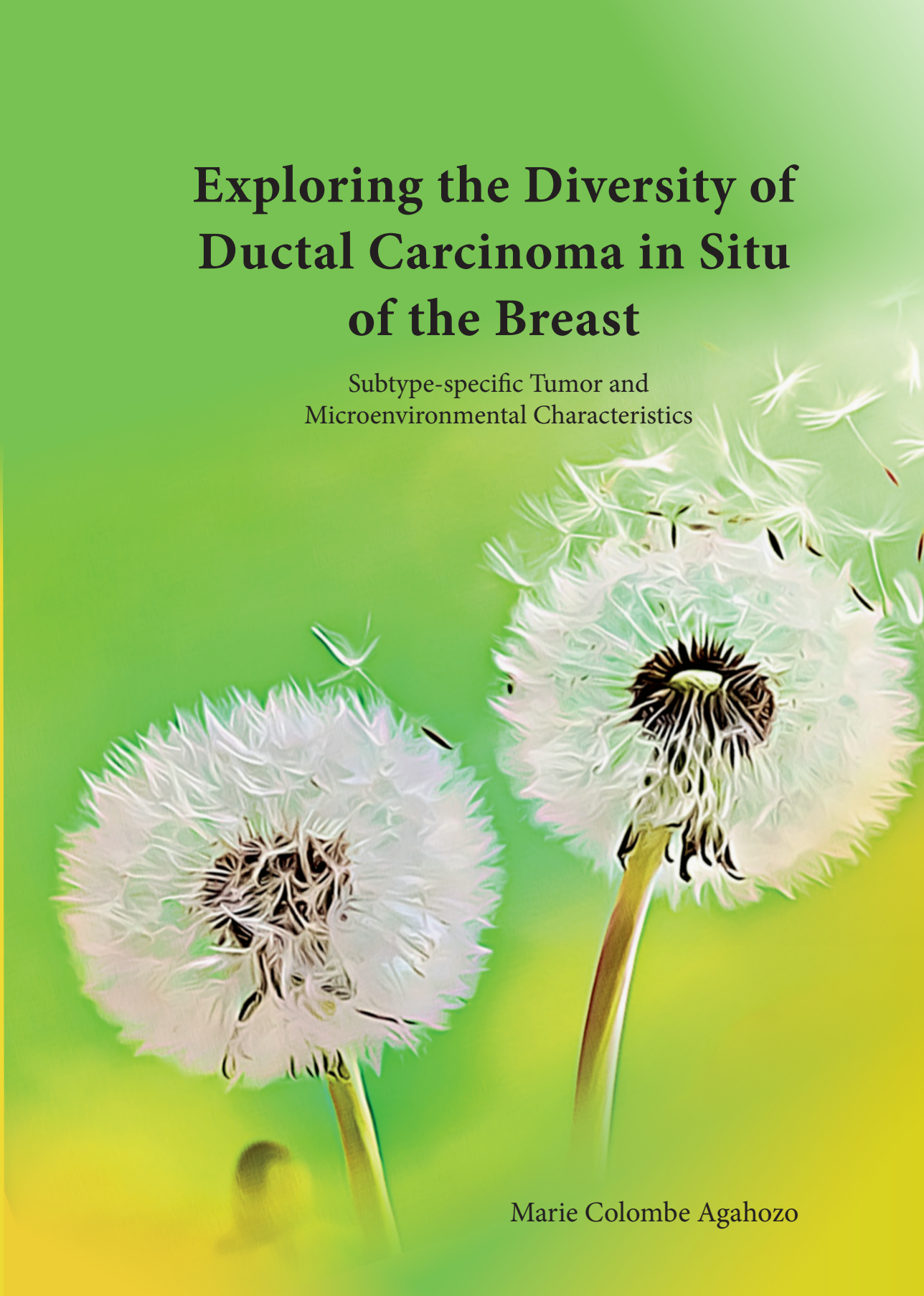
Exploring the Diversity of Ductal Carcinoma in Situ of the Breast

Subtype-specific Tumor and Microenvironmental Characteristics



Marie Colombe Agahozo

Exploring the Diversity of Ductal Carcinoma in situ of the Breast

Subtype-specific Tumor and Microenvironmental Characteristics

De verkenning van de diversiteit van ductaal carcinoom in situ van de borst

Subtype-specifieke tumor- en micro-omgevingskenmerken

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Maman, ce livre est pour toi merci.

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Chapter 1

Introduction

1.1 Breast Cancer Epidemiology

Breast cancer is the most common malignant neoplasm and the leading cause of cancer death amongst women worldwide[1]. In male, breast cancer is very rare, accounting for less than 1% of the yearly diagnosed cases[2]. Breast cancer is a heterogeneous disease, which is generally characterized by different molecular and histological characteristics, which are associated with a different clinical outcome. In the Netherlands, one in seven women will develop breast cancer during her life, meaning that roughly 17.300 women are yearly diagnosed with breast cancer[3]. Since the introduction of mammography and population based breast cancer screening programs, the detection rate of (early) breast cancer has increased[1,3,4]. The steepest increase is observed in ductal carcinoma in situ (DCIS). Currently, over 20% of all breast cancers is diagnosed at the DCIS stage, which accounted for only 5% three decades ago[4,5]. DCIS is considered to be a non-obligate precursor of invasive breast cancer (IBC) and is therefore included in the breast cancer registry[6]. However, the debate remains on whether all DCIS lesions are truly cancers or not[7]. Additionally, the most optimal DCIS specific treatment is yet to be established.

1.2 Classification of Ductal Carcinoma in Situ

The majority of DCIS lesions are non-palpable, presenting with microcalcifications on a mammogram[7,8]. Histologically, DCIS is characterized by proliferating neoplastic epithelial cells that remain confined within the myoepithelial layer[6,9,10]. However, heterogeneous DCIS biological behaviors have been described. While some DCIS cases are more likely to progress into invasive disease, others will have an indolent biological behavior. In 18% of patients who died of unrelated causes, DCIS has been observed postmortem, which suggests an indolent nature[11,12]. It was also reported that a proportion of well differentiated cases remains in situ up to 30 years without treatment[13,14]. Additionally, several cases of DCIS regression have been reported[15–18]. In order to describe DCIS subtypes and their potential behavior, several classifications have been proposed. These classifications are based on DCIS architecture, nuclear atypia, surrogate intrinsic molecular characteristics or gene-expression[19,20].

Historically, DCIS was classified as comedo-type, solid, cribriform, papillary and micropapillary, based on histological architecture[19]. It was suggested that the papillary subtype is more likely to remain indolent while the comedo subtype is more likely to progress into invasive disease. However, multiple architectural patterns can be recognized within one tumor, which reduces reproducibility and applicability[21]. Based on nuclear atypia and cellular differentiation, DCIS can be classified as low, intermediate or high grade[19,22]. High grade DCIS lesions are generally more likely to progress rapidly compared to low grade DCIS, which can stay indolent up to 30 years[13,14,23]. More recently, intrinsic molecular subtypes, which are based on gene-expression profiles, have been described for IBC[24]. Based on their immunohistochemical surrogates, DCIS can also be subtyped based on the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki-67. This results in the following surrogate subtypes: luminal A (ER+ and PR+/-, HER2-, low Ki-67), luminal B (ER+,HER2-, PR-or low and/or high Ki-67 index or ER+ HER2+ with any PR expression and Ki-67 index), HER2-overexpressed (ER-, PR- and HER2+) and basal/triple negative (ER-, PR- and HER2-)[25,26]. These subtypes are highly associated with histological grade

and biological behavior[25]. In IBC, intrinsic subtypes are used to determine (targeted) therapy. A gene-expression profile used to assess risk of re-occurrence and predict outcome is the Oncotype DX DCIS score[20]. This commercially available gene-expression assay, which is a quite robust technique with high reproducibility, is technically challenging and more often used in the USA. However, intrinsic subtypes and gene-expression assay are not implemented yet in daily practice for patients with DCIS, since their value remains unclear. Morphological grading, mainly based on nuclear atypia, therefore remains a widely used method for DCIS classification. Nevertheless, immunohistochemical surrogate subtypes are increasingly used in DCIS research.

1.3 (Over)Treatment of Ductal Carcinoma in Situ

As mentioned above, DCIS has the potential to progress into invasive disease when left untreated. Additionally, there is a risk for an invasive recurrence after treatment, which increases the mortality rate among patients with a primary DCIS diagnosis[27]. Proper DCIS treatment therefore aims to prevent progression and reduce the risk for recurrence, while also reducing overtreatment[28].

In the Netherlands, DCIS is generally treated with surgery and adjuvant radiotherapy depending on the choice of surgery[29]. DCIS surgery includes a mastectomy or breast conserving surgery. Since most DCIS lesions are non-palpable, the lesion has to be localized prior to breast conserving surgery. The most widely used localization methods are wire guided or radioactive seed guided localization[30]. The choice of a particular type of surgery (breast conservation or mastectomy) is based on several factors, including size of the DCIS, the breast size and the patients' choice[29,31]. Generally, a mastectomy is preferred when complete DCIS removal is not possible by breast conservation. The risk for local recurrence after mastectomy is limited (<2%)and the survival rates are around 99%[32–36]. Breast conserving surgery aims to remove the total lesion while preserving the breast. It is however associated with higher rates of local recurrence. An earlier study reported up to 30% recurrence at 15 years follow-up and a more recent study demonstrated a 9% to 51% recurrence rate at 5 year follow up without adjuvant treatment[37,38]. Adjuvant radiotherapy after breast conserving therapy halves the risk for ipsilateral recurrence, with survival rates around 96%[39]. Additionally, the risk for local recurrence is also dependent on surgical margins, whereby wider tumor-free margins are associated with low risk for recurrence[40,41]. On the other hand, increased margin compromises cosmetics[31]. A tumor-free margin >2mm is therefore advised, in combination with radiotherapy[42].

Nonetheless, current DCIS treatment could be considered excessive, since a proportion of all diagnosed DCIS lesions will not become invasive[43]. As a result, a substantial number of patients with DCIS are being overtreated, leading to potential preventable morbidity and costs[4,44,45]. On the other hand, the increased detection rate of breast cancer at the DCIS stage has not resulted in a substantial reduction of invasive or metastatic breast cancer diagnosis, which, compared to DCIS, are typically associated with poorer outcome[4,45]. Consequently, a reduction in breast cancer specific mortality is yet to be observed[27]. Novel preventive and therapeutic strategies are therefore needed to optimize early risk assessment and intervention. It is of major importance to find markers that differentiate low-risk patients from high-risk patients. These findings could reduce overtreatment of low-risk patients and provide effective treatment for high-risk patients. Current active clinical surveillance studies might give more insight in the biological behavior

of DCIS. However, different inclusion criteria are applied and reaching the required number of patients remains challenging.

1.4 From In Situ to Invasive Breast Cancer

In order to predict DCIS behavior in clinical practice, it is important to understand how these lesions progress from in situ to invasive disease. Histological changes of DCIS progression are apparent, which are characterized by a disrupted and eventually lack of the myoepithelial layer. Low grade IBC arises from low grade DCIS and high grade IBC arises from high grade DCIS[46,47]. Molecular features on the other hand are only mildly affected. Gene-expression levels and copy number aberrations of DCIS and its adjacent IBC remain similar[48–52]. However, several theoretical DCIS progression models, based on molecular profiles, have been reported[53]. Although these models describe a strong molecular overlap between DCIS and adjacent IBC, several heterogeneous genetic changes during progression have been reported. These changes include differences in somatic mutations and chromosomal instabilities such as deletions and amplifications.

PIK3CA is one of the most frequently mutated genes in breast cancer and occurs in 16-45% of all patients[54–58]. These mutations were also described in 24-43% of DCIS lesions adjacent to IBC, suggesting their early role in breast cancer[55,59–61]. The majority of PIK3CA mutations is found in ER+ IBC, regardless of the HER2 status[58,62–64]. PIK3CA mutations have been associated with good prognosis. However, their role in breast cancer progression is yet to be described. In male, a major chromosomal instability that is observed in cancer is the loss of Y-chromosome (LOY)[65–70]. It has been described in various types of cancer, including male breast cancer and was associated with increased cancer mortality[65,67,71,72]. A more recent study has also described LOY in male breast cancer and adjacent DCIS and suggested its role in tumor suppression[73]. However, little is known about its frequency in male breast cancer and its potential role during male breast carcinogenesis. Lastly, the amplification of the HER2 gene, which generally results in HER2 overexpression is a frequently observed chromosomal instability[74]. HER2 expression is routinely used in diagnostics and overexpression is observed in about 15% of all breast cancers[75–77]. These HER2 positive IBCs are frequently associated with DCIS and HER2 amplification status is generally similar in both lesions. However, a stronger HER2 expression is more often observed by immunohistochemistry in DCIS compared to the invasive component. Additionally, a heterogeneous HER2 expression pattern is observed in 5-41% of HER2-enriched IBC[78–80]. This does not only imply its role in DCIS progression, but also suggests alternative drivers of progression.

1.5 A Role for the Microenvironment during Progression

In recent years, a substantial role of the DCIS microenvironment during DCIS progression has been suggested[81,82]. DCIS is able to induce major changes in the microenvironment such as stromal changes and influx of immune cells[83,84]. These changes can interact with the DCIS cells to either enhance or suppress tumor growth[15,18,81,82,85]. They could therefore be important mediators of the propensity of DCIS to evolve to invasive disease.

Changes in the DCIS associated stroma, which primarily consists of fibroblasts, are mainly

observed in high grade, HER2-enriched DCIS, as sclerotic or myxoid stroma[15–18,83]. Stromal changes can also be reported by the expression of cancer associated fibroblasts (CAFs), a heterogeneous group of fibroblasts which can be characterized by immunohistochemical markers[86–88]. The presence of sclerotic stroma is regarded as part of DCIS regression, which is a multistep process that leads to a reduced number of neoplastic cells, leaving scar-like structures[15–18]. On the other hand, these stromal changes are associated with an increased risk for IBC following initial biopsy and ipsilateral recurrence[15,83]. However, stromal changes are subjected to high interobserver variability and the quantification and subtyping of CAFs is yet to be optimized[89,90].

