to tailor management decisions (Allegra et al., 2010). For this reason, some studies have endeavoured to risk stratify DCIS based on genetic and molecular factors including Oncotype DX® DCIS score (Gauthier et al., 2007, Zhou et al., 2010, Solin et al., 2013, Bartova et al., 2014, Perez et al., 2015). Although this score minimised the proportion of patients undergoing RT, the assay is not cost-effective, relatively expensive (Raldow et al., 2016), shows inconsistent results and therefore needs further validation (Knopfelmacher et al., 2015, Raldow et al., 2016). In addition, none of the investigated parameters have been translated to clinical practice outside the US. Therefore, there is a demanding need to identify a reliable and cost-effective biomarker(s) to predict outcomes for DCIS patients.

The role of TXNIP expression has previously been studied in different tissues (Goldberg et al., 2003, Tome et al., 2005, Sheth et al., 2006, Nishizawa et al., 2011, Morrison et al., 2014) including locally advanced IBC (Woolston et al., 2012). In IBC it was observed that loss of TXNIP expression was associated with features of aggressiveness. It is likely that the effect of TXNIP on cell growth and proliferation are cell-context dependent (Lu and Holmgren 2012), with TXNIP being a tumour suppressor (Morrison et al., 2014) that might be downregulated in cancer (Zhou et al., 2011).

To date, there is no well-established, robust, publicly available database for DCIS mRNA or copy number aberrations. To confirm the prognostic significance of TXNIP mRNA in breast cancer and assess the role of copy number alteration on its expression and function, the METABRIC cohort was used. Using a large annotated series of DCIS with uniform treatment at a single institution, it was demonstrated that TXNIP downregulation is associated with increased aggressiveness and poor outcome in terms of shorter LRFS. It was also found a downregulation of TXNIP expression in invasive disease compared to the co-existing DCIS. These results support the hypothesis that TXNIP is a tumour suppressor in breast cancer.

The results demonstrate that loss of expression of TXNIP in DCIS is not only associated with clinicopathological characteristic of poor prognosis but is also an independent predictor of recurrence. These results are in line with TXNIP functioning as a tumour suppressor that is reported to be commonly silenced in cancer cells (Zhou and Chng 2013, Nie et al., 2015).

In this chapter, a significant inverse association between TXNIP expression and local recurrence in DCIS was observed. A previous study indicated that TXNIP stabilises cyclin-dependent kinase inhibitor p27, which plays a crucial role in inhibiting cell proliferation and apoptosis resulting in G_1 arrest and inhibition of cell cycle (Elgort et al., 2010). This observation may explain our finding that high TXNIP

expression was associated with low-grade, low-proliferating ER-positive and HER2-negative DCIS and with longer LRFS. Independent of its interaction with TRX, TXNIP has the ability to inhibit cell cycle progression by indirectly inhibiting the mammalian target of rapamycin (mTOR), a regulator of cell growth and metabolism (Jin et al., 2011). Therefore, in addition to inducing a metabolic shift important to tumour biology, downregulation of TXNIP is likely to promote cell survival, growth, invasion, and metastasis. TXNIP can also reduce tumour invasion and angiogenesis through inhibition of TRX and can directly have an impact on cell survival by promoting a pro-apoptotic environment (Welsh et al., 2002, Dunn et al., 2010). The exact mechanisms by which TXNIP exerts its tumour-suppressive functions in DCIS cells are not yet fully understood. Future studies of the mechanisms by which TXNIP is expressed and functions in DCIS will improve our understanding of the progression to invasive disease.

In this study, a significant positive correlation between TXNIP expression and ER+/HER2- status was identified. This finding is consistent with previous reports indicating that TXNIP may interrupt HER2 mediated oncogenic effect (Nie et al., 2015). To my knowledge, few studies have reported an explicit impact of HER2 signalling on TXNIP expression. This might suggest that there is a direct link between ERBB2 and TXNIP. It is well known that cancer cells exhibit energetic stress and glucose deprivation, especially in HER2 positive cancer cells (Elgort et al., 2010). Glucose induces TXNIP expression, and ROS triggers the segregation of TXNIP from TRX, leading to

increased TXNIP availability (Schroder et al., 2010). Information regarding this mechanism will require further investigation in the future.

Differential expression of TXNIP between pure DCIS and DCIS associated with invasive disease was demonstrated in the current study, which may have potential application, after further validation, for predicting invasive disease in DCIS diagnosed on preoperative biopsy samples. In addition, the ability of TXNIP to inhibit the major ROS scavenger TRX, further regulate cell cycle progression and metastasis and promote apoptosis clearly identifies TXNIP as an important therapeutic target.

AURKA is one of the genes on this *RNA* expression panel of Oncotype DX both in invasive and *in situ* scores, and this study has shown that immunohistochemical detection of the protein is a robust and relatively inexpensive method that may be able to assist with DCIS prognostication.

An increased copy number of AURKA is often associated with progression from a colonic polyp to an invasive malignancy in colon cancer and is one of the common copy number alterations in cancer. This finding reveals that it has a role not only in tumour invasion and migration but also in tumour development (Carvalho et al., 2009, Nikonova et al., 2013). Similarly, although 20q gain is common in DCIS (~25%) (Rane et al., 2015), it has also been observed as an IBC-specific event in mixed DCIS, suggesting an association with

invasiveness in breast cancer (Johnson et al., 2012). It has furthermore been reported that AURKA is differentially expressed between normal breast tissue and invasive breast cancer (Moelans et al., 2010, Moelans et al., 2011), and here we have shown that AURKA expression was most prevalent in invasive tumour cells, in line with (Ingoldsby et al., 2013), followed by a lower level in DCIS coexisting with invasive carcinoma and much lower in pure DCIS. These findings corroborate the role of AURKA in DCIS progression. Moreover, using the METABRIC series, a significant association between aggressive behaviour of IBC and higher levels of AURKA mRNA was shown. These observations support the hypothesis that AURKA is a promising candidate as a biomarker that requires further functional studies to decipher its role in DCIS behaviour.

This study showed that high AURKA expression was a poor prognostic factor for DCIS that was independent from other clinicopathological factors. These findings were similar for all recurrent events and also when the analysis was restricted to invasive recurrences only, which strengthen the evidence that AURKA plays a crucial role in DCIS progression to invasive cancer.

There are a number of plausible biological mechanisms to explain the involvement of AURKA in breast carcinogenesis and cancer progression. AURKA overexpression in fibroblasts and epithelial cells of the breast and other carcinomas was reported (Goepfert et al., 2002, Staff et al., 2010, Beltran et al., 2018, Li et al., 2018, Zhou et al., 2018)

and this overexpression has been associated with centrosome amplification and DNA instability (Chou et al., 2013). The non-mitotic function of *AURKA* has been implicated in breast cancer progression and resistance to chemotherapy agents through epithelial to mesenchymal transition and the acquisition of stem cell-like characteristics (Regan et al., 2013, D'Assoro et al., 2014). Breast cancer cells with these stem cell-like properties have consequently been associated with tumour progression and onset of distant metastasis (Giancotti 2013, Piva et al., 2014).

In addition, AURKA acts as a transactivating factor to enhance the expression of MYC. The activation of MYC by AURKA is dependent on both AURKA nuclear localisation and its kinase activity (Zheng et al., 2014, Zheng et al., 2016). There is emerging evidence to suggest that AURKA also promotes cancer development through mechanisms independent of its kinase activity (Otto et al., 2009). The relationship of AURKA and p53 expression was previously highlighted (Chen et al., 2002) and is supported in this study by similar finding that high AURKA expression is associated with abnormal p53 expression. AURKA phosphorylates p53 to inactivate its transcriptional activity (Liu et al., 2004). Deregulation of this mechanism between AURKA and p53 might trigger DNA-damage induced G2 checkpoint abnormalities and chromosome instability (Marumoto et al., 2002). This hypothesis is supported by a clinical observation whereby AURKA overexpression was associated with p53 mutation in hepatocellular carcinomas, and tumours with both AURKA overexpression and p53 mutation had worse

prognosis than those with p53 mutation alone (Jeng et al., 2004). This finding also provides *in vivo* evidence that AURKA overexpression and p53 inactivation have a mutual effect that contributes to tumour formation.

TPX2 located on the commonly altered 20q chromosome in breast cancer (Rane et al., 2015) is one of the proteins involved in the complex process of mitosis and has been identified as one of the microtubule-associated proteins (Cocchiola et al., 2014). Overexpression of TPX2 was reported to induce the amplification of the centrosome and leads to DNA polyploidy. Its expression is tightly regulated by the cell cycle, and this protein is detected during the G1-S stage and disappears following the completion of mitosis (Tulu et al., 2006). Beyond TPX2 amplification, aberrant overexpression is reported in some tumours such as prostate (Zou et al., 2018), glioma (Gu et al., 2016), renal cell carcinoma (Chen et al., 2016), gastric (Tomii et al., 2017), colon (Wei et al., 2013), oral carcinoma (Shigeishi et al., 2009) and IBC (Yang et al., 2015).

Similar to AURKA, although 20q gain is common in DCIS (~25%) (Rane et al., 2015), it has also been observed as an IBC-specific event in mixed DCIS, suggesting an association with invasiveness in breast cancer (Johnson et al., 2012). It has furthermore been reported that TPX2 is differentially expressed between normal breast tissue and invasive breast cancer (Yang et al., 2015). The same observation was reported in the kidney where TPX2 showed higher expression in renal

cell carcinoma compared to adjacent normal tissue (Chen et al., 2016). Here, TPX2 expression was most prevalent in invasive tumour cells, in line with the previously mentioned studies, followed by a lower level in DCIS coexisting with invasive carcinoma and much lower in pure DCIS. These findings potentiate the role of TPX2 in DCIS progression. Moreover, using the METABRIC series, we have shown a significant association between aggressive behaviour of invasive breast cancer and higher levels of TPX2 mRNA. These observations corroborate the hypothesis that TPX2 is a promising candidate biomarker that requires further functional studies to decipher its role in DCIS behaviour.