DCIS associated immune cells generally consist of tumor infiltrating lymphocytes (TILs)[91]. Data on the presence, composition and clinical significance of TILs in DCIS is emerging. DCIS-associated TILs generally remain stromal and are associated with high grade, triple negative and/or HER2+ DCIS and stromal changes[92–96]. They are composed of high numbers of CD4+ T cells and CD20+ B cells and to a lesser extend CD8+ T cells and CD68+ macrophages[92,97,98]. Overall, a higher TIL density was reported to predict recurrence and a poorer disease free survival[99]. While high numbers of CD4+ T cells, FOXP3+ T cells, B cells and macrophages are associated with shorter recurrence free survival, high numbers of CD8+ T cells are associated with low risk for ipsilateral recurrence[17,97,99–102]. Additionally, CD8+ T cells were associated with regressive changes in HER2+ DCIS[17]. These associations suggest an active role of the different DCIS associated immune cell subsets, which offers opportunities in DCIS treatment. Vaccine strategies for prevention of invasive disease have also shown promising results[103–105]. Additionally, recent data demonstrated a crosstalk between TILs and stromal cells, which emphasizes the complexity of the role of the DCIS microenvironment during progression[106–110]. The DCIS microenvironment is depicted schematically in **Figure 1.1**.

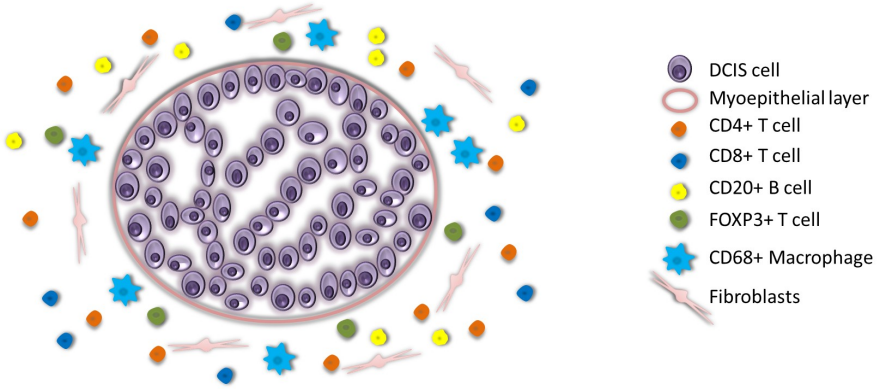

Figure 1.1: The DCIS (immune) microenvironment which consists of CD4+ T cells and CD20+ B cells and to a lesser extend CD8+ T cells, CD68+ Macrophages, FOXP3+ T cells and fibroblasts. These cells might be mediators of the DCIS behavior.

1.6 Thesis Aim and Outline

Based on current data and observations, there are three potential ways of DCIS behaviour; become invasive (1), remain indolent as DCIS (2) or go into regression (3), which is depicted in **Figure 1.2**. In case of evolution to invasive disease, the myoepithelial layer is disrupted and neoplastic cells invade the surrounding breast tissue. When DCIS remains indolent, neoplastic cells continue to proliferate and remain within the myoepithelial layer. During the process of regression, stromal regeneration around the DCIS increases. Hypothetically, it is then able to indulge proliferating neoplastic cells within the myoepithelial layer and push the DCIS into regression, which could make the DCIS disappear.

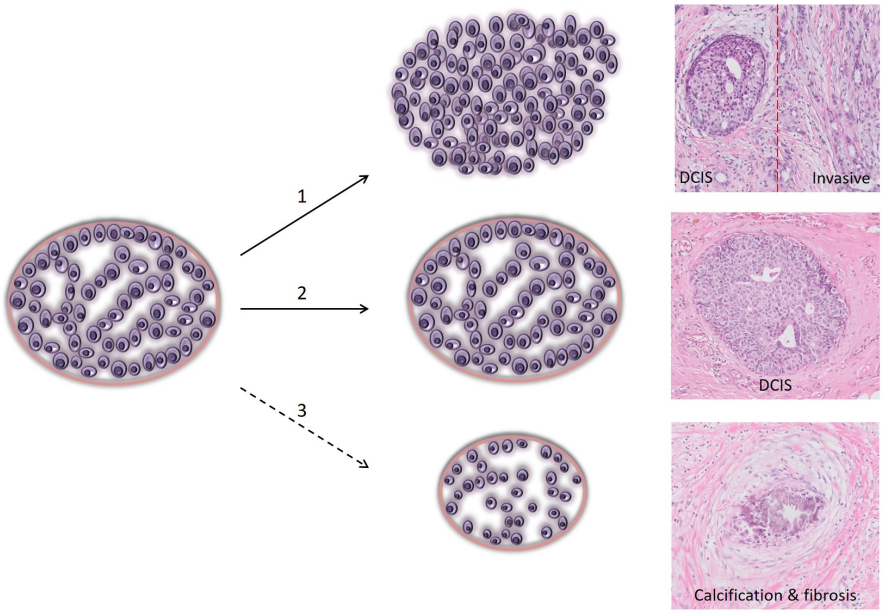

Figure 1.2: Three (potential) pathways of DCIS behaviour; DCIS can become invasive (1), remain in situ (2) or go into regression (3).

To date, markers that differentiate DCIS behavior are still limited due to the lack of data with respect to treatment-naïve DCIS cases. Research historically focused on intrinsic markers of the DCIS cells; however, environmental markers, such as the presence of tumor infiltrating lymphocytes (TILs) and DCIS-associated stroma are emerging. **The aim** of this thesis was therefore to identify internal and environmental DCIS markers that could contribute to DCIS behavior.

Accordingly, this thesis includes three major sections eluting these hypothetical DCIS behaviors, followed by one section including DCIS treatment and a proposed set-up for future research. The first section focuses on DCIS cases that progressed to invasive disease, whereby we tried to understand the mechanism behind the progression from in situ to invasive breast cancer. This section highlights the role of PIK3CA mutations (**chapter 2**), loss of Y-chromosome (**chapter 3**) and HER2 heterogeneity (**chapter 4**) during DCIS progression.

In the second section, we investigated the role of TILs in DCIS subtypes as possible biomarkers of DCIS behavior. First, we reviewed the literature with respect to the role of TILs in DCIS as a double-edged sword in **chapter 5**. **Chapter 6** describes how the different immune cells subsets associate with DCIS subtypes. In **chapter** 7, we elute on gene-expression characteristics of HER2 positive DCIS cells that are associated with the presence of TILs. In chapter 8 we describe a DCIS subtype specific interaction with stromal changes and localization/composition of immune cells.

In the third section, we compare pre-operative DCIS localization methods (**chapter 9**) and propose a retrospective alternative for currently ongoing active surveillance clinical trials (**chapter 10**). We finally conclude this thesis with a discussion of our current results and future perspectives (**chapter 11**).

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Part I

Progression from in situ to invasive breast cancer

Chapter 2

PIK3CA mutations in ductal carcinoma in situ and adjacent invasive breast cancer

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Abstract

PIK3CA is one of the most frequently mutated genes in invasive breast cancer (IBC). These mutations are generally associated with hyper-activation of the phosphatidylinositol 3-kinase signaling pathway, which involves increased phosphorylation of AKT (p-AKT). This pathway is negatively regulated by the tumor suppressor PTEN. Data is limited regarding the variant allele frequency (VAF) of PIK3CA, PTEN and p-AKT expression during various stages of breast carcinogenesis. Therefore, the aim of this study was to gain insight into PIK3CA VAF and associated PTEN and p-AKT expression during the progression from ductal carcinoma in situ (DCIS) to IBC.

We isolated DNA from DCIS tissue, synchronous IBC and metastasis when present. These samples were pre- screened for PIK3CA hotspot mutations using the SNaPshot assay and, if positive, validated and quantified by digital PCR. PTEN and p-AKT expression was evaluated by immunohistochemistry using the Histo-score (H-score). Differences in PIK3CA VAF, PTEN and p-AKT H-scores between DCIS and IBC were analyzed.

PIK3CA mutations were detected in 17 out of 73 DCIS samples, 16 out 73 IBC samples and 3 out of 23 lymph node metastasis. We detected a significantly higher VAF of PIK3CA in the DCIS component compared to the adjacent IBC component (p=0.007). The expression of PTEN was significantly higher in DCIS compared to the IBC component in cases with a wild type (WT) PIK3CA status (p=0.007), while it remained similar in both components when PIK3CA was mutated. There was no difference in p-AKT expression between DCIS and the IBC component.

In conclusion, our data suggest that PIK3CA mutations could be essential specifically in early stages of breast carcinogenesis. In addition, these mutations do not-co-occur with PTEN expression during DCIS progression to IBC in the majority of patients. These results may contribute to further unraveling the process of breast carcinogenesis and this could aid development of patient-specific treatment.

Keywords: PIK3CA; mutation; amplification; PTEN; ductal carcinoma in situ; invasive breast cancer; progression

2.1 Introduction

Invasive breast cancer (IBC) is a heterogeneous disease, consisting of several molecular subtypes, all with a specific tumor biology and clinical outcome[1]. Each of these molecular subtypes, which are based on microarray-based gene expression studies, also have an immunohistochemical surrogate subtype based on Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor (HER2) and Ki-67[2]. The somatic mutation spectrum varies widely across these different subtypes[1]. One of the most frequently described mutations in IBC are present in phosphatidylinositol-4, 5-biphospate-3-kinase, catalytic subunit alpha (PIK3CA) and occur in 16-45% of all patients[3–7]. The majority of these cases are E542K and E545K mutations, located in exon 9, and H1047R mutations, located in exon 20 of the gene[8]. These mutations are mainly observed in ER+ IBC subtypes, which generally have a good prognosis[7,9–11]. PIK3CA mutations are associated with hyper activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which involves increased phosphorylation of AKT (p-AKT) and results in increased cell growth and survival[12,13]. This pathway is negatively regulated by phosphatase and tensin homologue deleted on chromosome 10 (PTEN)[12]. Therefore, impaired PTEN function or loss of PTEN expression also results in uncontrolled activation of the PI3K signaling pathway. PTEN loss is generally associated with a worse disease free and overall survival[14–17]. On the other hand, PIK3CA mutations are generally associated with longer recurrence free survival in IBC patients, though associated with reduced therapy response in HER2+ IBC subtypes[9,18–20]. A reduced therapy response in HER2+ IBC subtypes was also shown in cases with an aberrant PTEN expression[21,22].

Approximately 50% of all IBCs are associated with a synchronous precursor lesion, of which ductal carcinoma in situ (DCIS) is the most common[23,24]. PIK3CA mutations already arise in these pre-malignant lesions. They are reported in 30% of patients with pure DCIS (without an invasive component) and in 24-43% of all DCIS cases with an adjacent invasive component[4,25–27]. These reported PIK3CA mutation frequencies in DCIS are similar to those reported in IBC[26,27]. Furthermore, the type of PIK3CA mutation remains the same throughout breast cancer progression. Next to these concordances, several studies also reported a discordant PIK3CA mutation status in synchronous DCIS and IBC[27–29]. However, data is limited with respect to the variant percentage of PIK3CA mutations during progression from DCIS to IBC.

Loss of PTEN expression has been described in 11-44% of cases with pure DCIS, in 35% of cases with synchronous DCIS and IBC and in 12-38% of cases with IBC[14,30–33]. In synchronous DCIS and IBC, PTEN expression was similar in the DCIS and the invasive component[31]. Studies correlating PIK3CA and PTEN mutations suggest mutual exclusivity[34,35], although data regarding PTEN expression during breast cancer progression is limited.

Current literature regarding p-AKT expression is mainly focused on IBC. Expression of p-AKT has been reported in up to 75% of IBC cases[20,31,36–39]. PIK3CA mutations are associated with high p-AKT expression in triple negative and metastatic breast cancer[28,36,37]. Expression of p-AKT has also been described in 42% of cases with pure DCIS and in 70% of cases with synchronous DCIS and IBC[31]. In matched DCIS and IBC, the expression was similar[31]. The effect of PIK3CA mutations on p-AKT expression at the DCIS stage has to be investigated.

Since PIK3CA mutations are early events in breast carcinogenesis, they might be critical in the earliest steps of breast cancer initiation. Furthermore, it is of interest to know how this might be associated with PTEN and p-AKT expression. Added knowledge on the variant allele fre-

quency (VAF) of PIK3CA and its association with PTEN and p-AKT expression during breast cancer progression could facilitate future development of early intervention studies. Therefore, the aim of this study was to have a better understanding of breast carcinogenesis by in depth analyses of the presence and VAF of PIK3CA mutations and their relation to PTEN and p-AKT expression during the progression from DCIS to IBC.

2.2 Materials and methods

2.2.1 Patient Selection

Patients diagnosed with synchronous DCIS and adjacent IBC and treated at the Erasmus Medical Center – Cancer Institute in the period between 2010 and 2015 were included (n=73). Patients with neoadjuvant treatment, previous breast irradiation or a history of breast cancer were excluded. The tumor cell percentage was estimated beforehand by a pathologist and only samples with an estimated tumor cell percentage $\geq$ 50% were included. DCIS grade was assessed primarily based on cytonuclear differentiation and IBC grade was assessed according to the modified Bloom Richardson score[40]. Immunohistochemistry (IHC) was performed on each IBC, using ER (ER SP1; Ventana Medical Systems, Inc.), PR (PR 1E2; Ventana Medical Systems, Inc.) and HER2 (HER2 4B5; Ventana Medical Systems, Inc.) antibodies. ER and PR status were scored positive when $\geq$10% of the tumor cells were positive, according to the Dutch Breast Cancer Guideline[41]. HER2 status was scored according to international guidelines[42]. For this study, coded leftover patient material was used in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands[43]. Therefore, there was no need for an informed consent or study approval by an ethical committee. **Figure 2.1** provides an overview of the workflow.

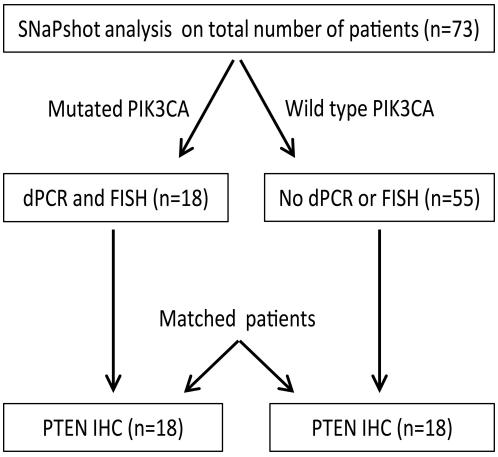

Figure 2.1: Flowchart of the analyzed samples. dPCR = digital PCR, FISH = Fluorescent in situ hybridization, IHC = Immunohistochemistry. Patients were matched on ER, PR, HER2 and tumor grade.

2.2.2 DNA and RNA isolation, sample enrichment and product validation

In addition to the estimated tumor cell percentage assessed by a pathologist, we analyzed *EPCAM, ESR1* and *ERBB2* gene-expression in all lesions. For this, 8 μm thick formalin-fixed paraffin-imbedded (FFPE) tissues of DCIS and adjacent IBC and, if available, corresponding lymph node and distant metastasis were dewaxed and rehydrated, followed by a haematoxylin staining. Subsequently, tissue was microdissected manually, using a sterile scalpel under a stereomicroscope (Zeiss).