High TPX2 expression was a poor prognostic factor for DCIS that was independent from other clinicopathological factors. These findings were similar for all recurrent events and also when the analysis was restricted to invasive recurrences only, which supports the evidence that TPX2 plays an essential role in DCIS progression to invasive cancer. Similar results have been reported by Colak et al., who showed that TPX2 was one of the 16 major genes identified to be involved in progression of breast cancer using integrative and cross-species comparative genomics analysis (Colak et al., 2013).

A number of mechanisms have been reported to show the mechanism by which TPX2 exerts its role in cancer progression. TPX2 was previously shown to upregulate MMPs, which are known to degrade the components of the ECM and are also particularly important for tumour invasion and metastasis (Crane et al., 2004, Ren et al., 2015). This

upregulation is attributed to activating the Akt signalling pathway (Engelman et al., 2006, Wei et al., 2013). Akt is a target protein of PI3K and phosphorylates several downstream mediators to enhance cell proliferation and survival. Akt activity can also regulate the phosphorylation of mTOR (Engelman et al., 2006). TPX2 increases the phosphorylation of Akt/mTOR in the cancer cells and these findings suggest that the Akt/mTOR pathway is a potential target of TPX2, and serves a key role in TPX2-induced proliferation and invasion (Chen et al., 2016).

The SRC proto-oncogene has been strongly implicated in the development, growth, progression, and metastasis of different human cancers including colon, breast, pancreas, and brain (Dehm and Bonham 2004) and may play a central role in the processes affecting cancer outcome. SRC deregulation primarily involves protein overexpression and abnormalities in SRC kinase activity. Different SRC expression patterns have been observed in lung, breast, pancreatic, colon and prostate cancer cells, compared with normal adjacent tissue, fibroblasts or normal mucosal cells (Roskoski 2004, Zhang et al., 2013, Cao et al., 2014, Gargalionis et al., 2014, Je et al., 2014, Saini et al., 2014). This goes in line with the finding that SRC expression was prevalent in invasive tumours followed by a lower level in the DCIS adjacent to invasion and much lower level in pure DCIS tumours.

These findings potentiate the role of SRC in DCIS progression. Moreover, using the METABRIC series, we showed a significant

association between aggressive behaviour of invasive breast cancer and higher levels of SRC mRNA, which corroborates its role as an oncogene.

High SRC expression was a poor prognostic factor for DCIS that was independent from other clinicopathological factors. These findings were similar for all recurrent events and also when the analysis was restricted to invasive recurrences only, which supports the evidence that SRC plays an essential role in DCIS progression to invasive cancer.

In conclusion, the difficulty in implementing predictive markers for DCIS remains the availability of large cohorts with consistent treatment for validation, and this study is an exception. TXNIP, AURKA, TPX2 and SRC are independent prognostic factors in DCIS that require further functional studies to extensively validate their role as predictors of progression to invasive disease.

6 Prognostic Significance of Tumour-Infiltrating Lymphocytes in DCIS

6.1 Introduction

TILs are signs of the adaptive immune response against tumours and play an essential role in cancer immunotherapy (Pardoll 2012, Sabatier et al., 2015). Evasion of immune surveillance and host immune response is a hallmark of carcinogenesis and cancer progression (Zitvogel et al., 2011). Furthermore, the intensity of tumour immune response influences the effectiveness of cancer therapy, and positively affects clinical outcome in several solid tumours (Zitvogel et al., 2011).

6.2 Prognostic significance of TILs

The association with patient outcome in the majority of the studies have focused on the role of TILs (Rohrer et al., 1999, Bates et al., 2006, Gobert et al., 2009, Ruffell et al., 2012, Gu-Trantien et al., 2013), showing that dense TILs augment the effect of chemotherapy, resulting in better prognosis, particularly in Triple Negative IBC (Adams et al., 2014, Ibrahim et al., 2014). Following demonstration of their prognostic significance in IBC and potential clinical application, guideline recommendations for the evaluation of TILs have been published (Salgado et al., 2015, Pruneri et al., 2017). However, the recommended method of TILs assessment in IBC may not be appropriate in DCIS as not all stromal TILs are in direct contact with malignant ducts and identification of the stromal area surrounding DCIS can be confounding and ill-defined. Although the role of TILs has minimally been reported in DCIS (Man et al., 2013, Thompson et al., 2016), studies utilising the IBC guidelines for TILs assessment in the context of DCIS did not find

any association with outcome (Pruneri et al., 2017). This is potentially related to the difference in distribution of TILs within DCIS compared to IBC and the sub-optimal method of their assessment. This study aims to identify the optimal method of TILs evaluation in DCIS in terms of reliability, reproducibility and prognostic significance by utilising a large, well-annotated DCIS series with long-term follow-up data.

6.3 Role of tumour infiltrating B lymphocytes

The critical role of tumour-infiltrating B lymphocytes (TIL-B) with patient survival has been suggested (Nielsen and Nelson 2012). B cells are commonly activated in cancer patients, supporting the possibility of a positive role in tumour immunity (Nelson 2010). In IBC, TIL-Bs are present in approximately 25% of cases and comprise up to 40% of the TILs population (Chin et al., 1992, Marsigliante et al., 1999, Coronella-Wood and Hersh 2003), appearing early during breast tumuorigenesis (Lee et al., 1997). However, assessment of TILs in cancer tissues remains a challenge (Brown et al., 2014). Despite the reports incriminating TIL-Bs in improving patient survival, the mechanisms, functional profiles and their allied antigens remain to be defined.

Of the common B cell antigens, CD20 is expressed on all mature B cells except plasma cells (DiLillo et al., 2008). T cell response is inhibited by resting B cells and facilitated by activated B cells (Watt et al., 2007). CD19, another B cell marker, is a member of the Ig superfamily that has a critical signal transduction function regulating the development, activation and differentiation of B lymphocytes (Watt et

al., 2007). The expression of CD19 is restricted only to B cells. Similar to CD20, it appears early during B cell maturation in the late pro-B cell stage and remains throughout maturation but is lost when B cells differentiate into plasma cells (Tedder and Engel 1994, Sato and Tedder 1997).

6.4 Hypothesis and aims

In view of the current debate arguing the role of TILs in progression of IBC, it is hypothesised that TILs play a similar role in DCIS progression to invasion. It is also hypothesised that the density of TILs is different between IBC and DCIS.

The aims of this chapter were to determine the optimum methodology to evaluate TILs in DCIS and to assess the role of TILs in prediction of DCIS outcome.

6.5 Materials and methods

6.5.1 Study cohort

This retrospective study included 614 cases, (a subset of the total 1249 DCIS cases included in the current thesis), diagnosed from 1990 to 2012 at Nottingham City Hospital, Nottingham, UK. Representative Haematoxylin and Eosin (H&E) stained whole sections from each case were prepared and chosen as those having the largest tumour burden.

The whole cohort was divided into two sets. A small set of 80 DCIS tumours (36 pure DCIS and 44 associated with invasion) was included

as a pilot study (training/test set). This set was completely excised with free surgical margins (10mm or more) to avoid the confounding effect of excision margin on the outcome analyses. The validation set is composed of 534 pure DCIS tumours.

6.5.2 Immunohistochemistry

IHC staining was performed for the training set, using the Novocastra Novolink Polymer Detection Kit (Leica Microsystems, Newcastle, UK) following the manufacturer's guidelines as previously described (**Chapter 2**). Tissue sections were stained by monoclonal anti-human CD20 antibody (clone L26, DAKO, Glostrup, Denmark, dilution 1:300) and monoclonal anti-human CD19 antibody (clone LE-CD19, DAKO, Glostrup, Denmark, dilution 1:75). CD19 and CD20 IHC were applied on sequential tissue sections from the same paraffin block and not on the same slide. FFPE sections of normal human tonsil were used as a positive control, which showed positive cells distributed mainly in the germinal centres and mantle zone (B cell area) with some scattered inter-follicular positive cells. Negative controls were included with staining runs by omitting the primary antibody.

6.5.3 Scoring of TILs

6.5.3.1 The training/test set

Evaluation of TILs was done by two observers; the author (Islam M Miligy) and a second pathologist (Michael S Toss).

TILs were evaluated around the DCIS lesions using seven different scoring methods (**Table 6-1 and Figure 6-1**) (Toss et al., 2018). These included:

1- Percentage of stromal TILs (defined as TILs in the area surrounding the DCIS duct within two high-power microscopic fields) (Pruneri et al., 2017, Hendry et al., 2017a). In numerous involved ducts, an evaluation of the area surrounding the whole lesion was performed, and the percentage of stromal TILs in the total stromal area of DCIS involved ducts was assessed (Thompson et al., 2016, Campbell et al., 2017, Pruneri et al., 2017);
2- Touching TILs mean number, defined by TILs touching or within one lymphocyte cell thickness from the malignant ducts' BM;
3- Mean number of TILs within 0.2mm distance from the ducts' BM;
4- Mean number of TILs present within 0.5mm distance from the ducts' BM;
5- Mean number of TILs that are within 1mm from the ducts' BM;
6- Hotspots of TILs defined as the largest number of lymphoid cells' aggregates directly surrounding or located between DCIS ducts within the boundaries of the lesion;
7- Assessment of lymphoid follicles with reactive germinal centres.

No assessment was done for the intra-tumoural lymphocytes because it was not obvious to assess in the *in situ* disease compared to the invasive tumours, owing to their potential similarity with some MECs at the H&E level.

Counting of TILs was carried out manually (eyeballing) with the aid of the Pannoramic Viewer software program scale v 1.15.4 (3D HISTECH®, Budapest, Hungary). In cases where TILs were overlapping

between adjacent ducts in each topographic area, TILs were counted once.

All recognisable lymphocytes and those marked by CD20 and/or CD19 positive cells, were counted (with the exclusion of polymorphonuclear cells). TILs were assessed around all DCIS duct profiles up to 20 ducts (the average number of ducts within the training set). In cases with more than 20 malignant ducts, the section was digitally divided into four identical quadrants and scoring of TILs was done around five ducts in each quadrant in order to keep the scoring more representative especially in cases with heterogeneous distribution of TILs. During scoring, very large (i.e. mass-forming papillary carcinoma, branching or confluent DCIS ducts) or very small (terminal duct-lobular units involved by DCIS) ducts were not considered in TILs' scoring to avoid skewing of the scores by the effect of extremes in duct sizes. TILs surrounding normal ducts, beyond the DCIS limits, surrounding the adjacent fat, associated LCIS, hyalinosis or at crushing artefacts were excluded from the scoring (Denkert et al., 2010, Salgado et al., 2015).

TIL-Bs were assessed in the invasive component separately to assess the pattern of distribution and the difference in density between components. TIL-Bs were assessed in the invasive component according to the previously published guidelines (Denkert et al., 2010, Salgado et al., 2015).

6.5.3.2 The validation set

The optimal method for TILs assessment was selected based on the reproducibility in terms of inter-observer concordance and association with patient outcome, which was then applied to the validation set.

Evaluation of TILs in DCIS was previously done by assessment of stromal TILs (Hendry et al., 2017a); therefore, stromal TILs were also assessed in the pure DCIS validation set using the same criteria applied for the training set.

6.6 Statistical analysis

X-tile bioinformatics software (Yale University, version 3.6.1) was used to define the optimal cut-off point for TILs density against LRFS, and therefore classification of TILs into sparse and dense infiltrates was performed (**Table 6-2**). To ensure consistency, the same selected cut-off point for the training set was applied for dichotomisation touching TILs in the validation set into sparse and dense groups. Inter-observer degree of agreement was assessed through inter-class correlation coefficient (ICC) for continuous data and Kappa test for categorical groups. All statistical analyses were performed by the author.

6.7 Results

6.7.1 Assessment of TILs using differential methods

The concordance rate of TILs assessment using different methods between the two observers is summarised in **Table 6-3**. The highest level of inter-observer concordance was observed when assessment of TILs was performed using touching TILs (ICC=0.96), whereas the least

concordance rate was recorded when using the percentage of stromal TILs as a method of evaluation (ICC=0.79), which was the recommended method to assess TILs in IBC.

Mean TILs counts within the different topographic areas surrounding DCIS are summarised in **Table 6-4**. The mean count of touching TILs increased from 37 cells/DCIS duct to 482 cells/DCIS duct within 1mm distance. Mean TILs density showed a four-fold increase between touching and within 0.2mm distance. The largest hotspot density ranged from 20 to 6,000 cells while the percentage of stromal TILs ranged from 1–65%. No cases with absent TILs were detected. Examples of dense and sparse TILs are shown in **Figure 6-2**. Histological assessment for DCIS and the corresponding IHC is demonstrated in **Figure 6-3**. The number of B-lymphocytes around the DCIS foci was higher in the pure group when compared to the DCIS foci or the invasive component in the mixed cases (p<0.001).

Dense TILs were observed in more than 60% of cases who developed local recurrence. A statistically significant association was observed between dense TILs infiltrate and shorter LRFS (p=4.7x10^{-6}, p=0.001, p=0.002, p=0.001 and 0.02 for touching TILs and TILs within 0.2mm, 0.5mm, and 1mm distances and stromal TILs density, respectively), with the highest p value observed for touching TILs. No significant association was observed between either hotspots or lymphoid follicle formation and the development of local recurrence (p=0.09 and p=0.15, respectively).

6.7.2 Validation set

The inter-rater concordance rate between the two observers evaluated touching TILs in the validation set was similar to the training set (ICC=0.95). Dichotomisation of touching TILs was performed using 20 cells/DCIS duct as a cut-off value, which showed that dense TILs infiltrate was detected in 239 cases (45%) while 295 cases (55%) showed sparse infiltrate.

6.7.3 Association between TILs and other clinicopathological parameters

Dense touching TILs were associated with younger age, symptomatic presentation and large tumour size. Only eight cases (3%) of dense TILs group were of low nuclear grade lesions in comparison with 19% and 78% of intermediate and high nuclear grade lesions, respectively (p<0.0001). Approximately 77% of dense TILs tumours were associated with comedo necrosis while this was observed in only 23% of cases without comedo necrosis (p<0.0001). Two-thirds of patients with dense TILs were treated with mastectomy (p=0.002). Dense TILs were associated with high tumour grade, Luminal B, HER2 and Triple Negative subtypes of DCIS (all p<0.0001). The association between TILs density and the different clinicopathological parameters is summarised in **Table 6-5**.

6.7.4 Outcome analysis

The ipsilateral local recurrence rate in the pure DCIS validation cohort was reported to be 12% (*n*=63). Recurrence occurred in 39/239 cases with dense TILs (62% from total recurrences), in comparison with 24/295 cases with sparse TILs (38% from total recurrences). In patients treated with BCS, local recurrence was reported in 24/80 cases with dense TILs (56%) compared to 19/139 cases with sparse infiltrate (44%). When recurrence was assessed based on the RT status, there were four (13%) recurrent events among RT-treated patients' DCIS with dense TILs (*n*=30) compared with 20 events (40%) among cases with dense infiltrate who did not receive adjuvant RT (*n*=50).

In univariate survival analysis, dense TILs showed a statistically significant association with shorter LRFS. This was observed when the analysis was done on the whole cohort (*p*=0.002), or restricted to patients treated with BCS (*p*=0.001). In the context of radiotherapy status, dense TILs were associated with shorter LRFS in non-RT-treated patients (*p*=0.001) but not in the RT-treated group (p=0.377) (**Figure 6-4**). No statistically significant association was detected between LRFS and TILs density when assessed based on the percentage of overall stromal TILs.

The multivariate Cox regression model including other clinicopathological parameters showed that dense touching TILs was the only independent predictor of shorter LRFS (*HR*=3.7, 95% *CI*= 1.7-7.7, *p*=0.001) (**Table 6-6**).

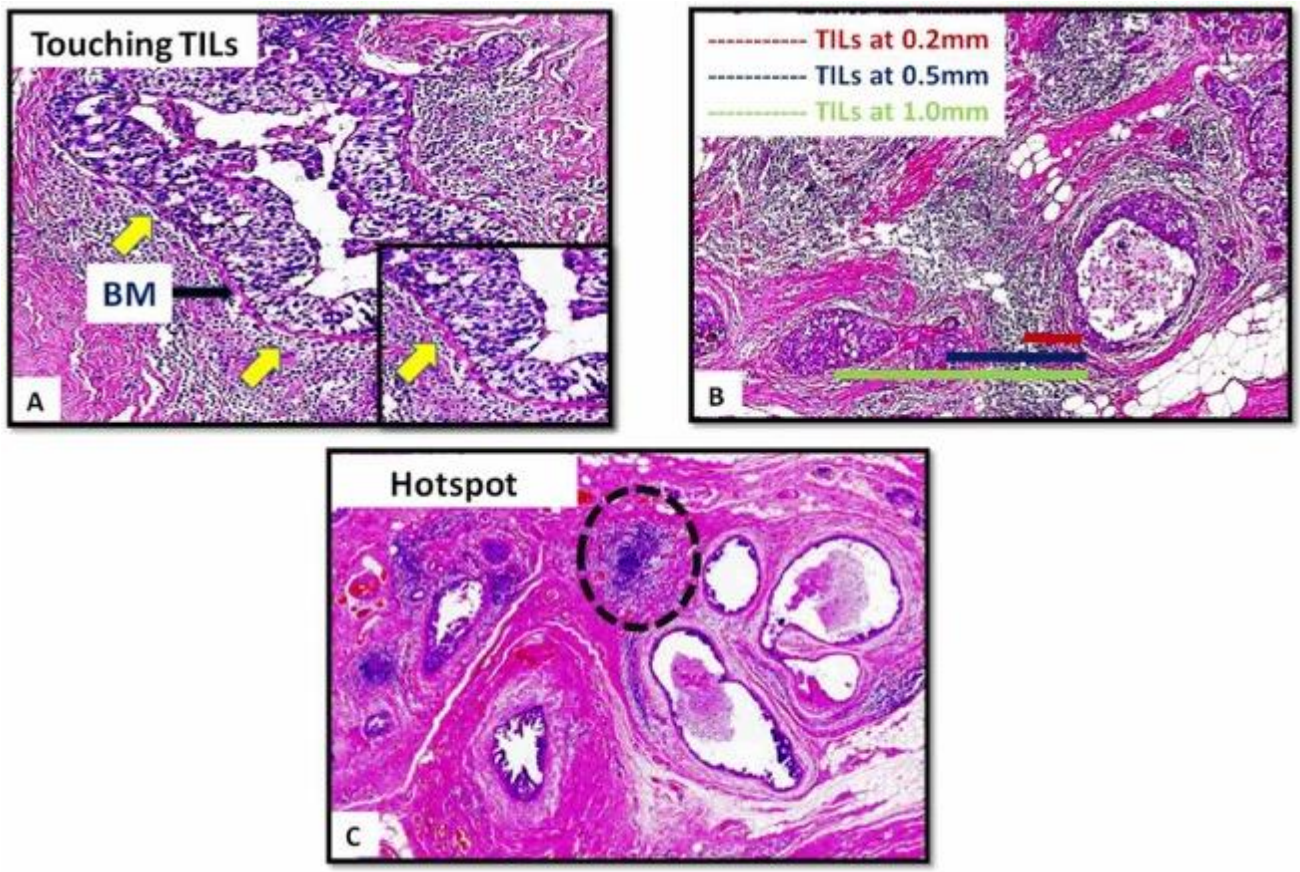


Figure 6-1: Parameters of TILs assessment: A) Touching TILs (x20) defined by lymphocytes that touch the basement membrane (BM) or present within one lymphocyte cell thickness from the BM (yellow arrows); inset (x40) for touching TILs; B) TILs within 0.2mm (red line), 0.5mm (blue line) and 1.0mm (green line) distance from the involved ducts; and C) hotspots (x4) as shown in area surrounded by the black circle) (Toss et al., 2018).