Microdissected cells were stored into RNAse/DNase free tubes containing RNALater (Thermo Fisher) at -80°C. RNALater was removed and replaced by xylene after which RNA and DNA isolation was performed with the AllPrep RNA/DNA FFPE isolation kit (Qiagen) according the manufacturer's instructions. RNA concentrations were measured by Nanodrop (Thermo Fisher Scientific) and DNA concentrations with picogreen by Qubit (Thermo Fisher Scientific) and stored at -30°C.

The isolated RNA was used to generate cDNA and analyze the *ESR1, ERBB2* and *EPCAM* expression by reverse transcriptase RT-PCR as previously described[44,45]. In brief, a gene-specific pre-amplification was performed for *ESR1* (Hs00174860_m1), *ERBB2* (Hs01001580_m1) and *EPCAM* (Hs00158980_m1) and three reference genes (*GUSB*, Hs9999908_m1, *HMBS*, Hs00609-297_m1, and *HPRT1*) using the Taqman PreAmp Master mix (Thermo Fisher Scientific) for 15 cycles, followed by Taqman probe-based real time PCRs according the manufacturer's instructions in a MX3000P Real-Time PCR System (Agilent). Gene expression levels were quantified relative to the average expression of *GUSB*, *HMBS*, and *HPRT1* using the 2^(average quantitative cycle (Cq) reference genes – Cq target gene) method.

Prior to use, DNA samples were diluted or vacuum dried to a working concentration of 1 ng/μL in case the original concentration was $\leq$0.5 ng/μL[45]. Next, the PIK3CA targets were enriched in a targeted PCR using Taqman PreAmp mastermix (Thermo Fisher Scientific) and PIK3CA specific exon 9 and exon 20 region primers at a final concentration of 10 nM in a Thermal cycler (Bio-Rad Laboratories, Inc.) (**Table S2.1**, see section on supplementary data given at the end of this chapter). These pre-amplified products were then diluted 10× in LoTE (3 mM Tris-HCl/0.2 mM EDTA, pH 8.0) before further amplification by SYBR-based quantitative PCR (qPCR), using the same primers at a final concentration of 100 μM and SensiFast low ROX mastermix (Bioline). The resulting qPCR products were used for fragment size validation and quantification of the fragment concentrations by microchip electrophoresis with the DNA-500 kit (MultiNA, Shimadzu) and SNaPshot analysis.

2.2.3 Detection of PIK3CA Mutations by SNaPshot Assay

SNaPshot is a rapid and simple assay to detect multiple PIK3CA mutations present in down to 5-10% of the tumor cells in a single analysis[46,47]. The SNaPshot Multiplex System for SNP Genotyping (Thermo Fisher Scientific) was therefore used to identify PIK3CA mutations in our cohort. The qPCR products were first diluted in LoTE to a working concentration of 2.5 ng/μL and were then amplified, using the PIK3CA primers at a final concentration of 1 μM for exon 9 and 0.7 μM for exon 20 (**Table S2.1**). Then, samples were purified using 3 U/Well FastAP and 0.45 U/Well Exonuclease 1 (Thermo Fisher Scientific) after which the SNaPshot ddNTP multiplex probe mix (**Table S2.2**) was used to extend and terminate the reaction as previously described[48]. Finally,

an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific) was used to detect the mutations by electrophoresis. Breast cancer cell lines with known PIK3CA mutation status were used as positive and negative controls. Mutation analysis was performed using GeneMarker V2.7.0 Software (SoftGenetics, LLC).

2.2.4 Quantifying PIK3CA Mutation by dPCR Assay

Digital PCR (dPCR) is a highly sensitive technique that can detect and, in addition to the SNaPshot assay, quantify mutations present in down to 0.1% of tumor cells[20,49]. We therefore used this technique to validate the SNaPshot results and quantify the relative number of PIK3CA mutated copies in both the DCIS and IBC component of those patients with a PIK3CA mutation identified by SNaPshot analysis. Additionally, we also performed a dPCR assay on samples with a wild type PIK3CA mutation (n=33 samples) outcome by SNaPshot to ensure we did not overlook mutations. Mutation specific TaqMan SNP Genotyping Assays (Thermo Fisher Scientific) were used for dPCR analysis. These assays consist of 2 TaqMan probes, one for the wild type sequence and the other one specific for E542K, E545K, H1047R or H1047L PIK3CA mutation (**Table S2.5**). Starting with 1 ng/μL DNA, the PIK3CA targets where first enriched using Taqman PreAmp mastermix (Thermo Fisher Scientific) and the mutation specific TaqMan SNP Genotyping Assays in a Thermal cycler (Bio-Rad Laboratories, Inc.), followed by a qPCR with the same assays and SensiFast Universal Mastermix (Bioline) to determine the optimum loading concentration in de dPCR chip (**Table S2.3**). The mutation specific TaqMan SNP Genotyping assay was then added to these pre-amplified DNA samples along with the Digital PCR V2 Master Mix (Thermo Fisher) (**Table S2.4**). Next, the samples were loaded to a Digital PCR 20K chip and sealed using the QuantStudio 3D Digital PCR Chip Loader, according to the manufacturer's instructions. After loading, a dPCR reaction was performed in a Thermo Fisher ProFlex PCR system. For optimal signal detection, two chip reads were performed using the Thermo Fisher QuantStudio 3D Digital PCR Chip Reader. The samples were individually analyzed online using the QuantStudio 3D AnalysisSuite. PIK3CA mutations in DCIS, IBC and lymph node metastasis were quantified as Variant Allele Frequency (VAF), i.e. defined as the number of PIK3CA mutated copies divided by the number of PIK3CA wild type copies plus the number of PIK3CA mutated copies. Samples were scored as PIK3CA mutant when the VAF was at least 5%.

2.2.5 Assessing PIK3CA Amplification by fluorescent in-situ hybridization (FISH)

Because the relative number of PIK3CA mutations might be influenced by amplification or deletion of the PIK3CA gene, we performed FISH on all patients with a PIK3CA mutation (n=18), using a PIK3CA specific FISH (ZytoLight SPEC PIK3CA/CEN 3 Dual Color Probe; Catalogue number: Z-2140-200, Zytovision). For this assay, consecutive slides were used from the same tissue blocks as previously used for microdissection. First, the 4 μm-thick FFPE tissues were dewaxed, dehydrated and cooked for 13 minutes in citrate buffer. Subsequently, tissue was treated with pepsin for 20 minutes and dehydrated, followed by probe hybridizing for 10 min. at 75°C and overnight at 37°C in a Hybridizer (Dako Agilent). The slides were washed in a stringent wash buffer at 73°C, rinsed in 2× SSC, dehydrated air-dried, sealed with a cover glass and stored at 4°C until further use.

For visualization and analysis, the stained slides were scanned by a wide field fluorescent

microscope (Zeiss, Jena). At least 3 areas of the hybridized region, with the highest tumor cell content were imaged at a 63X magnification using a Z-stack of 3 layers and a 3-by-3 tile. The images were analyzed using a Fiji (ImageJ 1.49s) cell counter plugin. The PIK3CA and CEN3 signals were counted in at least 100 cells containing both signals. PIK3CA amplification was defined as a PIK3CA/CEN3 ratio geq2.0 and/or when the average number of PIK3CA alleles per tumor cell nucleus was geq4.0[50,51].

2.2.6 Quantifying PTEN and p-AKT Expression by IHC

In order to assess potential negative regulation of the PI3K pathway during progression, we performed PTEN and p-AKT IHC on consecutive slides. We included all patients with a PIK3CA mutation (n=18) and matched patients with a WT PIK3CA status (n=18). Matching was based on ER, PR, HER2 expression and tumor grade. OptiView DAB IHC Detection Kit (v1.00.0117, Ventana Medical Systems, Inc.) was used to detect PTEN expression. For this assay, we used the same tissue blocks as previously used for microdissection. Initially, 4 µm-thick FFPE tissues of DCIS and adjacent IBC were incubated on a hot plate over the weekend for optimal sealing of the slides. The staining was then carried out by a BenchMark ULTRA instrument (Ventana Medical Systems, Inc.), using a customized protocol for the PTEN monoclonal antibody (D4.3 XP, 1:200, Cell Signaling Technology) and p-AKT monoclonal antibody (Ser473, D9E XP, 1:100, Cell Signaling Technology). The slides were incubated in citrate buffer at 97°C for antigen retrieval and then with the primary antibody, each for 32 min. Skin and breast epithelial cells were used as positive controls for PTEN. IBC and a breast cancer cell line (MM468) were used as positive controls for p-AKT. DCIS and IBC components were scored separately, blinded for PIK3CA status. PTEN and p-AKT expression was reviewed by two observers and assessed semi-quantitatively using the Histo-score (H-score)[52]. The total H-score varied from 0-300 and was calculated as the staining intensity (0-3) multiplied by the percentage of positive cells (0-100).

2.2.7 Statistical Analysis

SPSS Statistics 21 (IBM) was used for statistical analysis. After testing for normal distribution, we used non-parametric tests to analyze differences in PIK3CA VAF, PTEN and p-AKT expression. The Wilcoxon Signed Ranks Test was used to analyze differences between paired samples and the Mann-Whitney Test was used to analyze the differences in PTEN expression between patients with mutated and wild type PIK3CA. To correlate linear variables, the Spearman Rank Correlation test was used. A Chi-square test was used to analyze associations between groups. P-values (p) <0.05 were considered statistically significant.

2.3 Results

2.3.1 Clinicopathological Characteristics

A total number of 73 patients with paired DCIS and adjacent IBC were included (**Table 2.1**). Median age at diagnosis was 56 years, ranging from 28-102 years. Approximately half of the patients (53.4%) underwent breast-conserving surgery and the majority (94.5%) of patients underwent lymph node staging. Local metastasis was found in half of the examined lymph nodes

and 6 patients developed a distant metastasis. In total, 23 lymph nodes metastasis and 3 distant metastatic lesions were included for PIK3CA mutation analysis.

Table 2.1: Patient and tumer characteristics.

Patient and tumor characteristics (n=73)	*Frequency (%)*
Age at diagnosis (in years)	
- Median (range)	56.7 (28-102)
Diameter IBC (cm)	
- Median (range)	2.10 (0.60 - 11.0)
IBC histological grade	
- Low	2 (2.7)
- Intermediate	28 (38.4)
- High	43 (58.9)
Diameter DCIS (cm)	
- Median (range)	3.00 (0.10-15.0)
DCIS histological grade	
- Low	1 (1.4)
- Intermediate	23 (31.5)
- High	49 (67.1)
Surrogate IHC subtype	
- ER+ HER2-	34 (46.6)
- ER+ HER2+	15 (20.5)
- HER2+	9 (12.3)
- ER- PR- HER2-	15 (20.5)
Lymph node status	
- No metastasis or isolated tumor cells	41 (58.9)
- Micro- or macro-metastasis	26 (35.6)
- No lymph node procedure	4 (5.5)
Distant metastasis	
- Yes	6 (5.5)
- No	67 (94.5)

2.3.2 Presence of PIK3CA Mutations in DCIS and adjacent IBC by SNaPshot Analysis

Overall, a PIK3CA somatic hotspot mutation was detected in 24.7% (18 out of 73) of patients (**Figure 2.2**). The detected mutations were mainly located in H1047R (n=9), followed by E545K (n=5) and E542K (n=4). A PIK3CA mutation was identified in both the DCIS and the IBC component of 14 patients. A discordant PIK3CA mutation status was found in 4 patients, whereby 3 patients had a PIK3CA mutation in the DCIS component only and 1 patient had a PIK3CA mutation in the IBC component only. A PIK3CA mutation was detected in 3 out of the 23 patients with a lymph node metastasis. In these 3 patients, a PIK3CA mutation was also present in both the DCIS and IBC stage. In our cohort, none of the patients with a PIK3CA mutation in either the DCIS component and/or the invasive component developed a distant metastasis. Furthermore, no PIK3CA mutations were identified in the 3 available distant metastases of 3 patients without a PIK3CA mutation in the DCIS and IBC component. When a PIK3CA mutation was detected in the DCIS and/or IBC component of a patient, this concerned the same mutation in all cases. We never observed 2 or more different mutations in the material of one patient.

There were no significant associations between the presence of a PIK3CA mutation in either the DCIS or IBC component and clinicopathologic characteristics. However, the majority of samples with a PIK3CA mutation, 11 out 18 patients, were ER+ and HER2-. This result was not affected by using a cut-off for ER/PR positivity of 1%.

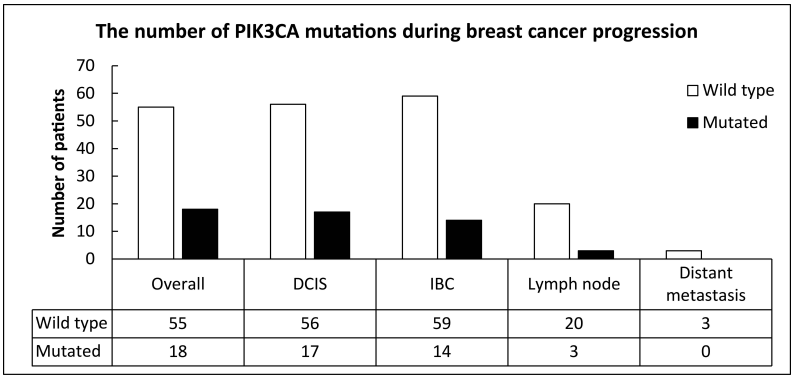

Figure 2.2: The number of PIK3CA mutations during breast cancer progression. Overall, 18 patients with a mutated PIK3CA status (black bars) and 55 patients with a wild type PIK3CA status (white bar) were identified.

2.3.3 VAF of PIK3CA in DCIS and adjacent IBC by dPCR Analysis

All PIK3CA hotspot mutations, as previously detected by SNaPshot analysis, were also detected by dPCR. However, dPCR detected one additional patient with a 6.15% PIK3CA VAF in an IBC lesion which was not detected by the SNaPshot assay. In this patient, the SNaPshot assay only detected a mutation in the DCIS component. Other samples with a wild type PIK3CA outcome by SNaPshot remained wild type after sample check with dPCR. In summary, after validation by dPCR, the total number of discordant PIK3CA mutation status between DCIS and IBC was reduced from 4 to 3 patients: 2 patients had a PIK3CA mutation in the DCIS component only and 1 patient had a mutation in the invasive component only.