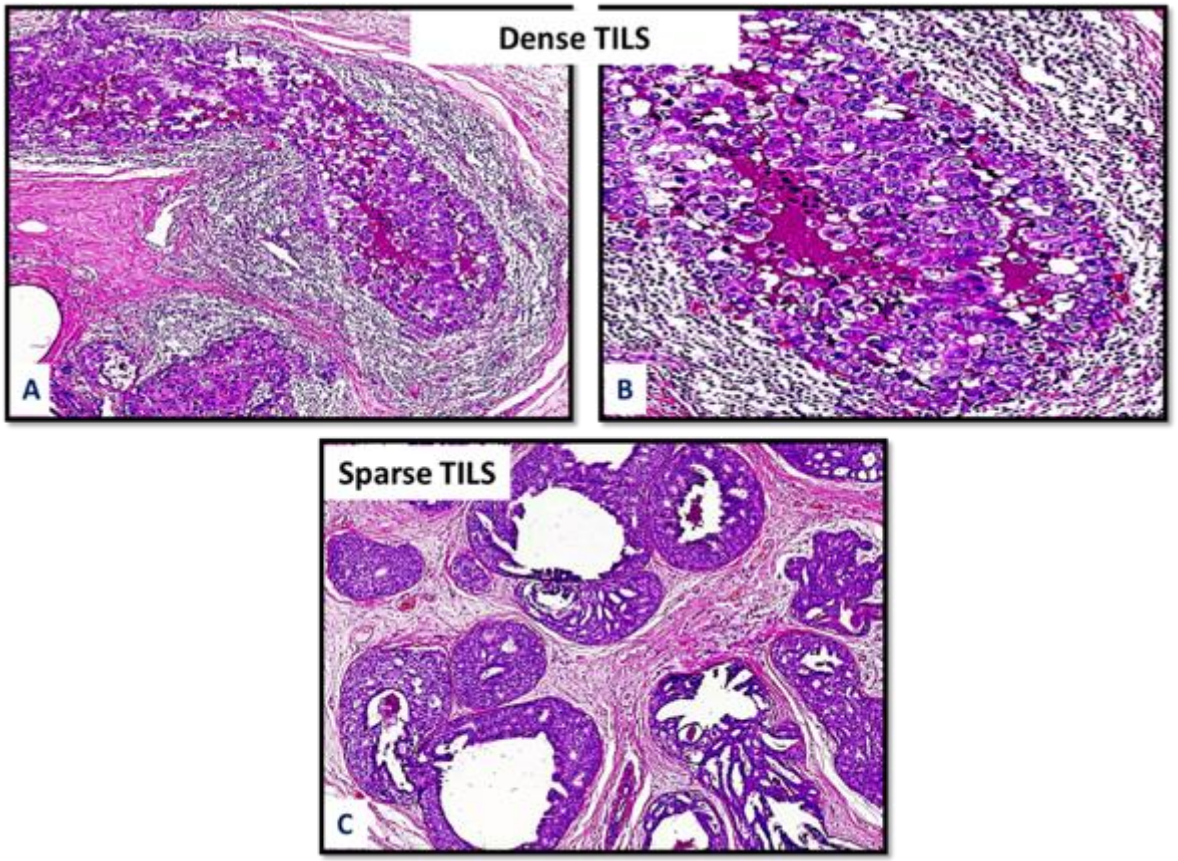


Figure 6-2: Touching TILs density around DCIS: A) Dense TILs: mean number of touching TILs is > 20 cells/DCIS duct; B) Dense touching TILs at 40x magnification; and C) Sparse TILs: mean number of touching TILs within the lesion is ≤ 20 cells/DCIS duct (Toss et al., 2018).

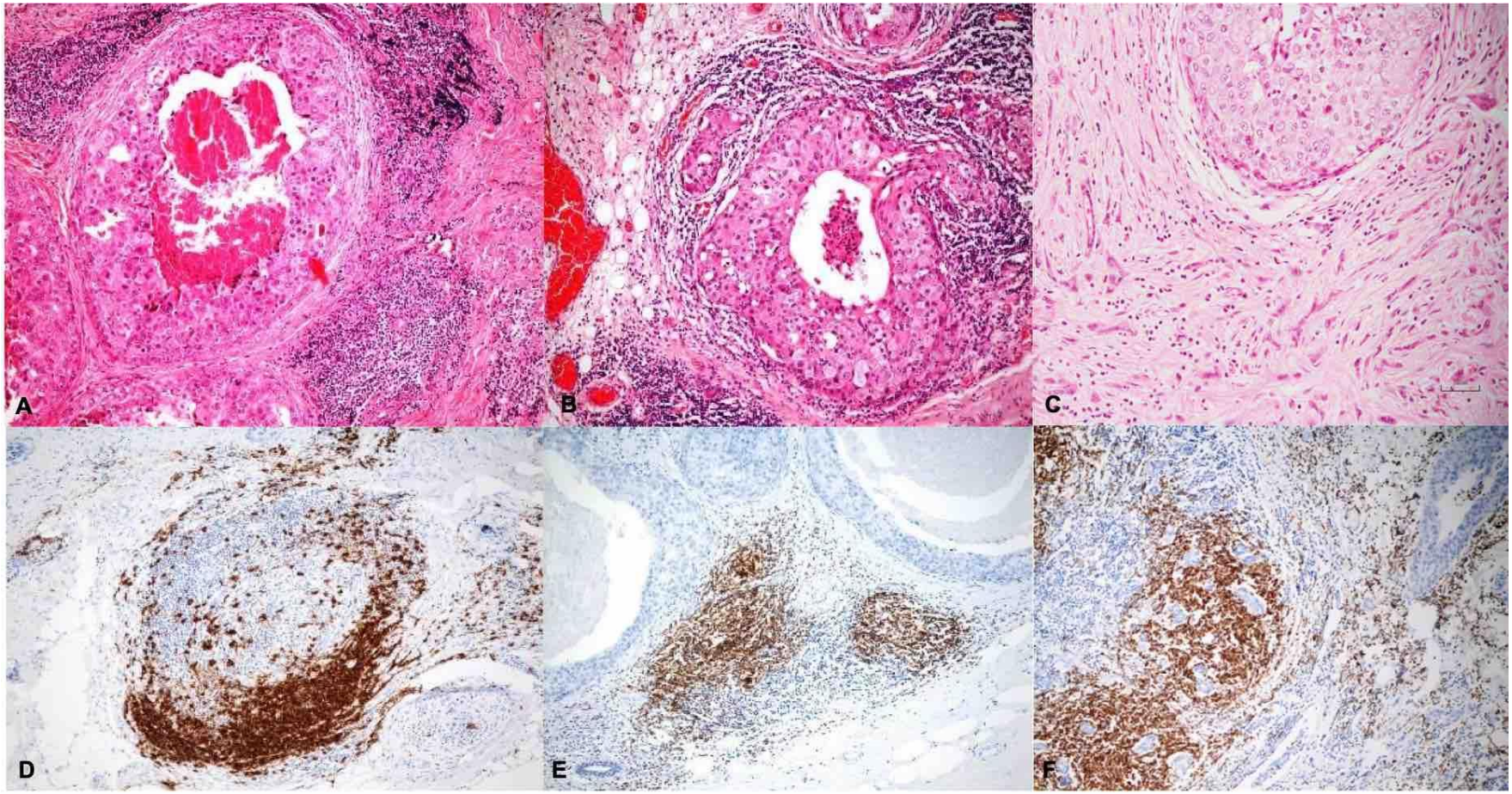


Figure 6-3: A) High-grade comedo DCIS with central necrosis and adjacent touching lymphocytic aggregates (H&E x10). B) DCIS with micro-invasion and surrounding touching lymphocytes and some intra-tumoural lymphocytes (H&E x10). C) Solid DCIS mixed with invasive component and scattered stromal lymphocytes (H&E x10). D) A case of DCIS with peri-tumoural CD20 positive lymphocytes forming aggregates (IHC x10). E) Peri-tumoural and stromal CD19 positive lymphocytes (IHC x10). F) CD19 positive lymphocytic aggregate in a case mixed with invasive component (IHC x 4) (Miligy et al., 2017).

Table 6-1: Methods of assessment of tumour-infiltrating lymphocytes (TILs) in DCIS (Toss et al., 2018)

Methods for evaluation of TILs in DCIS
1. TILs were evaluated in full-face H&E-stained sections. Lesions diagnosed on core biopsy were excluded. 2. For each patient, one representative 4μm thick section, which had the maximum tumour burden, was selected for TILs assessment. 3. All mononuclear inflammatory cells excluding polymorphonuclear leukocytes were counted. 4. TILs within the boundaries of the DCIS were assessed. TILs surrounding normal ducts/lobules, beyond the tumour border, adjacent to fatty tissue, LCIS, hyalinosis, crushed artefacts or sites of previous biopsies were excluded. 5. TILs within tumour cells (intra-tumoural TILs) were not assessed. 6. TILs were assessed around all malignant ducts up to 20 ducts. For lesions with more than 20 malignant ducts, assessment is done for TILs surrounding only 20 ducts (five ducts from each quadrant of the lesion). 7. Average-sized ducts only are assessed (case specific). TILs surrounding large DCIS ducts, such as mass forming papillary carcinoma, branching or confluent DCIS ducts or too small ducts such as terminal duct-lobular system involved by DCIS, were excluded. 8. Overlapping TILs between adjacent ducts were counted once.
Topographic parameters used for TILs assessment*
A. Estimation of stromal TILs (as previously published): The stromal area (defined as the area surrounding the DCIS duct within two high power microscopic fields) is used for evaluation of stromal TILs percentage (Pruneri et al., 2017, Hendry et al., 2017a). In cases with numerous involved ducts, an evaluation of the area surrounding the whole lesion was performed, and percentage of stromal TILs in the total stromal area of all DCIS involved ducts was determined (Thompson et al., 2016, Campbell et al., 2017, Pruneri et al., 2017). B. Estimation of the periductal TILs (based on counting TILs around all DCIS duct profiles up to 20 ducts). 1. Evaluation of the mean number of TILs touching DCIS involved ducts (defined as TILs touching or within one lymphocyte cell thickness from the ducts' BM). 2. Evaluation of the mean number of TILs within 0.2mm distance from the ducts' BM. 3. Evaluation of mean number of TILs within 0.5mm distance from the ducts' BM. 4. Evaluation of mean number of TILs with 1.0mm distance from the ducts' BM. 5. Evaluation of the TILs hotspot defined by largest number of lymphoid aggregates directly surrounding or located between DCIS ducts within the boundaries of the lesion. 6. Evaluation of lymphoid follicles formations with reactive germinal centres in the stroma directly surrounding or located between DCIS ducts within the boundaries of the lesion.

H&E: Haematoxylin and Eosin, LCIS: lobular carcinoma *in situ*
*All parameters were assessed in the training set while touching and stromal TILs assessment were conducted for the validation set.

Table 6-2: X-tile determined cut-off points of mean TILs count/DCIS duct based on association with local recurrence free survival

Parameter	Definitions of TILs density	
	Sparse	**Dense**
Mean Touching TILs count*	≤20 cells	>20 cells
Mean TILs count within 0.2mm distance*	≤60 cells	>60 cells
Mean TILs count within 0.5mm distance*	≤100 cells	>100 cells
Mean TILs count within 1mm distance*	≤120 cells	>120 cells
Hotspots	≤ 1200 cells or less	> 1200 cells
Stromal TILs	≤ 5% from the total surrounding stromal area	> 5% of total surrounding stromal area

*Represent mean number of TILs count within the specified areas/DCIS duct.

Table 6-3: TILs observers' concordance results in the training set

Parameter	Inter-cluster correlation coefficient (ICC) between observers
Mean touching TILs count	0.96
Mean TILs count within 0.2mm distance	0.89
Mean TILs count within 0.5mm distance	0.92
Mean TILs count within 1mm distance	0.90
Hotspots	0.89
Lymphoid follicles*	0.86
Stromal TILs percentage	0.79

* Kappa test is used for the analysis

Table 6-4: TILs count assessment in different topographic areas

	Mean	Median	Minimum	Maximum
Touching TILs count/ duct	37	20	4	120
TILs at 0.2mm distance/ duct	144	80	10	800
TILs at 0.5mm distance/ duct	319	135	20	2000
TILs at 1.0mm distance/ duct	482	250	50	3000
Hotspot	1010	500	20	6000
Percentage of stromal TILs (%)	13	5	1	65

Table 6-5: Correlation between TILs density (based on mean cut-off 20 touching cells/DCIS duct) and clinicopathological parameters in the validation set

Parameter	Total (n=534) n (%)	TILs Density Sparse (n=295) n (%)	Dense (n=239) n (%)	χ^2 (*p* value)
Age (years)*				
<40	23 (4.3)	8 (2.7)	15 (6.3)	8.501
40 to 60	323 (60.5)	170 (57.6)	153 (64.0)	**(0.014)**
>60	188 (35.2)	117 (39.7)	71 (29.7)	
Presentation				
Screening	280 (52.4)	166 (56.3)	114 (47.7)	3.89
Symptomatic	254 (47.6)	129 (43.7)	125 (52.3)	**(0.049)**
DCIS Size*				
<16mm	161 (30.3)	108 (36.7)	53 (22.4)	23.441
16 to 40mm	210 (39.5)	121 (41.2)	89 (37.6)	(**$8x10^{-6}$**)
>40mm	160 (30.1)	65 (22.1)	95 (40.0)	
Grade				
Low	57 (10.7)	49 (16.6)	8 (3.3)	41.180
Intermediate	132 (24.7)	88 (29.8)	44 (18.4)	**($1.1x10^{-9}$)**
High	345 (64.6)	158 (53.6)	187 (78.3)	
DCIS histologic type				
Single	267 (50.0)	147 (49.8)	120 (50.2)	0.008
Mixture**	267 (50.0)	148 (50.2)	119 (49.8)	(0.931)
Comedo necrosis				
Yes	367 (68.7)	182 (61.7)	185 (77.4)	15.16
No	167 (31.3)	113 (38.3)	54 (22.6)	**($9x10^{-6}$)**
Associated LCIS				
Yes	43 (8.1)	25 (8.5)	18 (7.5)	0.159
No	491 (91.9)	270 (91.5)	221 (92.5)	(0.690)
Associated Paget's				
Yes	26 (37.1)	8 (4.3)	18 (10.3)	5.030
No	336 (62.9)	180 (95.7)	156 (89.7)	**(0.023)**
Management+				
Mastectomy	314 (58.9)	156 (52.9)	158 (66.4)	9.926
BCS	219 (41.1)	139 (47.1)	80 (33.6)	**(0.002)**
Radiotherapy***				
Yes	80 (36.5)	50 (36.0)	30 (37.5)	0.051
No	139 (63.5)	89 (64.0)	50 (62.5)	(0.821)
ER Status				
Positive	278 (72.0)	177 (84.3)	101 (57.4)	34.382
Negative	108 (28.0)	33 (15.7)	75 (42.6)	**($4.5x10^{-9}$)**
PR Status				
Positive	217 (53.2)	152 (67.3)	65 (35.7)	40.284
Negative	191 (46.8)	74 (32.7)	117 (64.3)	**($2.2x10^{-10}$)**
HER2 Status****				
Negative	355 (76.5)	229 (88.8)	126 (61.2)	48.529
Positive	109 (23.5)	29 (11.2)	80 (38.8)	**($3.2x10^{-12}$)**
Ki67 Index				
Low (<14%)	284 (75.9)	160 (81.2)	124 (70.1)	6.356
High (≥14%)	90 (24.1)	37 (18.8)	53 (29.9)	**(0.012)**

P53 Mutation				
Inactivating (0)	93 (26.6)	49 (27.1)	44 (26.2)	5.715
Normal (1-50%)	181 (51.9)	102 (56.3)	79 (47.0)	(0.057)
Missense (>50%)	75 (21.5)	30 (16.6)	45 (26.8)	
Molecular Classes				
Luminal A	235 (56.6)	171 (74.7)	64 (34.4)	
Luminal B (HER2-)	37 (8.9)	20 (8.7)	17 (9.1)	78.807
Luminal B (HER2+)	37 (8.9)	9 (3.9)	28 (15.1)	**($3.1x10^{-16}$)**
HER2+	57 (13.8)	12 (5.3)	45 (24.2)	
Triple negative	49 (11.8)	17 (7.4)	32 (17.2)	

DCIS: Ductal carcinoma *in situ*; n: number
***p* value in bold: significant**, TILs: Tumour infiltrating lymphocytes, LCIS: Lobular Carcinoma *in situ*, BCS: Breast conserving surgery, ER: Oestrogen receptor, PR: Progesterone receptor
*Age and size: categorised according to the Van Nuys Prognostic Index (VNPI), **Histologic type is a mixture of more than one morphologic type, ⁺Management is according to the final operation, ***Radiotherapy status is for cases treated with BCS
****HER2 final status is achieved using combination of IHC and chromogenic *in situ* hybridisation (CISH).