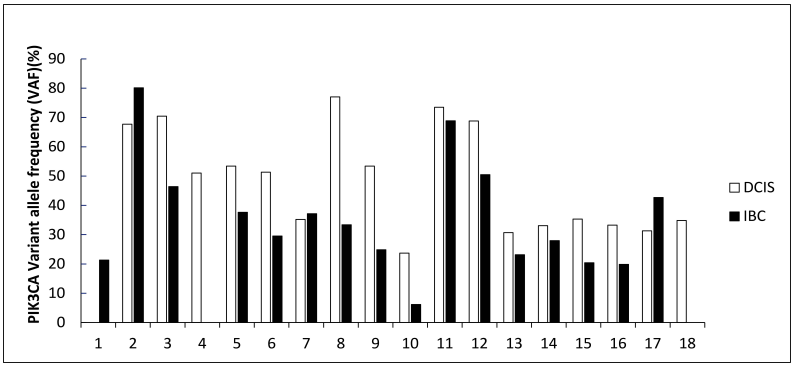

Figure 2.3: The percentage PIK3CA VAF in paired DCIS (white bars) and IBC (black bars). In total, 2 patients (patient number 4 and 18) had a PIK3CA mutation in the DCIS component only and one patient (patient number 1) had a mutation in the invasive component only. The X-axis displays the number of analyzed patients and the Y-axis displays the PIK3CA VAF in %. DCIS, ductal carcinoma in situ; IBC, invasive breast cancer.

After quantification, we found a higher PIK3CA VAF in the DCIS stage (45.8%) than in the IBC stage (31.7%), p=0.007 (**Figure 2.3**) which could not be caused by differences in tumor cell percentage as the mean tumor cell percentage in DCIS was similar to that in IBC (p=0.24) and results were not affected by correction for tumor cell percentage (p=0.018). Additionally, the expression of the epithelial marker EPCAM, ESR1 expression and ERBB2 expression were similar in DCIS and IBC (p=0.910, p=0.177 and p=0.381 respectively).

All 3 patients with a PIK3CA mutation in the lymph node metastases also had a PIK3CA mutation in both the DCIS and the IBC component. The PIK3CA VAF in lymph node metastases were similar to that found in the IBC stage (33.8% vs 30.9%). In one patient with a PIK3CA mutation in the DCIS stage only, no PIK3CA mutation was detected in the lymph node metastasis (**Figure S2.1**).

2.3.4 Assessing PIK3CA Amplification by fluorescent in-situ hybridization (FISH)

Using FISH we detected PIK3CA amplification in 3 out of 18 patients (**Figure 2.4**; patients 3, 4 and 8). The PIK3CA VAF by dPCR in these patients was 70.5%, 51.1% and 77.0% respectively. The number of mutant PIK3CA DNA copies in patients 3 and 8 were up to 3 times higher than the number of wild type PIK3CA DNA copies. In patient 4, the number of wild type and the mutated PIK3CA DNA copies were similar (data not shown). With regard to breast cancer progression, PIK3CA amplification was observed only in the DCIS component. No PIK3CA amplification was observed in IBC lesions or lymph node metastasis. The differences in VAF remained significant after removal of PIK3CA amplified cases.

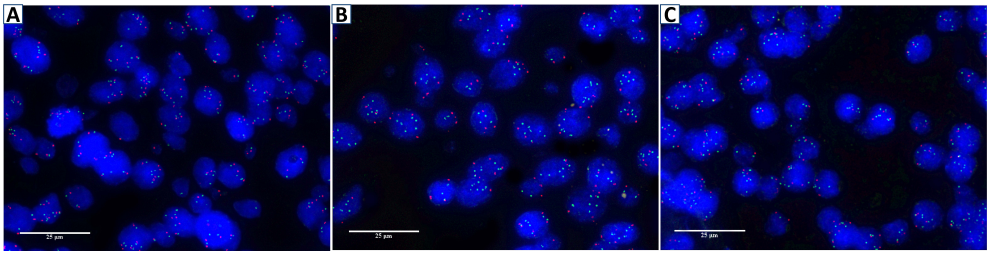

Figure 2.4: Representative FISH images (63× magnification) of cases with a PIK3CA amplification (number of PIK3CA/nucleus $\leq$4) in DCIS of patient 3 (A), patient 4 (B) and patient 8 (C). Blue represents the cell nuclei, red dots represent CEN3 and the green dots represent PIK3CA.

2.3.5 PTEN Expression during Breast Cancer Progression

PTEN was expressed in the cytoplasm in the majority (29 out of 36) of examined patients. Nuclear PTEN expression was observed in 2 cases. Both patients had a wild type PIK3CA status. Overall, PTEN expression had a significantly lower H-score in IBC compared to DCIS (mean ± SD of 111.11 ± 112.43 versus 138.57 ± 120.78 respectively; p=0.004). **Figure 2.5** illustrates a case with a higher PTEN expression in the DCIS component with a wild-type PIK3CA status as compared to the adjacent invasive component. When stratified by PIK3CA mutation status, PTEN expression was lower in the IBC component compared to the DCIS component in patients with

a wild type PIK3CA status (mean H-score ± SD of 90.28 ± 105.20 versus 129.72 ± 118.61, respectively; p=0.007). In patients with a PIK3CA mutation on the other hand, there was no significant difference between PTEN expression in the DCIS component and the IBC component (p=0.14).

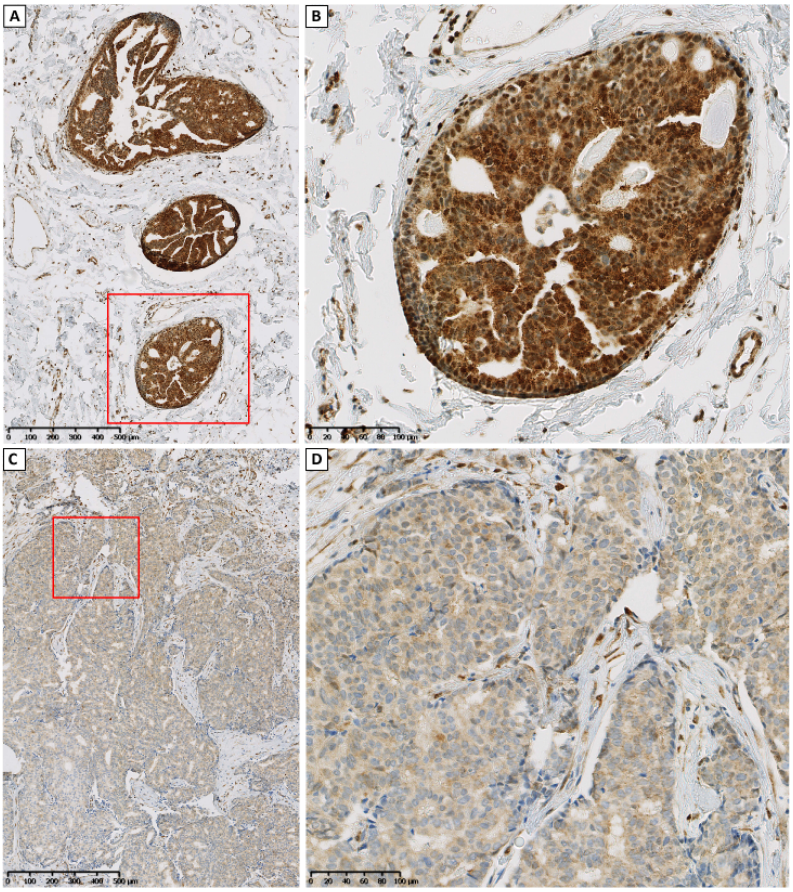

Figure 2.5: The percentage PIK3CA variant allele frequency (VAF) during breast cancer progression. The VAF of PIK3CA mutation in lymph node metastasis was similar to that found in the IBC stage. The X-axis displays the number of analyzed patients and the Y-axis displays the PIK3CA VAF in %. DCIS = ductal carcinoma in situ, IBC = invasive breast carcinoma, LN = lymph node

2.3.6 pAKT Expression during Breast Cancer Progression

P-AKT expression was evaluated on 36 patients (two DCIS samples were excluded due to tissue exhaustion). In total, there was cytoplasmic p-AKT expression in 15 out of 36 patients. Overall, p-AKT expression was low with a median H-score of 0 (range: 0-205 in DCIS and 0-45 in IBC). Seven out of the 15 patients with p-AKT expression had a PIK3CA mutation; the remaining 8 cases were wild type. Six out of 15 patients had p-AKT expression in DCIS and adjacent IBC.

There was no difference in the level of p-AKT expression between these two components. The remaining 9 cases showed a discordant p-AKT expression; 4 patients had expression in the DCIS component only and 5 patients had expression in de IBC component only. **Figure 2.6** provides a heat map of these analyses.

2.4 Discussion

PIK3CA mutations have been frequently described in IBC. However, data is limited regarding its presence and quantity during progression from DCIS to IBC. Therefore, the aim of this study was to have a better understanding of PIK3CA mutations during breast cancer progression. We identified a PIK3CA mutation in 24.7% of our cohort (18 out of 73 patients) by SNaPshot analysis. The majority of these patients (14 out of 18) had a PIK3CA mutation in both the DCIS and the IBC component. A discordant PIK3CA mutation status was found in 4 patients by SNaPshot analyses. This number was reduced to 3 patients after validation and quantification by dPCR. Our results are in line with previous reports on PIK3CA mutations in DCIS and adjacent IBC, regardless of the different detection methods used in these studies[4,26,27,31,53]. Our data supports the presence of PIK3CA mutation heterogeneity in synchronous DCIS and IBC as previously demonstrated[4,27]. Besides, this underlines the early development of PIK3CA mutations during breast cancer carcinogenesis, specifically in ER+ tumors. However, late development of PIK3CA mutations have also been observed[20,28,54]. In those cases, a PIK3CA mutation was found in the metastases and not in the primary tumor, suggesting a role in maintenance of breast cancer metastatic lesions. In our cohort, the low frequency of PIK3CA mutations in lymph node metastasis and the absence of a PIK3CA mutations in distant metastasis suggests otherwise, although the number of metastases was too small for define conclusions.

After pre-screening by SNaPshot analyses, we used dPCR to validate and quantify our results. All mutations identified by the SNaPshot assay were confirmed by dPCR. However, we identified one additional mutation in the IBC stage, which was not detected by the SNaPshot assay. This detection failure by the SNaPshot assay is most likely due to its detection limit of 5-10%[47]. The PIK3CA VAF in this sample was rather low (6.2%). We further quantified the PIK3CA mutation frequency in synchronous DCIS and IBC. Our data showed a significantly higher PIK3CA VAF in the DCIS component compared to the adjacent IBC component, which could not be contributed to differences in the EPCAM, ESR1 or ERBB2 expression as quantified by RT-qPCR. Previous studies on this topic either focused on the IBC stage only or on high-grade DCIS and synchronous DCIS and IBC[20,31]. Sakr and colleagues suggested that PIK3CA mutations in high-grade synchronous DCIS and adjacent IBC are present in a non-modal population of the DCIS component versus a modal population of the IBC cells. These results are rather contradictory to ours, since our data shows the opposite. This outcome could be explained by the use of different detection methods or inclusion criteria. Our results suggest that PIK3CA mutations could be essential in early stages of breast carcinogenesis but might not be necessary for progression from DCIS to IBC. Furthermore, a higher PIK3CA VAF in DCIS compared to IBC suggests subclonal heterogeneity, whereby different clones of malignant cells might have progressed into IBC. However, since PIK3CA mutations are present in both the DCIS stage and the IBC stage in the majority of patients, it could be needed for maintenance of the tumor in the majority of breast cancers.

patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
PIK3CA muation	E542K				E545K					H1047R								
PIK3CA %VAF (dPCR)	0.0	67.71	70.48	51.05	53.4	51.35	35.2	77.02	53.43	23.7	73.5	68.78	30.67	33.06	35.33	33.25	31.29	34.86
PIK3CA/CEN3 ratio (FISH)	1.1	1.1	1.3	1.7	1.0	1.1	1.4	1.9	1.0	0.8	n.a.	1.9	1.0	0.9	1.7	1.1	1.1	1.0
PIK3CA signal / Nucleus (FISH)	1.7	2.1	4.4	5.0	1.8	1.7	2.7	5.9	1.5	1.7	n.a.	3.0	2.3	1.7	1.9	2.4	1.8	1.9
PTEN H-score (IHC)	60.0	25.0	200.0	105.0	20.0	280.0	300.0	300.0	0.0	0.0	20.0	300.0	100.0	n.a.	300.0	15.0	270.0	220.0
p-AKT H-score (IHC)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	205.0	5.0	0.0	0.0	15.0	5.0	0.0	0.0	0.0
ESR1-expression (dCq-qPCR)	-1.93	4.98	9.09	3.87	-3.94	4.38	3.35	3.70	5.01	-1.41	-3.74	4.06	4.11	7.18	2.72	3.84	5.88	13.12
ERBB2-expression (dCq-qPCR)	-2.43	-0.58	-1.33	-1.48	-0.58	-0.31	-1.49	-2.20	-0.52	2.18	2.01	0.74	1.23	1.28	-2.35	-0.85	-0.16	-10.0
EPCAM-expression	-6.07	-10.0	-10.0	-7.36	-10.0	-10.0	-7.38	-8.10	-10.0	-10.0	-8.90	-7.33	-7.28	-10.0	-10.0	-7.95	-7.38	-10.0
PIK3CA %VAF (dPCR)	21.32	80.14	46.42	0.0	37.62	29.56	37.17	33.36	24.85	6.15	68.87	50.46	23.13	27.97	20.38	19.88	42.66	0.0
PIK3CA/CEN3 ratio (FISH)	1.4	1.0	1.3	1.0	1.0	1.1	1.2	1.4	1.0	0.9	1.6	1.1	1.4	0.9	1.8	1.1	1.1	1.0
PIK3CA signal / Nucleus (FISH)	2.0	1.6	3.2	1.8	1.5	1.9	2.3	2.9	2.0	1.7	3.0	1.8	3.5	1.6	2.7	1.8	1.2	1.8
PTEN H-score (IHC)	15.0	30.0	130.0	300.0	0.0	190.0	300.0	140.0	0.0	0.0	0.0	220.0	80.0	300.0	90.0	50.0	300.0	230.0
p-AKT H-score (IHC)	0.0	0.0	n.a.	0.0	0.0	1.0	0.0	n.a.	0.0	45.0	13.0	0.0	0.0	9.0	1.0	0.0	2.0	0.0
ESR1-expression (dCq-qPCR)	-2.96	2.21	5.35	4.38	-1.76	3.29	2.33	4.14	3.36	-3.57	-3.96	-2.17	3.60	7.38	2.26	3.11	5.60	16.28
ERBB2-expression (dCq-qPCR)	-1.42	0.21	0.11	-1.63	-0.59	-1.80	-1.60	-2.73	-2.03	-2.36	1.87	0.48	2.50	2.96	-2.54	-1.52	-1.65	-10.0
EPCAM-expression	-9.69	-9.44	-8.54	-8.55	-8.97	-10.0	-6.20	-6.82	-9.21	-9.36	-9.65	-6.16	-9.53	-10.0	-9.27	-8.14	-9.21	-10.0

Figure 2.6: Heat map of DCIS and IBC results with an overview of performed assays. %VAF = % variant allele frequency of PIK3CA, dPCR= digital polymerase chain reaction FISH = fluorescent in situ hybridization, IHC= immunohistochemistry, qPCR= quantitative PCR.