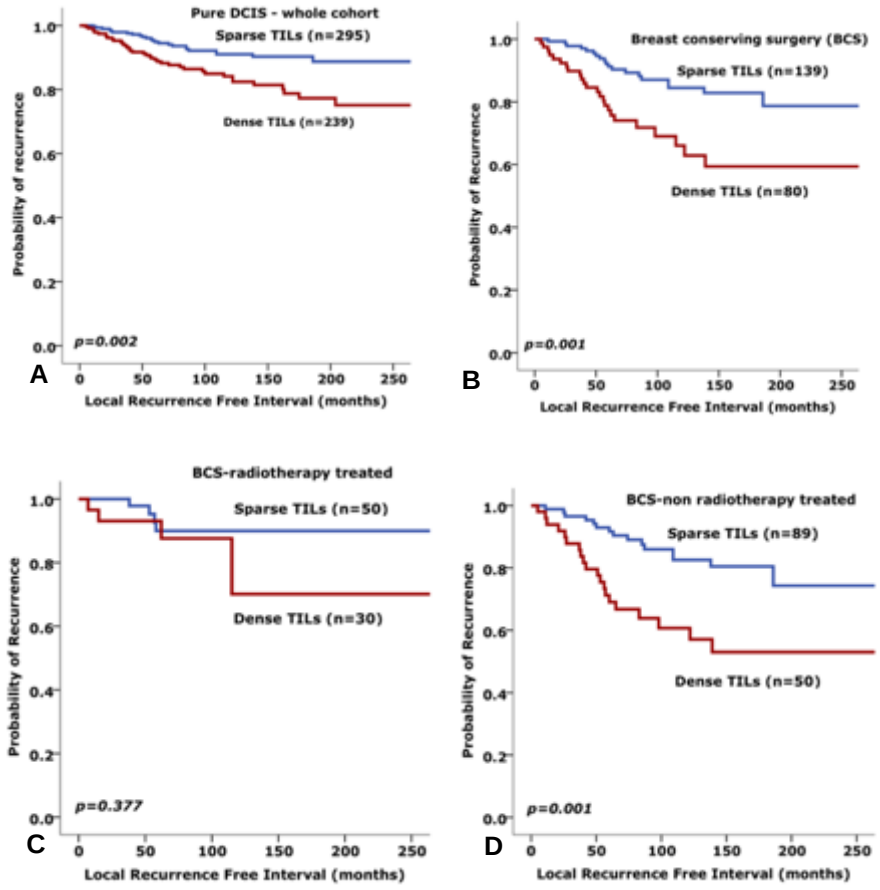


Figure 6-4: Kaplan-Meier curves for association of touching TILs density with recurrence-free interval (in months): A) all cases; B) cases treated with breast conserving surgery (BCS); C) for BCS-treated patients who received adjuvant radiotherapy; and D) for non-radiotherapy-treated patients. Cases were dichotomised into dense and sparse according to the median of 20 touching TILs.

Table 6-6: Multivariate Cox proportional hazards for predictors of local recurrence in pure DCIS patients*

Parameters	Hazard ratio (HR)	95% confidence interval (CI)		*p*-value
		Lower	Upper	
Dense TILs	3.7	1.7	7.7	**0.001**
Patient Age	0.6	0.1	6.1	0.706
DCIS size	1.7	0.4	1.6	0.453
DCIS nuclear Grade	2.5	0.5	12.4	0.271
Comedo necrosis	0.8	0.4	1.8	0.642
Molecular classes	1.3	0.5	3.7	0.628
Radiotherapy	0.4	0.1	1.1	0.051
Margin status	0.8	0.4	1.3	0.752

*Performed only for breast conservative surgery (BCS) treated patients.

6.8 Discussion

Current clinicopathological parameters used for DCIS risk stratification are not sufficient to precisely estimate recurrence risk associated with DCIS (Furnival 2006, Virnig et al., 2010). It is hypothesised that TILs in DCIS have a role in tumour progression; however, there are no consensus guidelines for evaluating TILs in clinical practice.

TILs are an essential immune component of the response to cancer (Gutkin and Shurin 2014). It is now accepted that the immune system has a dual role in cancer development and progression. It can eradicate emerging malignant cells by an orchestrated action of innate and adaptive branches, thus preventing tumour growth. On the other hand, it can paradoxically promote growth of malignant cells, their invasive capacity

and their ability to metastasise (Stanton and Disis 2016, Agahozo et al., 2018, Saint-Jean et al., 2018).

Previous studies of TILs evaluation in DCIS did not achieve the desired success as they have neither used uniform definition of the stromal area surrounding DCIS for TILs assessment nor identified clear cut-off points that can predict DCIS patients' outcomes. The International Working TILs Group's guidelines have been used by some researchers, with modification to DCIS considering the stroma within the boundaries of the whole lesion (Thompson et al., 2016, Campbell et al., 2017) as a representative of stromal TILs. Pruneri et al. (Pruneri et al., 2017) defined stromal TILs as those within two high power fields form the DCIS ducts and this method was adopted by other authors (Hendry et al., 2017a, Hendry et al., 2017b, Dieci et al., 2018). However, these studies have reported a lack of associations between TILs density and DCIS recurrence. In the current chapter, TILs assessment was performed in a large cohort of DCIS cases with long-term follow-up data using different scoring methods to best determine the most reproducible method that can additionally provide prognostic information.

Assessment of TILs in the training set revealed that touching TILs was the optimal method for TILs assessment in DCIS in terms of predicting patient local recurrence. This conclusion is based on some criteria: first, they had the highest inter-rater concordance rate; second, assessment of touching TILs was the fastest and easiest method that can be performed without the necessity for accurately measuring the distance around the ducts;

third, no uncertainty/variability in scoring cells, particularly in overlapping areas between the adjacent breast ducts, occurred during assessment of touching TILs; lastly, touching TILs showed the strongest association with other clinicopathological prognostic parameters and as a predictor of DCIS recurrence.

Herein, touching TILs showed significant association with LRFS, with dense touching TILs being associated with shorter LRFS in the whole cohort or in patients treated with BCS. It is noteworthy that touching TILs is a prognostic factor for DCIS recurrence in patients treated with BCS independent of other known determinants of tumour behaviour (Stallard et al., 2001, Kerlikowske et al., 2003, Mechera et al., 2009, Kong et al., 2014).

Surprisingly, when assessment of the percentage of overall stromal TILs was done using the same methods for TILs evaluation described in previous studies (Pruneri et al., 2017, Hendry et al., 2017a, Hendry et al., 2017b), it showed not only the least concordance rates but also variability in association with patient outcomes.

Studies of TILs in IBC reported the association between TILs and good prognosis, particularly in Triple Negative breast cancer, and supported their synergistic effect with chemotherapy (Adams et al., 2014, Ibrahim et al., 2014). However, the current study showed that dense TILs in DCIS were associated not only with potential risk factors for aggressive DCIS behaviour but also with increased risk of tumour recurrence and progression (Miligy et al., 2017, Toss et al., 2018). Supporting these

results, dense TILs were associated with more aggressive DCIS (Pruneri et al., 2017), high tumour grade, comedo type necrosis, ER-negative and Her2-positive DCIS lesions (Hendry et al., 2017a, Hendry et al., 2017b). Some studies failed to provide a significant association between TILs density and tumour outcome, and this might be due to a difference in the assessment methods. Recent studies showed that dense TILs were associated with aggressive DCIS and that copy number changes in DCIS with dense TILs are more profound than lesions with low density, making them more liable to progress or recur (Hendry et al., 2017a, Hendry et al., 2017b). In addition, dense TILs were associated with high DCIS Oncotype DX score (Knopfelmacher et al., 2015), providing evidence to support the results that dense TILs are associated with increased recurrence risk in DCIS. Association between dense TILs and poor prognosis has also been described in oral, colonic, prostatic and pancreatic preinvasive lesions (Gannot et al., 2002, Hiraoka et al., 2006).

High TILs density was detected in DCIS lesions compared with IBC. This finding supports the proposed role of inflammatory cells in DCIS progression and aggressiveness (Man et al., 2003, Jiang et al., 2013, Man et al., 2013, Thompson et al., 2016). TILs density assessment and reporting in the routine practice for DCIS diagnosed by core biopsy may provide a predictive factor for presence of invasion in these lesions, as previously observed (Lee et al., 2006, Doebar et al., 2016a).

It is speculated that different immune cell subpopulations are involved in DCIS behaviour. Regulatory T cells (T-regs) play key roles in tumour evasion from the immune system (Gobert et al., 2009). Homeostasis of

the immune response in the body is regulated by T-regs; however, a paradoxical action may occur through over-suppression of the immune cells attacking the tumour cells, leading to tumour progression (Curiel et al., 2004). It was reported that high-grade DCIS lesions harbour a higher percentage of FOXP3+ cells (Campbell et al., 2017). Moreover, tumour cells cuffed by dense TILs might produce some proteins to evade the host immune system. Thompson et al. have reported that DCIS with dense TILs show a higher level of PDL-1-positive tumour cells (Thompson et al., 2016). This was supported by two studies that characterised the immune microenvironment in DCIS (Campbell et al., 2017, Hendry et al., 2017b).

Although the role of B-lymphocytes in tumour immunity and behaviour is unclear, Miligy et al. showed that increased B lymphocytes infiltrate was associated with increased recurrence liability and with other poor outcome parameters (Miligy et al., 2017). In this chapter, the density of CD19 was much lower than that of CD20 and the number of follicles identified by the CD19 antibody was a subset of those identified by the CD20 antibody (Miligy et al., 2017). This finding goes in line with findings in other tumours such as chronic lymphocytic leukaemia, where Ginaldi et al. in 1998 showed that CD19 had low density and a lower number than CD20-positive cells (Ginaldi et al., 1998). This can be explained as a reflection of an early stage of maturation of tumour-infiltrating B lymphocytes in comparison to their counterparts in normal blood (Ginaldi et al., 1998).

In conclusion, assessment of touching TILs using H&E-stained DCIS sections is a reproducible and feasible method to predict tumour

behaviour. Implementation of this method in the clinical setting would help in risk-stratifying DCIS patients for improved and individualised management.

7 General Discussion

The current study demonstrated the importance of a constellation of different clinicopathological factors, proteomics and tumour microenvironment in prediction of DCIS behaviour in terms of the development of local recurrence.