We detected PIK3CA amplification in the DCIS component of 3 out of 18 patients by FISH. The PIK3CA VAF of 2 out of these 3 patients suggests a PIK3CA amplification of the variant PIK3CA allele, since the mutant versus wild type allele ratio was up to 3. Additional investigations in other cohorts have shown important clinical consequences, since acquired PIK3CA amplifications have been associated with resistance to selective phosphoinositide 3-kinase inhibitors in IBC[55].

With respect to PTEN and p-AKT scoring, there is no standardized scoring system, nor a standardized cut-off point for positive versus negative. We semi-quantified PTEN and p-AKT expression immunohistochemically using the H-score. In general, PTEN expression was significantly lower in the IBC component compared to the DCIS component. Previous studies reported either a similar PTEN expression or a higher frequency of PTEN loss in the IBC stage compared to the DCIS stage using a dichotomous scoring system[31,56]. The latter study is in line with our finding, suggesting decreased PTEN expression at the IBC stage. When we stratified by PIK3CA mutation status, a lower PTEN expression in IBC compared to DCIS was only observed in wild type PIK3CA. In patients with a mutated PIK3CA on the other hand, no significant difference in PTEN expression was observed between these components. In our cohort, we observed co-occurrence of PTEN loss and a PIK3CA mutation in a minority of cases (4 IBC samples). Based on these results, we hypothesize that a PIK3CA mutation could contribute to preservation of PTEN expression during progression from DCIS to IBC, since PIK3CA driven progression does not seem to co-occur with PTEN loss in the majority of patients. However, we did not observe significant differences in PTEN expression between the PIK3CA mutated and wild type cases, which might be due to the small cohort. In our cohort, p-AKT was expressed in 15 out of 36 patients. There was no association between PIK3CA mutation status and p-AKT expression: 7 patients with p-AKT expression had a PIK3CA mutation, 8 patients were wild type. There was a strong overlap between p-AKT expression in the DCIS component and the adjacent IBC component. In those patients with p-AKT expression in both components, there was no difference in the level of expression between DCIS and IBC, which is in line with a previous study[31]. To the best of our knowledge, this is the first study focusing on the presence and quantity of PIK3CA mutations during the progression from DCIS to IBC, including the potential association with PTEN and p-AKT expression. We are also one of the first to report PIK3CA amplification in breast cancer, specifically in DCIS. Nevertheless, our study also has several limitations. First, the number of mutated cases in our series was rather small for further subgroup analyses. Next, our study is restricted to hotspot changes in the PIK3CA gene only. It is also likely that changes in the PIK3CA gene can further interact with other genes, in and outside the PI3K pathway[57–60]. Besides, we only included patients with a substantial DCIS component to be able to isolate enough DNA to perform our analyses, which could have resulted in a selection bias.

In conclusion, we detected a significantly higher PIK3CA VAF in the DCIS component compared to the adjacent IBC component, which suggests that PIK3CA mutations could specifically be essential in early stages of breast carcinogenesis rather than progression from DCIS to IBC. Moreover, we have shown maintained PTEN expression in DCIS and loss of PTEN expression at the IBC stage, which is associated with a wild type PIK3CA. This association suggests that PIK3CA mutations and loss of PTEN expression do not co-occur during DCIS progression to IBC in the majority of patients. In our cohort, there was no association between PIK3CA mutation status and p-AKT expression. Our data might contribute to further unraveling breast carcinogenesis and heterogeneity, which could facilitate patient-specific treatment.

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Supplementary information

Table S2.1: PIK3CA specific PCR primers and working concentrations

Target	Location	Product size (bp)	Forward strand sequence (5'-3')	Reverse strand sequence (5'-3')	Concentration Pre-amplification PCR mix (μM)	Concentration qPCR PCR mix (μM)	Concentration SNaPshot PCR mix (μM)
PIK3CA	Exon 9	139	AGTAACA GACTAGC TAGAGA	ATTTTAG CACTTAC CTGTGAC	0.01	100	1
PIK3CA	Exon 20	109	GACCCTA GCCTTAG ATAAAAC	GTGGAA GATCCAA TCCATTT	0.01	100	0.7

Table S2.2: SNaPshot multiplex probe mix and working concentration

Probe	Location	Mutation	Size (bp)	Sequence (5'-3')	Concentration in probe mix (μM)
E542K	Exon 9	G → A	35	T17 ACACGAG ATCCTCTCTCT	0.15
E545G	Exon 9	A → G	40	T21 CCTCTCTC TGAAATCACTG	0.5
E545K	Exon 9	G → A	45	T25 ATCCTCTCT CTGAAATCACT	0.3
H1047R/L	Exon 20	A → G /A →T	50	T30 GAAACAAAT GAATGATGCAC	0.3

Table S2.3: PCR programs

Assay	Number of cycles	Duration and temperature	Final DNA concentration in Reaction
Pre-amplification PCR program	1 cycle 15 cycles Hold	5 min 95°C 15 sec 95°C 4 min 55°C 0°C	0.25ng/µL
SYBR-based, Sensivast low ROX quantitative PCR program	1 cycle 40 cycles	3 min 95°C 5 sec 95 C 30 sec 55°C 30 sec 72°C, read 30 sec 76°C, read	Not determined
SNaPshot Amplification PCR program	1 cycle 35 cycli 1 cycle	5 min 95°C 45 sec 95°C 45 sec 55°C 45 sec 72°C 10 min 72°C	0.33 ng/µL
Sensifast Universal Mastermix quantitative PCR program	1 cycle 45 cycles	3 min 95°C 10 sec 92°C 30 sec 55°C	Not determined
Digital PCR program	1 cycle 40 cycles 1 cycle Hold	10 min 96°C 2 min 55°C 30 sec 98°C 2 min 55°C 10°C	Not determined

Table S2.4: dPCR dilutions

Reagent	Start dilution	Dilution pre-amp	Dilution qPCR	Dilution dPCR
Taqman PreAmp master mix			n.a.	n.a.
Mutation specific TaqMan SNP Genotyping Assay mix	40×	100×	n.a.	n.a.
Sensifast universal PCR master mix		n.a.	0.5×	0.5×
Mutation specific TaqMan SNP Genotyping Assay	40×	n.a.	0.5×	0.5×

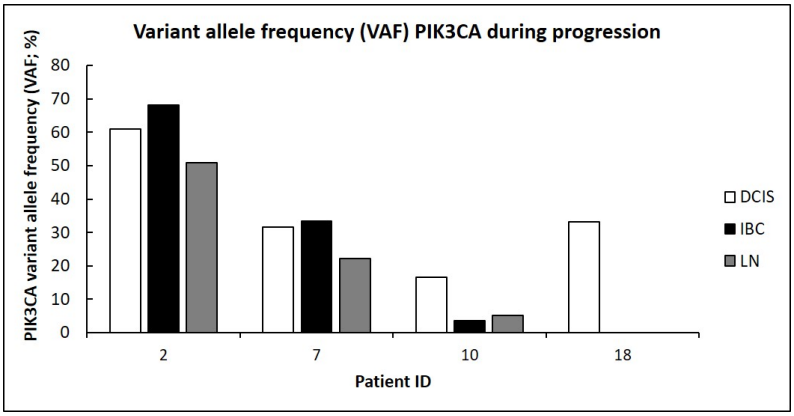

Figure S2.1: The percentage PIK3CA variant allele frequency (VAF) during breast cancer progression. The VAF of PIK3CA mutation in lymph node metastasis was similar to that found in the IBC stage. The X-axis displays the number of analyzed patients and the Y-axis displays the PIK3CA VAF in %. DCIS = ductal carcinoma in situ, IBC = invasive breast carcinoma, LN = lymph node

Table S2.5: Mutation specific TaqMan SNP Genotyping Assays for Digital PCR and working dilutions

Assay name	Product code	Amino acid change	Nucleotide change	Product Size (bp)	Start dilution	Concentration Pre-amplification PCR mix	Concentration qPCR PCR mix	Concentration dPCR mix
PIK3CA_760	Hs000000085_rm	p.E542K	c.1624G>A	252	40x	100x	n.a	0.5x
PIK3CA_763	Hs000000086_rm	p.E545K	c.1633G>A	119	40x	n.a.	0.5x	0.5x
PIK3CA_775	Hs000000088_rm	p.H1047R	c.3140A>G	106	40x	n.a.	0.5x	0.5x
PIK3CA_776	Hs000000089_rm	p.H1047L	c.3140A>T	106	40x	100x	n.a.	0.5x

Chapter 3

Loss of Y-Chromosome during Male Breast Carcinogenesis

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Abstract

Loss of Y-chromosome (LOY) is associated with increased cancer mortality in males. The prevalence of LOY in male breast cancer (BC) is unknown. The aim of this study is to assess the presence and prognostic effect of LOY during male BC progression.

We included male BC patients diagnosed between 1989 and 2009 (n = 796). A tissue microarray (TMA) was constructed to perform immunohistochemistry and fluorescent in situ hybridization (FISH), using an X and Y probe. We also performed this FISH on a selected number of patients using whole tissue slides to study LOY during progression from ductal carcinoma in situ (DCIS) to invasive BC.

In total, LOY was present in 12.7% (n = 92) of cases, whereby LOY was associated with ER and PR negative tumors (p = 0.017 and p = 0.01). LOY was not associated with the outcome. Using whole slides including invasive BC and adjacent DCIS (n = 22), we detected a concordant LOY status between both components in 17 patients.

In conclusion, LOY is an early event in male breast carcinogenesis, which generally starts at the DCIS stage and is associated with ER and PR negative tumors.

Keywords: male breast cancer; loss of Y-chromosome; ductal carcinoma in situ; invasive breast cancer; progression

3.1 Introduction

Male breast cancer (BC) is a relatively rare disease that accounts for less than 1% of all BCs[1,2]. It is generally diagnosed at a later stage compared to female BC, which worsens the outcome[2,3]. Male BC is generally estrogen-receptor (ER) positive, progesterone receptor (PgR) positive, androgen receptor (AR) positive, and human epidermal growth factor receptor 2 (HER2) negative[1,3–5]. Historically, male BC literature was restricted to small single-center studies, thus data regarding male BC biology are relatively limited.

Loss of Y-chromosome (LOY) has previously been described in several solid tumors, including esophageal carcinoma, pancreatic cancer, urothelial bladder cancer, colorectal cancer, and prostate cancer[6–11]. Early case reports described the presence of LOY in male BC tissue[6,8,12]. A larger, more recent study reported LOY in 5 out of 31 patients with male BC. They suggested that LOY could lead to the loss of a candidate tumor suppressor gene (TMSB4Y), resulting in increased cell proliferation[13]. Additionally, they reported LOY in the corresponding ductal carcinoma in situ (DCIS) component in one male BC case, suggesting that LOY could be an early event in male breast carcinogenesis[13]. In the peripheral blood of males, LOY has been reported to be associated with an increased risk of all-cause mortality and non-hematological cancer mortality[14]. Although this study does not mention male BC, they conclude that LOY could become a predictive biomarker for male carcinogenesis. Nonetheless, the presence and role of LOY during breast carcinogenesis remain poorly understood.

The aim of this study was, therefore, to assess the presence and potential prognostic effect of LOY in male BC using a large cohort of patients. Secondary, we studied the presence of LOY during the progression from DCIS to invasive BC by performing fluorescent in situ hybridization (FISH) on paired DCIS and invasive BC.

3.2 Patients and Methods

3.2.1 Patients

This work was approved by the Medical Ethics Committee of the Erasmus MC (approval number MEC 02.953). According to national guidelines, no informed consent was needed for this study. This study included all Dutch male BC cases diagnosed with invasive BC between 1989 and 2009. Central pathology review was performed based on whole tissue slides, including histologic subtype, grade (according to Bloom and Richardson), the presence and type of BC precursor lesions, and density of tumor-infiltrating lymphocytes (TILs), as described previously[4,15,16]. Other clinicopathological data were collected by the Netherlands Comprehensive Cancer Organization (IKNL), including age at diagnosis, tumor size, nodal status, and outcome.

Overall survival was defined as the time between initial diagnosis and death due to any cause. Relapse free survival was defined as the time between initial diagnosis and ipsilateral recurrence, metastasis, or death due to any cause. Breast cancer specific survival was defined as the time between diagnosis and breast cancer specific death.

3.2.2 Immunohistochemistry on Tissue Micro-Array

A tissue microarray (TMA) of all invasive male BC cases was constructed and used to assess ER, PR, AR, and HER2 status. An overview of these antibodies is depicted in **Table 3.1**. Briefly, 4 µm-thick formalin-fixed paraffin-embedded (FFPE) TMA slides were dewaxed, and heat-induced antigen retrieval was performed at antibody specific pH, varying from 6.0 to 9.0. The tissue samples were then incubated with the primary antibody, followed by a hematoxylin counterstain, whereby DAB was used as a chromogen. ER and PR status was classified as positive when the percentage of positive tumor cells was at least 10%, according to Dutch guidelines[17]. The cut-off for AR positivity was also set at 10%[3]. HER2 status was scored according to international guidelines[18]. For this study, coded leftover patient material was used in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands[19].

Table 3.1: Antibody characteristics and used protocol for immunohistochemistry

Antibody	Type	Company	Clone	Lot Number	Dilution	Antigen Retrieval pH	Incubation Time
ER	Anti-mouse	Dako	1D5	M7047	1:40	9	60 min
PR	Anti-mouse	Dako	PgR 636	M3569	1:50	9	60 min
AR	Anti-mouse	ErasmusMC	F39.4	Trapman	1:50	9	Overnight
HER2neu	Anti-rabbit	Dako	Herceptest	K5204	ready to use	ready to use	60 min

3.2.3 Fluorescent In Situ Hybridization on Invasive BC Using Tissue Micro-Array

We performed an XY specific FISH on a TMA of invasive male BC to determine LOY using the Satellite Enumeration (SE) X (DXZ1)/ Y (DYZ3) FISH probe (catalog number: PI-KBI-20030 D1.1, Kreatech™FISH probes, Leica Biosystems, Wetzlar, Germany). This dual-color probe contained a green-labeled (DXZ1) probe for the X chromosome (at Xp11.1-q11.1 with PlatinumBright™495) and a red-labeled (DYZ3) probe for the Y chromosome, (at Yp11.1-q11.1 with PlatinumBright™550). First, 4 µm-thick FFPE TMA slides were dewaxed and dehydrated, cooked for 13 min in citrate buffer, and then treated with pepsin for 20 min. Hereafter, hybridization followed first at 75 °C for 10 min and then at 37 °C overnight in a Hybridizer (Dako Agilent, Stanta Clara, CA, United States). Non-specific binding was removed by a stringent wash buffer at 73 °C. The slides were rinsed in a 2× SCC, dehydrated, air-dried, and sealed with a cover glass. They were stored at 4 °C until further use.