7.1 Background

Incidence of ductal carcinoma *in situ* (DCIS) has increased as screening programmes are widely implemented (Virnig et al., 2010). Given the increasing proportion of screening-detected tumours, clinicians are encountering DCIS with size of <1cm more often than in the past (Cho et al., 2018). A number of clinicopathological factors have been suggested to predict the risk of recurrence and therefore help individualise treatment in DCIS patients (Sagara et al., 2016), although these remain inadequate.

Our inability to elucidate DCIS lesions that will progress to invasion and that will remain indolent culminate in recommendations that women with DCIS undergo treatment (Rakovitch et al., 2018). Most women are treated by BCS followed by adjuvant whole breast RT, which has been postulated to lower the risk of local recurrence (DCIS or invasive) (Correa et al., 2010). Some women will derive little or no absolute benefit from RT, resulting in unnecessary exposure to radiation and its potential toxicities (over-treatment), while in others the omission of RT may result in a higher risk of DCIS or invasive LR that might have been avoided by treatment (under-treatment) (Rakovitch et al., 2013). To reduce over- or under-treatment of DCIS, ascertainment of more

precise estimates of individualised LR risk after BCS is advised to help clinicians and patients more accurately assess the risks of LR with potential absolute benefits of treatment (Rakovitch et al., 2018).

Despite the widely accepted notion that DCIS is a non-obligate precursor of IBC, no reliable biomarkers or clinical tests are available to predict which DCIS cases are most likely to progress (Doebar et al., 2018).

In addition, neither molecular subtype nor histological features/grades are consistent or reproducible predictors of DCIS behaviour. For this reason, it is hypothesised that DCIS becomes invasive as a result of either intrinsic factors (genetic aberrations of the neoplastic cells) or extrinsic factors within the microenvironment or tumour stroma, independent of or in association with genetic changes within the tumour cells (Cowell et al., 2013).

It is widely accepted that in the engagement between histopathological classification, assessment of molecular prognostic biomarkers, ER and HER2 status remains the mainstay of BC management and is currently contributing to DCIS management (Di Cesare et al., 2017). HER2 and ER are important pathways involved in BC development and it is well known that HER2 is an oncogenic factor that is responsible for driving adverse DCIS prognosis (Di Cesare et al., 2017). HER2 amplification in primary DCIS is identified more frequently in younger patients and hypothesises a high risk of recurrence in HER2-positive DCIS perhaps related to histopathological predictors of overall relapse as high nuclear

grade, high Ki67 expression, low ER and PR levels, and other identified clinicopathological factors (Williams et al., 2015).

Different pathways have been recognised to have interactions with HER2, ER, Ki67 and/or p53 including proliferation (Lesurf et al., 2016), oncogenes such as kinases (Finn 2008) and tumour suppressor genes (Nie et al., 2015). Evidence as to whether the identified biomarkers can overwhelm the driving effect on well-established different proteins, especially when both are co-expressed and the potential effect on therapeutic response is insufficient, and needs further investigation.

7.2 Hypothesis, aims and methods of the study

In this thesis, it was hypothesised that the progression of DCIS and the development of ipsilateral LR, particularly invasive recurrence is multifactorial and it is unlikely that a single factor will act as a prognostic determinant; rather, it will be a panel of synergistic clinicopathological and molecular tissue markers that can be used in synergism with each other.

A set of aims were put forward to explore the hypotheses and identify biological markers or molecular predictors for LR able to stratify women with DCIS into high- and low-risk populations and improve risk stratification and treatment recommendations for DCIS patients.

Therefore, this thesis aimed to characterise DCIS patients and determine the clinicopathological and molecular characteristics associated with LR in women with DCIS. This was achieved through

assessment of a large cohort of DCIS cases treated in a single institution with attached all relevant clinical, pathological and outcome information, together with a comparator set of cases mixed with invasive disease and construction of a TMA. This was followed by characterisation of the pure DCIS cohort via IHC and CISH for molecular markers. Studying the expression pattern of a proposed biomarker panel of adverse clinical outcome in DCIS was done by selection of markers spanning important biological changes responsible for reflecting DCIS progression upon their deregulation. As the microenvironment is a major effector on DCIS behaviour, the role of tumour-infiltrating lymphocytes was also assessed as a main constituent of the tumour microenvironment.

7.3 Cardinal findings of the study

7.3.1 Morphological and clinical characterisation of DCIS

In Nottingham, approximately two thirds of DCIS patients were initially treated with BCS, in particular, those with screen-detected lesions. However, mastectomy was the choice for patients with features of aggressive tumour behaviour as high tumour grade with comedo necrosis and large lesion size. The improvement in imaging quality, better evaluation of DCIS size and extent, surgery quality and adjuvant radiotherapy have contributed to the reduction in mastectomy, re-excision after BCS and local recurrence rates.

Despite the surgical margin being adopted as an essential factor in determining the rate of re-excision and recurrence afterwards, the

current study showed that the change in surgical margin width in BCS-treated patients did not, in fact, affect the recurrence rate. Therefore, it is proposed that better risk assessment, rational use of radiotherapy and individualised patient management based on constellation of different clinicopathological factors are essential to tailor DCIS treatment.

This study reported a comparable incidence of LR to previous studies and this rate was associated with high-grade tumours involving more than one breast quadrant and where RT was not an option for treatment.

In addition, it was shown that VNPI was a reasonable risk index for determining the treatment modalities but might not be the best index to predict tumour recurrence.

Therefore, certain clinicopathological factors for DCIS patients remain important in the assessment of patients and determining the treatment modality.

7.3.2 Molecular taxonomy of DCIS using well-established biomarkers

This study demonstrated that cases of Luminal A DCIS subtype were associated with features of good outcome, in contrast to HER2+, Luminal B/HER2+ and Triple Negative phenotypes. HER2 expression was higher in DCIS compared with invasive tumours but at a lower level than the published literature (Rakovitch et al., 2012, Borgquist et al., 2015).

The current study postulated that lower HER2 detection rate might be related to detection of DCIS by imaging and HER2 positivity was shown in the earlier period of the study and declined over time. It therefore suggests a potential role of HER2 as a prognostic marker that needs further studies to prove its precise role, as it was previously addressed by other studies (Borgquist et al., 2015, Mustafa et al., 2017).

This study suggested the need to identify potential predictors of recurrence, as the identification of molecular predictors in DCIS is challenging because the number of recurrences is low, and the expression levels of molecular biomarkers are often correlated. HER2 was shown to predict the DCIS local recurrence.

The current study showed that assessment of HER2 gene amplification was complementary to protein expression, as HER2 2+ cases required to be confirmed their status and CISH was a robust technique for such cases.

7.3.3 Molecular characterisation of DCIS

It was hypothesised that the progression of DCIS to invasion is multifactorial and requires the interplay between genetic factors within tumour cells and the surrounding microenvironment; some identified aberrated genes were studied in terms of their protein expression. In this study, some genes were selected based on the gene expression data for IBC due to the lack of DCIS datasets. These genes have a

distinct role in IBC progression and correlated with adverse outcome, so they were candidates to study in terms of their role in DCIS progression.

In this thesis, TXNIP was shown to have a role as a tumour suppressor gene in the progression of DCIS to IBC, confirming previous studies (Zhou et al., 2011, Morrison et al., 2014). TXNIP is an inhibitor of cell cycle progression as well as interrupting the HER2 oncogenic pathway, evidenced by the inverse association between TXNIP expression, HER2 and Ki67. TXNIP also inhibits the ROS scavenger TRX with direct impact on cell survival by promoting a pro-apoptotic environment, which suggests its potential importance as a therapeutic target.

AURKA was another candidate to study as it is one of the Oncotype DX panel in invasive and *in situ* scores and is located on the commonly mutated 20q. AURKA was reported to have differential expression patterns between normal breast tissue and invasive disease (Moelans et al., 2010, Ingoldsby et al., 2013). The present results were consistent with what has previously been published and showed that AURKA expression was higher in invasive tumours than DCIS. AURKA was a poor prognostic marker for DCIS that acted independently from other clinicopathological factors and it was a predictor of invasive recurrence.

A number of mechanisms were reported to explain the role of AURKA in cancer progression. AURKA overexpression was associated with centrosome amplification and DNA instability (Chou et al., 2013) and the non-mitotic function of AURKA was implicated in breast cancer progression and resistant to chemotherapy treatment via epithelial to

mesenchymal transition and acquisition of stem cell-like characteristics (D'Assoro et al., 2014, Chen et al., 2017). There was evidence that AURKA promotes expression of MYC and aberrant P53 mutation (Marumoto et al., 2005, Zheng et al., 2016). The deregulated mechanism between AURKA and P53 might trigger DNA damage and hence contribute to an adverse clinical outcome.

TPX2, a microtubule-associated protein and a co-factor for AURKA, was a candidate to study. Its overexpression was reported to be associated with centrosome amplification, similar to AURKA (van Gijn et al., 2018). The current work showed, in line with others (Yang et al., 2015, Chen et al., 2016), that TPX2 expression was higher in invasive tumours compared with *in situ* carcinoma, which strengthened the hypothesis that TPX2 was a promising biomarker that requires further functional studies to translate its role in DCIS. The results showed that TPX2 was a marker of poor prognosis in DCIS independent from other studied clinicopathological factors and it was also a predictor of invasive local recurrence.

Different possible mechanisms were reported to explain the role of TPX2 in cancer progression. TPX2 promotes MMPs via the Akt signalling pathway (Wei et al., 2013, Ren et al., 2015) and TPX2 increases the phosphorylation of Akt/mTOR in cancer cells and this suggests that the Akt/mTOR pathway is a potential target of TPX2.