For visualization and analysis, the stained slides were scanned by the Vectra 3 automated quantitative pathology imaging system (Akoya biosciences, Malborough, MA, USA). Selected cores were manually scored using Inform (Akoya biosciences, Malborough, MA, USA), whereby LOY was defined as the absence of Y-chromosome in at least 75% of tumor cells. **Figure 3.1** depicts representative images scored as XY (A) or LOY (B).

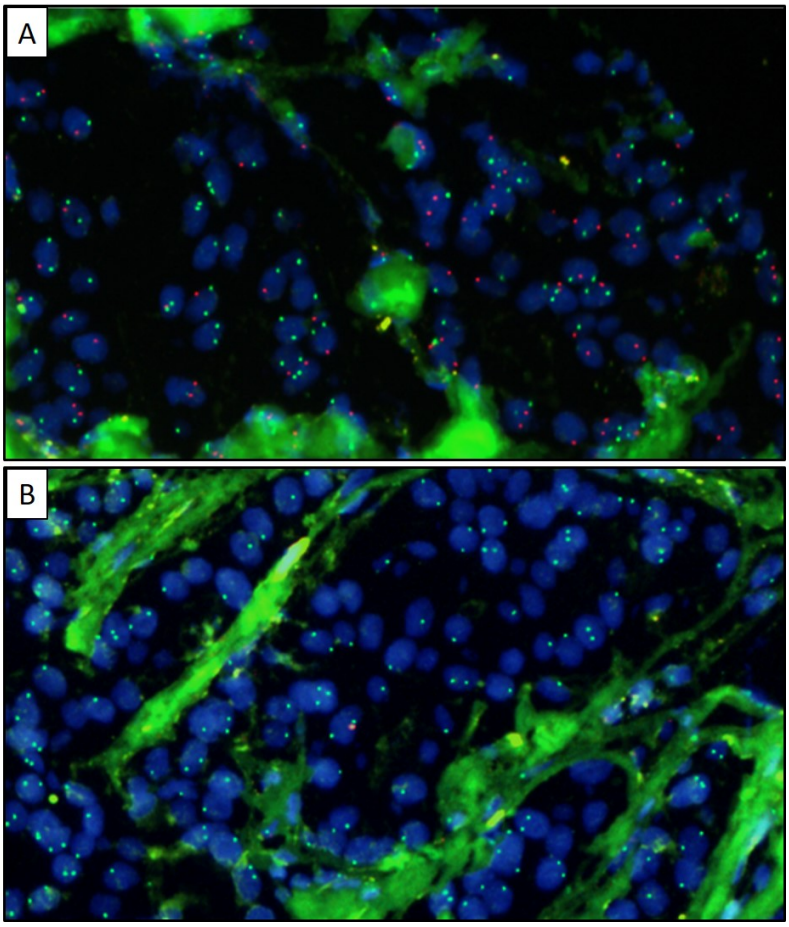

Figure 3.1: Representative images of male BC cases with XY (A) and LOY (B), both at a 40 magnification. The cell nuclei are depicted in blue (dapi), the X-chromosome is depicted in green (FITC), and the Y-chromosome is depicted in red (Texas red).

3.2.4 Fluorescent In Situ Hybridization on Invasive BC and Adjacent DCIS Using Whole Tissue Slides

In total, 22 cases with LOY and 20 cases without LOY in the invasive component on TMA were selected for further analyses to study the pattern of LOY during progression from DCIS to invasive BC. For this purpose, we included patients with a DCIS component in the whole tissue section. The XY FISH was performed on whole tissue sections to assess the presence of LOY in paired DCIS and invasive BC cells. **Figure 3.2** depicts the workflow of our study.

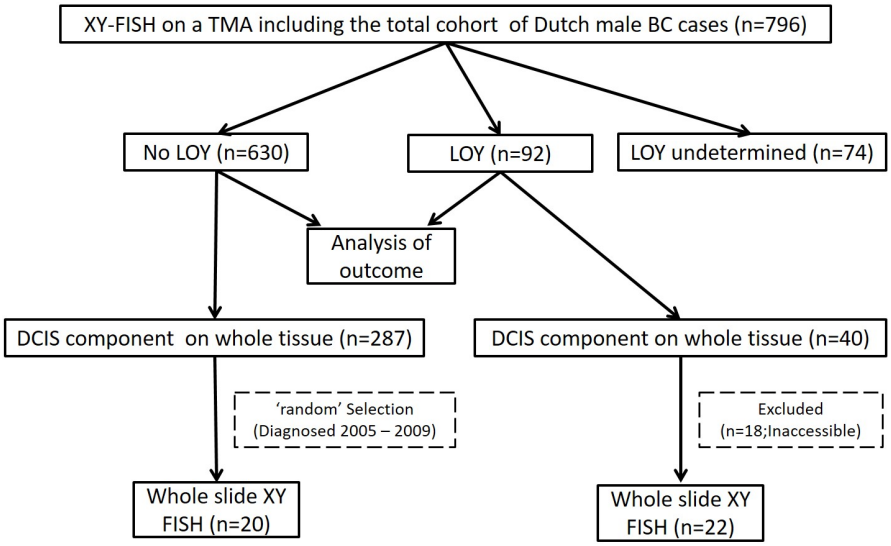

Figure 3.2: Study flowchart. TMA: tissue microarray

3.2.5 Statistical Analysis

The Chi-square test was used to analyze associations between LOY and clinicopathological features. The Mann-Whitney U test was used to compare continuous variables between patients with LOY and those without LOY. A Cox proportional hazards regression was used to examine the overall survival, recurrence-free survival, and breast cancer-specific survival. P-values <0.05 were considered significant.

3.3 Results

3.3.1 General Patients and Tumor Characteristics

A total number of 796 patients were included. **Table 3.2** includes general clinicopathological features of this cohort. The median age at diagnosis was 67 years (range 25 to 98 years). Within this cohort, 46.0% of cases had a breast cancer precursor lesion adjacent to the invasive component. The majority of these precursor lesions (98.4%) were classified as DCIS. Median follow up time was 89 months (range 0 to 323 months), in which 62.1% of the patients died. Recorded breast cancer specific survival was available for only 96 patients, of which 49 patients died due to breast cancer progression.

Table 3.2: Clinic pathological baseline characteristics (n = 796).

Patients and tumor characteristics	n	Range / %
Age at diagnosis (in years)		
- Median-range	67	25-98
Tumor size (in mm) (missing; n=413)		
- Median-range	20	1-110
- Median-range	20	1-110
Grade (%) (missing; n = 30)		
- Low	190	24.8
- Intermediate	401	52.3
- High	175	22.8
Precursor lesion (missing; n = 27)		
- None	403	52.4
- DCIS	360	46.8
- Other	6	0.8
TIL density (missing; n = 32)		
- Minimal-Mild	652	81.9
- Moderate-Severe	112	14.1
ER status (missing; n = 54)		
- ER+	694	93.5
- ER-	48	6.5
PR status (missing; n = 42)		
- PR+	548	72.7
- PR-	206	27.3
AR status (missing; n = 45)		
- AR+	533	71.0
- AR-	218	29.0
HER2 status (missing; n = 24)		
- HER2+	34	4.4
- HER2-	738	95.6
IHC subtype (Undermined; n = 77)		
- ER+PR+/-HER2-	649	90.3
- ER+PR+HER2+	32	4.5
- ER-PR-HER2+	1	0.1
- ER-PR-HER2-	37	5.1
Loss of Y (Undetermined; n = 74)		
- XY	630	87.3
- X_	92	12.7
Distant metastasis (missing; n= 494)		
- Yes	66	21.9
- No	236	78.1
Survival (missing; n = 6)		
- Alive	296	37.5
- Dead	494	62.5

3.3.2 LOY in Male BC and Outcome

The presence of LOY was detected in 12.7% (92 out of 722) of patients. These patients had a median age of 64 years (range 34-98 years). There was a significant association between LOY and ER and PR status (p=0.017 and p=0.01, respectively), whereby LOY was associated with ER and PR negative tumors. There was no significant association between LOY and other tumor characteristics, although there was a trend for an association with tumor grade (p=0.056) (**Table 3.3**). In addition, there was no association between LOY and overall survival, recurrence-free survival or breast cancer-specific survival (Hazard Ratio: 1.23 (95% Confidence Interval (CI) 0.86–1.48), 1.12 (95%CI 0.86–01.46) and 0.47 (95%CI 0.17–1.33) respectively).

Table 3.3: Association of loss of Y-chromosome (LOY) with male breast cancer (BC) clinicopathological characteristics (n = 722).

Patient and tumor characteristics	XY Status XY (range/%)	X_ (range/%)	P-value
Age at diagnosis			0.311
- Median (years)	67.0 (25-95)	64.5 (34-98)	
Tumor size (missing; n = 362)			0.093
- Median (mm)	20.0 (1-110)	21.0 (0-90)	
Grade (missing; n = 26)			0.056
- Low	161 (26.5)	13 (14.7)	
- Intermediate	306 (50.3)	53 (60.2)	
- High	141 (23.2)	22 (25.0)	
Precursor lesion (missing; n = 24)			0.605
- None	317 (52.0)	48 (54.5)	
- DCIS	287 (47.0)	40 (45.5)	
- Other	6 (1.0)	0 (0.0)	
TIL density (missing; n = 28)			0.268
- Minimal-Mild	515 (85.0)	13 (54.2)	
- Moderate-Severe	91 (15.0)	11 (45.8)	
ER status (missing; n = 41)			0.017
- ER+	561 (94.4)	76 (87.4)	
- ER-	33 (5.6)	11 (12.6)	
PR status (missing; n = 34)			0.01
- PR+	448 (74.4)	53 (61.6)	
- PR-	154 (24.6)	33 (38.4)	
AR status (missing; n = 32)			0.327
- AR+	428 (67.9)	67 (72.8)	
- AR-	174 (27.6)	21 (22.8)	
HER2 status (missing; n = 17)			0.542
- HER2+	29 (4.7)	3 (3.3)	
- HER2-	585 (95.3)	88 (96.7)	
IHC subtype (undetermined; n = 60)			0.242
- ER+/-PR+/-HER2-	525 (90.7)	72 (86.7)	
- ER+PR+/-HER2+	27 (4.6)	3 (3.6)	
- ER-PR-HER2+	1 (0.2)	0 (0.0)	
- ER-PR-HER2-	26 (4.5)	8 (9.6)	
Distant metastasis (missing; n = 451)			0.157
- Yes	54 (22.6)	3 (10.8)	
- No	189 (79.4)	25 (89.2)	
Survival (missing; n = 5)			0.469
- Alive	240 (38.4)	30 (33.0)	
- Dead	386 (61.6)	61 (67.0)	

3.3.3 LOY during Progression from DCIS to Invasive BC

Out of the 92 patients with LOY based on TMA, 40 had an adjacent DCIS component in the whole tissue slide. In 22 patients, LOY was analyzed on whole tissue slides, including DCIS and adjacent invasive disease (remaining 18 cases were excluded due to limited tissue availability). **Table 3.4A** depicts the results of the whole slide analysis of these 22 patients. All invasive BC cases (n=22) with LOY based on TMA also had LOY in the whole tissue slide. We did not find LOY in non-tumor breast tissue.

A concordant LOY status between IBC and adjacent DCIS was found in 17 out of 22 patients. In these 17 patients, LOY was detected in both DCIS and invasive BC. A discordant LOY status was detected in four patients. LOY was detected in these four invasive BCs, whereby the corresponding DCIS component did not have LOY. The LOY status of the DCIS component of one case remained undetermined. **Figure 3.3** illustrates a case with a concordant (A and B) and a discordant (C and D) status between the DCIS and the invasive component, respectively. From patients without LOY in the invasive component based on TMA, we selected 20 cases with adjacent DCIS

in the whole tissue slide. None of these cases had LOY, neither in the invasive component nor the DCIS component. **Table 3.4B** depicts the results of the whole slide analysis of the 20 patients without LOY.

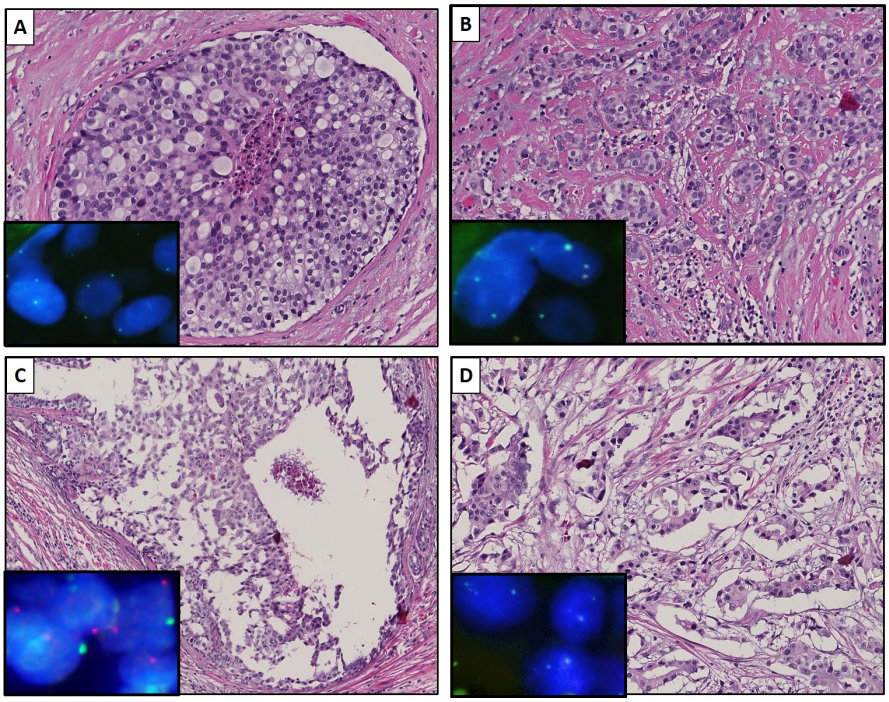

Figure 3.3: Representative images of a male breast cancer (BC) case with a concordant loss of Y-chromosome (LOY) status between ductal carcinoma in situ (DCIS) (A) and invasive BC (B) and a case with a discordant LOY status between DCIS (C) and invasive BC (D). Hematoxylin and eosin staining, at a 15× magnification and insets of the corresponding XY fluorescent in situ hybridization (FISH) images at a 63× magnification. The cell nuclei are depicted in blue (dapi), the X-chromosome is depicted in green (Fluorescein-5-isothiocynate;FITC), and the Y-chromosome is depicted in red (Texas red).

3.4 Discussion

The aim of our study was to assess the presence and prognostic effect of LOY during male BC progression. Our study is the first to describe LOY in a large cohort of male BC patients. Previous studies of LOY in male BC were restricted to blood samples, analyses of the invasive component only, or included small numbers[6,8,12,13]. In the current study, LOY was detected in 12.7% of male BC cases, whereby tumors with LOY were more likely to be ER and/or PR negative. This association suggests that patients with LOY might have more aggressive tumors since ER-negative breast tumors are generally associated with shorter overall survival[3,5,20]. Additionally, the presence of LOY in peripheral blood was also recently associated with all-cause mortality and non-

Table 3.4: Whole slide LOY analysis of patients with LOY in the invasive BC component and adjacent ductal carcinoma in situ (DCIS).

Patient	DCIS	Invasive BC	Patient	DCIS	Invasive BC
A: Patients with LOY in the invasive component (n=22)			B: Patients without LOY in the invasive component (n=20)		
1	X_	X_	1	XY	XY
2	X_	X_	2	XY	XY
3	X_	X_	3	XY	XY
4	X_	X_	4	XY	XY
5	XY	X_	5	XY	XY
6	X_	X_	6	XY	XY
7	X_	X_	7	XY	XY
8	X_	X_	8	XY	XY
9	X_	X_	9	XY	XY
10	XY	X_	10	XY	XY
11	X_	X_	11	XY	XY
12	XY	X_	12	XY	XY
13	X_	X_	13	XY	XY
14	Undetermined	X_	14	XY	XY
15	X_	X_	15	XY	XY
16	X_	X_	16	XY	XY
17	X_	X_	17	XY	XY
18	X_	X_	18	XY	XY
19	X_	X_	19	XY	XY
20	X_	X_	20	XY	XY
21	X_	X_			
22	XY	X_			

hematological cancer mortality[14,21]. However, in our series, there was no association between LOY and survival. A possible explanation for the lack of association between LOY and outcome in our series are missing breast cancer-specific outcome data for the majority of patients in this cohort. Furthermore, our data were restricted to the presence of LOY in breast cancer tissues, whereas data regarding LOY in peripheral blood cells was missing.

In our series of invasive male BC samples with LOY, about 43% were associated with an adjacent DCIS component. We did not detect any LOY in non-tumor breast epithelia by XY analysis on whole tissue slides. This is consistent with the results of Wong and colleagues, who also demonstrated that LOY was restricted to malignant lesions[13]. We detected LOY in the DCIS component in the majority (17 out of 22) of cases with LOY in the invasive component. This suggests that LOY in male BC tissue is a process that is mostly already present in the DCIS component, resulting in LOY in the invasive component. Furthermore, this further supports that DCIS is a precursor lesion of invasive male BC, which is in line with other studies that demonstrated similar molecular aberrations in DCIS and paired invasive male BC[22,23]. This early role for LOY in male carcinogenesis was also previously suggested by Wong and colleagues[13]. Using a functional assay, they showed that clonal LOY contributes to breast carcinogenesis through the deletion of a Y-chromosome expressed tumor suppressor gene. Together with our data, showing an early LOY, this suggests that LOY might contribute to male breast carcinogenesis through dysregulation of the cell proliferation and differentiation mechanism.

The strength of our study is the size of our cohort. This study is by far the largest study reporting on LOY in male BC, including the evolution of LOY during the progression of DCIS to invasive BC. However, our study also had several limitations. First, outcome data are incomplete, as mentioned above, which limits the analyses of the clinical impact of LOY. Second, we included BC samples between 1989 and 2009, thus a substantial proportion of samples were relatively old. This hampered the FISH analysis, resulting in the exclusion of 9.3% of cases due to undetermined XY status. Future studies could also use in vitro models to confirm our findings. However, male BC is rare, making the use of in vitro models, specifically for DCIS, challenging. Additionally, a comprehensive and integrated genomic analysis could be performed to shed more light on the molecular mechanisms that affect or might be affected by LOY in male BC.

In conclusion, we demonstrated that LOY is present in a substantial proportion (12.7%) of male BC cases and that it was associated with ER and PR negative tumors. With regard to progression, there was a concordant LOY status between the DCIS component and the paired invasive component in the majority of cases. We, therefore, suggest that LOY is an early event, which starts in the DCIS stage and mostly results in LOY at the invasive stage.

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Chapter 4

Somatic mutations and copy number variations in breast cancers with heterogeneous HER2 amplification

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Abstract

Intra-tumour heterogeneity fuels carcinogenesis and allows circumventing specific targeted therapies. *HER2* gene amplification is associated with poor outcome in invasive breast cancer. Heterogeneous *HER2* amplification has been described in 5-41% of breast cancers. Here, we investigated the genetic differences between HER2-positive (HER2+) and HER2-negative (HER2-) admixed breast cancer components. We performed an in-depth analysis to explore the potential heterogeneity in the somatic mutational landscape of each individual tumour component.

Formalin-fixed, paraffin-embedded breast cancer tissue of ten patients with at least one HER2- and at least one HER2+ component was micro-dissected. Targeted next-generation sequencing was performed using a customized 53-gene panel. Somatic mutations and copy number variations were analysed.

Overall, the tumours showed a heterogeneous distribution of 12 deletions, 9 insertions, 32 missense variants, and 7 nonsense variants in 26 different genes, which are (likely) pathogenic. Three splice site alterations were identified. One patient had an *EGFR* copy number gain restricted to a HER2- in situ component, resulting in EGFR protein overexpression. Two patients had *FGFR1* copy number gains in at least one tumour component. Two patients had an 8q24 gain in at least one tumour component, resulting in a copy number increase of *MYC* and *PVT1*. One patient had a *CCND1* copy number gain restricted to a HER2- tumour component. No common alternative drivers were identified in the HER2- tumour components.

This series of ten breast cancers with heterogeneous *HER2* gene amplification illustrates that HER2-positivity is not an unconditional prerequisite for maintenance of tumour growth. Many other molecular aberrations are likely to act as alternative or collaborative drivers. This study demonstrates that breast carcinogenesis is a dynamically evolving process characterized by a versatile somatic mutational profile, of which some genetic aberrations will be crucial for cancer progression, and others will be mere 'passenger' molecular anomalies.

Keywords: Breast cancer; HER2 amplification; Intra-tumour heterogeneity; Copy number variations; Somatic mutation; Next-generation sequencing

Conflicts of Interest

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4.1 Introduction

Cancer is a genetic disease, resulting from an accumulation of successive somatic gene mutations that drive cancer cell proliferation[1]. Invasive breast cancer is heterogeneous and comprises different molecular subtypes[2]. Around 12-20% of invasive breast cancers have a *HER2* gene amplification, which generally results in overexpression of the HER2 protein[3–5]. The *HER2* gene, located at 17q12-21, encodes a 185 kDa transmembrane tyrosine kinase receptor. The HER2 receptor has no known ligand of its own but activates other receptors of the HER family by heterodimerization[6]. *HER2* amplification is associated with shorter disease-free and overall survival in patients with node-negative and node-positive invasive breast cancer treated with adjuvant chemotherapy and/or local radiation[7,8]. With the advent of the humanized monoclonal anti-HER2 antibody trastuzumab (Herceptin, Genentech, San Francisco, CA, USA), HER2 has evolved from a mere prognostic marker to a predictive marker and a target for therapy[9]. Since then, the anti-HER2 treatment arsenal has substantially expanded, and current therapeutic options include trastuzumab, pertuzumab (Perjeta, Genentech), trastuzumab emtansine or T-DM1 (Kadcyla, Genentech) and lapatinib (Tykerb, GlaxoSmithKline, Brentford, UK).

Most HER2-positive (HER2+) carcinomas, both in situ and invasive, present with homogeneous HER2 overexpression and amplification, implying that it is a key molecular event that propels cancer cell proliferation. Such genetic events occur early in the process of carcinogenesis and are designated 'truncal' somatic events[10]. However, an intra-tumoural heterogeneous pattern of HER2 amplification is not uncommon. Heterogeneity has been described in 5-41% of HER2+ breast cancers, depending on its definition[11–13]. The latest ASCO/CAP guidelines do not define intra-tumour heterogeneity[14], but previous studies discerned regional from genetic heterogeneity[15–18]. Genetic HER2 heterogeneity is defined as >5% and <50% of infiltrating tumour cells presenting with a *HER2* copy number ≥ 6[18,19]. Regional heterogeneity comprises an amplified tumour component admixed with a negative and/or equivocal tumour component based on immunohistochemistry and ISH studies[11,15,17]. The observed heterogeneity suggests that in some tumours, not all cancer cells are depending on the *HER2* oncogene. Other genomic aberrations might act as potent alternative drivers of cancer cell proliferation and invasion in HER2-negative(HER2-) subclones, such as the previously identified *BRF2* and *DSN1* gene amplification and the *HER2* p.I767M somatic mutation[12].

In the current study, we aimed to further explore the landscape of somatic mutations and copy number variations (CNVs) in HER2-heterogeneous breast cancers. We performed an in-depth analysis of ten breast cancers containing at least two distinct components with different HER2 expression and copy number profiles, designated regional HER2 heterogeneity. We investigated whether these immunohistochemically distinct components were clonally related, and whether the HER2- components were associated with specific molecular aberrations that might act as alternative drivers of carcinogenesis.

4.2 Materials and methods

4.2.1 Patient samples

This retrospective study collected formalin-fixed, paraffin-embedded (FFPE) tissue samples from ten breast cancer patients who were treated between 2010 and 2018 at the Erasmus Medical Center - Cancer Institute (Rotterdam, The Netherlands). Coded leftover patient material was used in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands[20], as previously described[21]. Both core biopsies and resection specimens were eligible. Any histological type of breast cancer was included, provided that the tumour presented with regional heterogeneous HER2 amplification and corresponding heterogeneous HER2 protein overexpression, as previously described[17]. Heterogeneous HER2 status was defined as the presence of at least one HER2+ in situ and/or invasive component and at least one HER2- in situ and/or invasive component, as demonstrated by immunohistochemical and in-situ hybridization (ISH) analysis. These different components had to be in close proximity of one another: all components were present in a single tissue block (with the exception of axillary lymph node metastases, if present). One representative tissue block was selected for all subsequent analyses.

4.2.2 Immunohistochemistry

Four-µm-thick FFPE tissue sections were mounted on Superfrost plus slides (Menzel-Gläser, Germany). Immunohistochemical stainings for oestrogen receptor (ER), progesterone receptor (PR), the myoepithelial cell marker p63, E-cadherin, HER2, FGFR1 and EGFR were performed using an automatic immunostainer (Benchmark XT, Ventana Medical Systems, Arizona), according to the manufacturer's instructions (**Table S4.1**, see section on supplementary data given at the end of this chapter). HER2 expression was assessed according to the ASCO/CAP guidelines[14]. ER and PR expression were scored as percentages, regardless of the intensity. Hormone receptor status was determined according to the ASCO/CAP guidelines[22]. Surrogate molecular intrinsic subtyping was based on the combined ER/PR/HER2 status.

4.2.3 HER2 in situ hybridisation (ISH) analysis

Automated HER2 ISH analysis was performed on all cases using the Benchmark ULTRA (Ventana Medical Systems). Four-µm-thick FFPE tissue sections were deparaffinized and incubated with Cell Conditioning 2 (CC2) buffer at 86 °C for 28 minutes. Tissue sections were treated with ISH-Protease-3 at 36°C for 12 minutes, followed by HER2 probe denaturation at 96 °C for 8 minutes and hybridization at 80 °C for 6 minutes. UltraView SISH was used for detection and haematoxylin II was used as counterstain. Tumour components were considered HER2 amplified when a mean HER2 copy number of $\geq$6 per cell was observed, in accordance with the ASCO/CAP guidelines[14].

4.2.4 DNA extraction

All tissue sections were first reviewed by two breast pathologists (MRVB and CHMVD) who selected tumour areas with an estimated minimum tumour cell percentage of 30%. Ten consecutive FFPE 5-µm-thick tissue sections were deparaffinized and haematoxylin-stained prior to

microdissection. Selected tumour areas and normal tissue areas were micro-dissected manually into 5% Chelex 100 Resin (Biorad) Cell lysis solution (Promega), using a sterile scalpel. DNA was extracted by proteinase K (Roche) digestion by overnight incubation at 56°C. Proteinase K was inactivated at 95°C for 10 minutes. Finally, the samples were centrifuged for 5 minutes at 20.000x g to remove remaining cell debris and Chelex resins. The DNA was collected into new tubes and stored at -80°C until further use. DNA concentrations were measured by a Qubit 2.0 fluorometer (Thermo Fisher Scientific).

4.2.5 Targeted Next-Generation Sequencing

For targeted next-generation sequencing (NGS) a custom-made amplicon panel was applied. This panel comprised 2778 amplicons covering 53 genes (**Table S4.2**), including single nucleotide polymorphisms (SNPs) and hotspot mutation regions. Gene selection for this panel was based on two large tumour profiling studies (ICGC/TCGA and METABRIC), as well as frequently found driver mutations in breast cancer[23,24]. The Ion AmpliSeq Designer tool was used to design amplicons for the multiplex PCR assay, thereby aiming for 150-bp amplicons and allowing efficient amplification of fragmented DNA isolated from FFPE tissue. Full sequence coverage of large exons required amplification and sequencing of overlapping amplicons. Therefore, the multiplexed PCR was split into two reactions, using 10 ng of DNA for each reaction. The Ion AmpliSeq Library Kit Plus (ThermoFisher Scientific, Waltham, USA) protocol was used to process the samples analysed by the Ion AmpliSeq custom 53-gene panel, according to the manufacturer's instructions. Each sample was barcoded using IonXpress barcoded adapters, allowing multiplexed sequencing. A total of 18 PCR cycles were performed. Ten samples were multiplexed on an Ion 540 Chip and sequenced on the Ion S5XL Semiconductor sequencer.

4.2.6 Mutation Analysis

The Variant Caller v5.6.0.4. (ThermoFisher Scientific, Waltham, USA) was used for variant calling. Filtering was performed by the 'somatic low stringency' default of the Torrent Variant Caller. Variants were annotated in a local Galaxy pipeline (`www.galaxyproject.org`) using ANNOVAR[25] (Wang et al., 2010). Exonic and splice site variations were selected for analysis. Synonymous point mutations, as well as variants identified as common polymorphisms in the 1000 Genomes-database (with a frequency of >1%), were removed from the dataset. Variants were kept in the dataset if they had a minimum read depth of 100 reads and if they were present within a tumour component with a frequency higher than 10%. Variants were excluded if a strand artefact was suspected (forward/reverse or reverse/forward ratio of <1/10). For each case, a patient-matched normal tissue sample was analysed to verify whether the identified variants were somatic or germline. Pathogenic and likely pathogenic variants were considered germline if their variant allele frequency (VAF) ranged within 45-55% in the normal tissue sample.