SRC, as a proto-oncogene, showed a similar pattern of expression to AURKA and TPX2, where higher levels were observed in invasive

cancer followed by lower levels in DCIS. It was also associated with poor prognostic parameters and an independent predictor of recurrence, particularly as invasive tumour. SRC was reported to have many biological functions such as promoting tumour cell proliferation, cell growth, differentiation, motility, migration, angiogenesis, and survival (Wheeler et al., 2009). Preclinical evidence showed that SRC binds to HER2 and is activated in HER2-positive breast cancer cells, allowing PI3K signalling (Nagata et al., 2004). Furthermore, anti-HER2 antibody trastuzumab causes dissociation of SRC from HER2, inactivating SRC, and consequently inhibiting HER2-mediated PI3K pathway signalling. This evidence supports the rational combination of SRC inhibitors and trastuzumab in HER2-positive breast cancer, with subsequent implications in DCIS.

7.3.4 Prognostic significance of tumour-infiltrating lymphocytes in DCIS

As the tumour microenvironment is an important contributing factor to DCIS progression, it was important to study TILs as major component of the microenvironment. This thesis showed the detailed optimal methodology for TILs assessment in DCIS and concluded that touching TILs was the most robust type of TILs in predicting patient outcome as well as being the easiest, fastest and most accurate method without the need to measure the distance around the ducts. The current results also showed a significant association between dense TILs and shorter LRFS as well as potential risk factors for aggressive DCIS behaviour, contrary to the previous results, shown in invasive carcinoma (Ibrahim

et al., 2014). This finding might be attributed to more profound copy number changes in DCIS with dense TILs than lesions with low TILs density, making them more aggressive in behaviour (Hendry et al., 2017a). In addition, it was reported that dense TILs were associated with high Oncotype DX DCIS score (Knopfelmacher et al., 2015), supporting the current results of association between dense TILs and poor outcome in DCIS.

A subset of TILs, B lymphocytes, was studied in this thesis and results were similar to whole TILs in terms of their association with increased local recurrence and aggressive tumour behaviour.

7.4 Final conclusions

This study identified several predictors of DCIS progression. In addition to some clinical and pathological variables including tumour size, grade and radiotherapy use, expression of TXNIP, AURKA, TPX2 and SRC, together with TILs density were independent predictors of DCIS outcome. These variables can potentially be used in combination to refine the prognostic stratification of DCIS patients in routine practice **(Table 7-1)**.

Table 7-1: Recommended clinicopathological and biological markers to redict the DCIS outcome based on the final conclusion of the study.

Clinical Features	Multifocal disease Tumour size Radiotherapy status
Histopathological Features	Nuclear grade Comedo necrosis Positive or close surgical margins

Protein Markers	ER HER2 Cell cycle markers Ki67, AURKA, TPX2 SRC TXNIP
Others	Touching TILs

TILs: Tumour infiltrating lymphocytes

The outcome of DCIS dramatically improved during the period of the study. The study demonstrated that the prediction of DCIS outcome is a result of a constellation of different phenotypic and genotypic factors, including the clinicopathological, intrinsic tumour cell and microenvironment factors. The availability of a large cohort of cases was the mainstay of getting robust results and in risk stratifying DCIS patients and predicting disease behaviour. Immunohistochemical assessment of the cellular and molecular derangements is cost-effective, reproducible and clinically applicable; therefore, targeting these molecular allies, through specific therapies, could halt DCIS progression, particularly those targeting specific oncogenes such as AURKA, TPX2 and SRC or tumour suppressor genes such as TXNIP. Characterisation of DCIS using a similar classification system to IBC via well-characterised biomarkers such as ER, PR, HER2 and Ki67 would be helpful in understanding the biology of DCIS. The routine assessment of touching TILs is a powerful predictor of DCIS behaviour that would be helpful tailoring DCIS patients' management.

7.5 Strengths of the study

1- The study was designed to use a large cohort of DCIS patients. In addition, clinicopathological and outcome data were individually

collected, validated and prospectively maintained. Therefore, findings of different studied tissue markers were linked to the robust clinical outcome and IHC-defined intrinsic biological subtypes.

2- The availability of a large collection of FFPE blocks facilitated the proper selection of tumour-containing blocks used for TMA construction of a large cohort of DCIS.

3- The use of automated TMA production allowed for the careful selection and insertion of the tumour cores, proper alignment of the tissue cores, magnificent recording of the samples' information and facilitated the subsequent scoring.

4- In the current study, antibodies were used with positive and negative controls in addition to Western blotting for some of the studied antibodies. In addition, for adequate staining interpretation and statistical correlations, strict adherence to published guidelines of prognostic marker studies was followed throughout (McShane et al., 2006, Sauerbrei et al., 2018).

5- The use of different statistical approaches in determination of the optimal cut-off points of staining positivity of the used markers, rather than using arbitrarily chosen ones.

6- The use of data as both continuous and categorical was helpful in confirmation of the results.

7- Validation of the semi-qualitative results of HER2 protein expression by using CISH which confirms HER2 status in HER2 2+ cases.

8- Using the pathologist's eye approach in markers' expression scoring rather than using automated image analysis methods. An important concern is that automated methods might count *in situ* lesions or some foci of normal mammary epithelial cells entrapped in some tissue cores as hyperplasia, whereas a pathologist's score/count would more likely exclude this potential error.

7.6 Limitations and weakness of the study

1- This study could have possible limitations; for instance, the subjectivity of staining interpretation and qualitative results of immunohistochemical detection of marker expression (Oyama et al., 2007). However, I have followed a standard approach to keep these to the minimum.

2- The data were only assessed in one centre and no multicentre validation was considered.

3- To facilitate studying of tissue markers in large cohorts of samples, the biomarker panel was trained on subsets of full-face sections and studied on TMAs, which has been shown to have specific technical limitations such as core loss. However, TMA results have repeatedly been reported to show excellent concordance with full-face sections of cancer tissues including breast cancer. In addition, previous studies have validated the use of one TMA core to study the expression of tumour marker (Camp et al., 2000, Torhorst et al., 2001).

4- Most of the biomarkers studied showed either nuclear or cytoplasmic expression; however, this does not exclude the

possibility that these biomarkers are expressed in other parts of the malignant cells such as membrane, mitochondrial or Golgi. Therefore, future studies can highlight the importance of subcellular localisation of these proteins.

5- For this historical patient cohort, relatively old paraffin blocks were used to study tissue marker expression, which might be exhausted or consumed in some blocks. Moreover, therapeutic regimens used at the time when patients of this study were treated tended to be different from those currently in practice and many modalities are continually evolving.

7.7 Suggestions and future investigations

1- Assessment of a subset of prospective cases of DCIS in addition to the current series would be of value as a comparator set. This would be regarding different clinicopathological factors, particularly patient age, presentation, tumour size, grade, operative procedure and margin assessment together with hormone receptor and HER2 status.

2- Multicentre assessment of the DCIS cases will be more robust as this will provide the opportunity to rely on the obtained results if deemed parallel with the possibility for their clinical application.

3- Further functional studies are required to validate the protein expression results. *In vitro* knockdown of the identified genes, particularly the oncogenes, would be of value to explore the practicality of therapeutic consideration of the target genes.

4- To highlight the role of subcellular localisation, measuring different cellular components separately, known as subcellular fractionation, is warranted.
5- Using more than one core per case in the TMA could be more beneficial for better assessment, to compensate for the core loss and overcome the heterogeneity if there is any.
6- Genetic and copy number analysis could be a useful tool in predicting DCIS lesions with likelihood of recurrence.

7.8 Ongoing and future work

7.8.1 DCIS and clonal recurrence

A more powerful approach would be to compare DCIS in its pure state with the clonally related invasive recurrence. Such an approach will bypass the difficulties of comparing pure DCIS with unrelated IBC, where inter-tumour heterogeneity reduces the power of the analysis. This novel strategy has yet to be undertaken in more than one case using genome-wide approaches.

Currently, a retrospective study is conducted on a consecutive series of breast conservative surgery treated primary pure DCIS cases and their matched local recurrences diagnosed and treated between 1989 to 2012 with extensive follow-up from three sources: Nottingham City Hospital (UK); Peter MacCallum Cancer Centre (Australia) and Royal Melbourne Hospital (Australia). This study aims to determine the clonal local recurrence rate in DCIS using genome-wide sequencing approaches, compare primary DCIS with their clonal recurrences to

understand the genetic evolution of recurrence and to compare features of DCIS with clonal and non-clonal recurrences.

The study is performed on a carefully selected cohort, comprising tumours treated only with wide local excision but all with clear margins and either with at least seven years of follow-up or with a recurrence at least 12 months but less than five years after initial surgery. The cohort has helped to undertake a comparison of copy numbers between DCIS that have recurred and those that have not. This is the first multicentre study to be undertaken on a large number of DCIS cases and only the second study to report CN changes in DCIS with follow-up data (Gorringe et al., 2015).

7.8.2 Digital image analysis

As current clinicopathological markers are insufficient to accurately predict recurrence risk and guide optimal treatment, a novel method is developed to predict risk of ipsilateral recurrence based on whole-slide, high-content image analysis of digitised H&E stained sections of DCIS tumours removed via BCS, in collaboration with Georgia State University, USA.

The model is able to predict the ten-year recurrence risk in patient cohorts from Nottingham University Hospital, UK. The tool vastly outperforms common clinicopathological variables in predicting DCIS recurrence and identifies patients that might benefit from mastectomy and/or adjuvant radiotherapy.

This easy-to-use machine-learning-based model fills an unmet clinical need for accurately predicting recurrence risk and has tremendous promise for DCIS patients. In the future, this model could potentially be applied to other cancer types that are in need of prognostic markers.

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