Four prediction algorithms, MutationTaster (`www.mutationtaster.org/`), Provean (`http://provean.jcvi.org/index.php`), UMD-Predictor (`http://umd-predictor.eu/`) and Sift (`https://sift.bii.a-star.edu.sg/`), were used to predict the effects of coding non-synonymous variants. The Cataloque of Somatic Mutations in Cancer (COSMIC; `https://cancer.sanger.ac.uk/cosmic`) was interrogated to assess for previous reports on the selected variants. Variants were selected when at least three of the four prediction algorithms indi-

cated that the variant was pathogenic or probably pathogenic. If this criterion was not met, the variant was retained only if the COSMIC database indicated it was a known pathogenic or likely pathogenic variant. All variants were reported at the cDNA level (c. annotation) and the protein level (p. annotation) according to the Human Genome Variation Society (HGVS) nomenclature[26].

4.2.7 Copy Number Variation Analysis

The presence of high level gene copy number gains was investigated by using the relative coverage, as previously described[27]. Sample normalization was performed to correct for differences in the number of total reads. The normal tissue samples of all patients constituted the reference series. The normalized coverage of the reference series was calculated by dividing the number of reads for each amplicon by the total number of reads for each normal tissue sample. The arithmetic mean was calculated for each amplicon, based on all samples in the reference series. The coverage of each amplicon from the tumour tissue samples was normalized by dividing the number of reads by the total number of reads per tumour tissue sample. The relative coverage for each amplicon of the tumour tissue samples was calculated by dividing the normalized coverage of the sample by the mean normalized coverage of the reference series[27]. Copy number gains were suspected when at least five amplicons clustered together, provided that the Log2 scale of the relative coverage amounted >1,5. Copy number losses were not investigated as the presence of background (due to the use of FFPE tissue samples) hampered reliable interpretation of the presence of copy number losses. Visualisation of CNVs was achieved by the construction of scatter plots in MS Office Excel (Windows).

4.3 Results

4.3.1 Patient Population

Ten patients with a breast cancer with spatially heterogeneous *HER2* amplification were included in this study. This series included eight patients with invasive carcinoma of no special type (NST) and associated DCIS, one patient with invasive lobular carcinoma and associated lobular carcinoma in situ (LCIS), and one patient with metaplastic carcinoma (spindle cell type) and associated DCIS. **Figure 4.1** illustrates the presence of a HER2+ and a HER2- DCIS component, associated with a HER2+ invasive metaplastic carcinoma (patient #1). **Figure 4.2** demonstrates the presence of a HER2+ and HER2- LCIS component associated with a HER2- invasive component (patient #3). In some patients, heterogeneous HER2 amplification was associated with heterogeneous hormone receptor status as well (**Figure S4.1**; **Table 4.1**). All patients underwent nodal staging. Six patients had no sentinel lymph node metastases. Patient #10 had seven axillary macrometastases, with sufficient tissue available for targeted NGS. Patients #4, #9 and #5 had a sentinel lymph node with isolated tumour cells, a single micrometastasis and a single macrometastasis, respectively. These metastases were not analysed due to insufficient amounts of available tumour tissue.

4.3.2 Coverage and Mutation Analysis

Sufficient DNA for sequencing was extracted from all but two tissue samples (**Table 4.1**). The mean percentage of amplicons ≤ 100 and ≤ 500 reads was 94.4% and 78.5%, respectively.

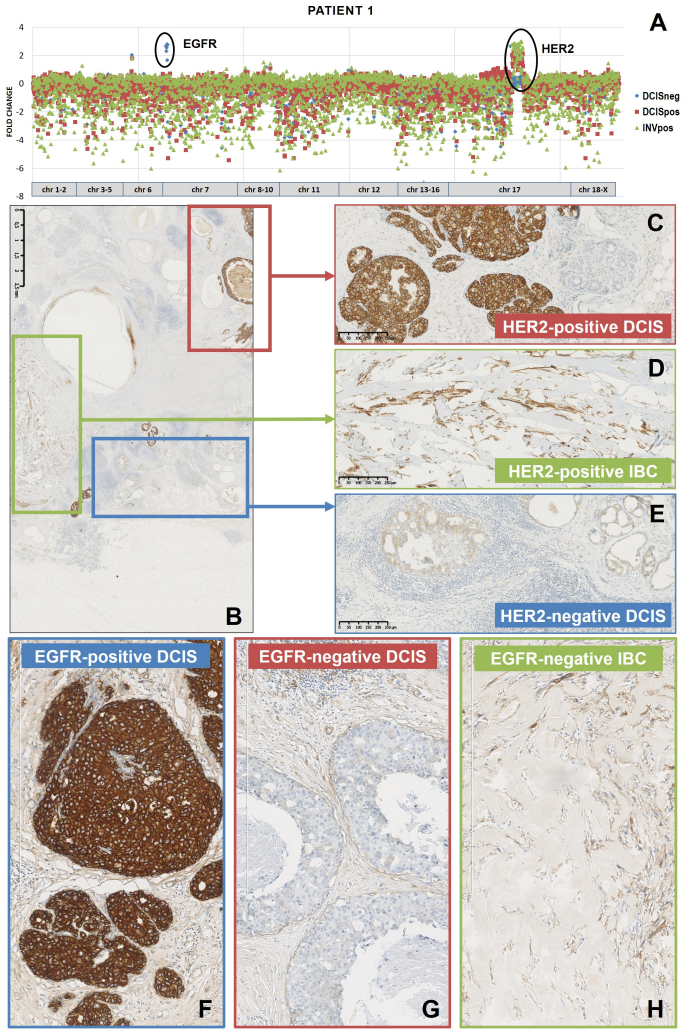

Figure 4.1: Heterogeneous copy number variations and heterogeneous HER2 and EGFR expression in the tumour of patient #1. The scatter plot illustrates the presence of a HER2 copy number gain in one DCIS component and in the metaplastic carcinoma, as well as an EGFR copy number gain in the HER2- DCIS component (A). Immunohistochemistry for HER2, with an overview of breast cancer #1 (B; original magnification 12,5×), and detailed microphotographs of the HER2+ DCIS (C), the HER2+ metaplastic carcinoma (D), and the HER2- DCIS (E; original magnification 100×). Immunohistochemistry for EGFR, which was positive in the HER2- DCIS component (F) and negative in the HER2+ DCIS (G) and in the HER2+ metaplastic carcinoma (H; original magnification 100×).

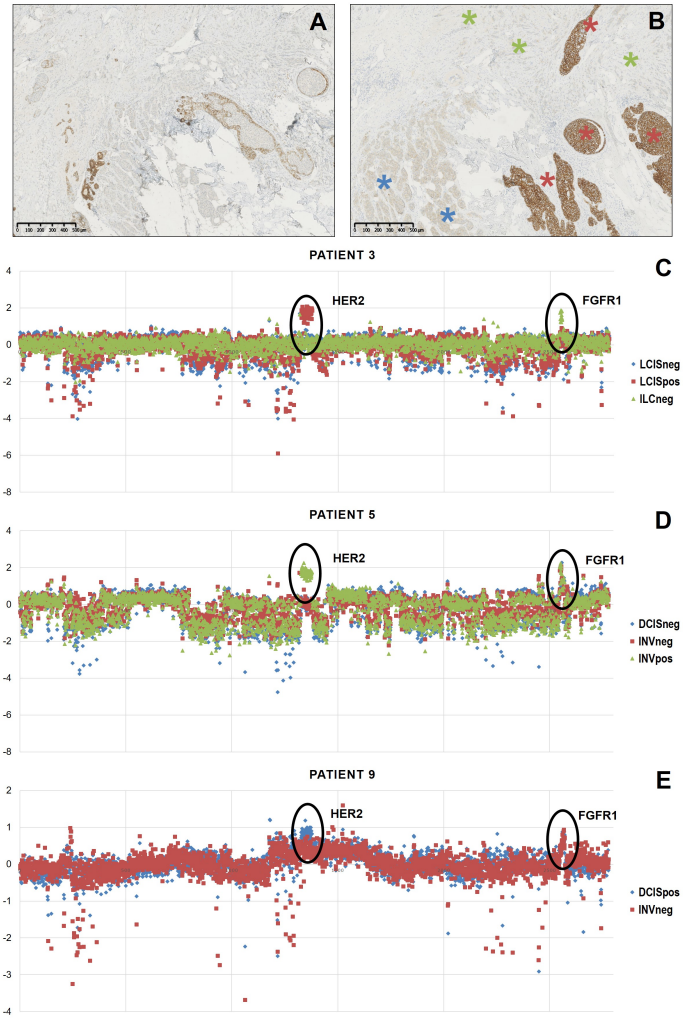

Figure 4.2: Heterogeneous HER2 overexpression and copy number variations in the breast cancer of patient #3. Immunohistochemistry for E-cadherin (A) illustrates the absence of expression in all tumour components (original magnification 50×). Immunohistochemistry for HER2 (B) demonstrates a positive 3+ score in the *HER2* amplified LCIS component (orange asterisks), and an equivocal 2+ score in the *HER2* non-amplified LCIS component (blue asterisks) and the invasive lobular carcinoma of classic type (grey asterisks; original magnification 50×). The scatter plot confirms the *HER2* copy number gain in the HER2+ LCIS (indicated by orange squares) and its absence in the HER2- components (C). Additionally, the presence of an *FGFR1* copy number gain in the invasive lobular carcinoma is noted (indicated by grey triangles).

Table 4.1: Detailed patient and tumour characteristics. Hormone receptor status and HER2 receptor status are indicated for each in situ and invasive tumour component.

ID	Patient age at diagnosis (years)	Nottingham grade	Invasive tumour size (mm)[£]	Tumour and node stage	DCIS grade	Analysed specimen	NAC	In situ component		Invasive component	
								Hormone receptor status		*Hormone receptor status*	
								HER2-	HER2+	HER2-	HER2+
1	50	3	7	pT1bN0	3	Resection	No	DCIS *ER- PR-*	DCIS *ER- PR-*	-	MC *ER- PR-*
2	35	3	16	ypT1c(2) N0	3	Resection	Yes[$]	-	DCIS *ER- PR-*	NST *ER+ PR+*	NST *ER- PR-*
3	51	2	15	pT1c N0	-	Resection	No	LCIS *ER+ PR+*	LCIS *ER+ PR+*	ILC *ER+ PR+*	-
4	51	2	11	pT1c N0(i+)	3	Resection	No	-	DCIS *ER+ PR+*	NST *ER+ PR+*	NST *ER+ PR+*
5	55	3	24	pT2 N1a	3	Resection	No	DCIS *ER+ PR-*	-	NST *ER+ PR+*	NST *ER+ PR-*
6	50	2	12	ypT1c N0	3	Biopsy	Yes	-	DCIS* *ER+ PR-*	NST *ER+ PR+*	NST *ER+ PR-*
7	51	1	21	pT2 N0	2	Resection	No	-	DCIS *ER+ PR-*	NST *ER+ PR+*	-
8	56	3	18	pT1c N0	3	Resection	No	DCIS *ER+ PR+*	DCIS *ER+ PR-*	NST *ER+ PR+*	NST *ER+ PR-*
9	55	2	18	pT1c N1(mi)	3	Resection	No	DCIS* *ER+ PR+*	DCIS *ER- PR-*	NST *ER+ PR+*	-
10	42	3	19	pT1c N2b	3	Resection	No	-	DCIS *ER- PR-*	NST *ER- PR-* Axillary metastasis *ER- PR-*	-

* Single duct, which disappeared during tissue sectioning; not included in this study because of insufficient material for targeted sequencing.
[£] As measured in the resection specimen.
[$] Miller-Payne response grade 3
DCIS: ductal carcinoma in situ; ER+: oestrogen receptor-positive; ER-: oestrogen receptor-negative; ID: patient pseudonym; ILC: invasive lobular carcinoma; LCIS: lobular carcinoma in situ; MC: metaplastic carcinoma; NAC: neoadjuvant chemotherapy; NST: invasive carcinoma of no special type; PR+: progesterone receptor-positive; PR-: progesterone receptor-negative.

The average base coverage depth was 2216 (Supplementary Table 3 in reference[28]). No pathogenic or likely pathogenic somatic variants were detected in *ARID1B, BRCA2, CCND3, CHECK2, ERBB2, ERBB3, MAP2K4, MLL, NCOR1, NOTCH1, PBRM1,* and *PDGFRA*. Overall, germline pathogenic variants were not observed.

We identified sixty-three pathogenic or probably pathogenic variants in 26 different genes (**Table S4.3**), based on four prediction tools and the COSMIC database. These variants included twelve deletions, nine insertions, thirty-two missense variants, seven nonsense variants (with introduction of a stop codon) and three splice site alterations. These somatic aberrations were commonly found in *ARID1A*, *MLL3, NF1, PIK3CA* and *TP53* (**Figure 4.3**). The tumour suppressor gene *TP53* was mutated in at least one component in seven out of ten breast cancers (70%). The *TP53* aberrations included five missense variants, two deletions and one splice site change. The presence of a *TP53* mutation was homogeneously present in all components of the breast cancers of patients #4, #5, and #8. Patients #1, #2, #7 and #10 each presented with a tumour with heterogeneous presence of a *TP53* mutation (**Table 4.2**). Patient #1 presented a p.R248W *TP53* mutation in the HER2- DCIS component and the HER2+ invasive component, which was absent in the HER2+ DCIS component. However, the latter presented with a p.Y234H *TP53* mutation. Patient #2 presented with a p.S241fs deletion in both the HER2+ DCIS and the HER2+ invasive component, whereas the HER2- DCIS component harboured a p.R273C missense variant. Patient #7 showed a p.R209fs *TP53* deletion in the HER2+ DCIS component, which was not detected in the associated HER2- invasive component. Patient #10 showed a p.D259V missense variant in the HER2- invasive component, which was not detected in the HER2+ DCIS, nor in the HER2- axillary metastasis.

Seven out of ten (70%) breast cancers harboured a *PIK3CA* mutation in at least one tumour component. Patients #1, #4, #5, #7 and #9 presented with a breast cancer with homogeneous presence of a *PIK3CA* mutation in each individual tumour component, whereas *PIK3CA* mutations were heterogeneously distributed in the tumours of patients #3 and #6 (**Table 4.2**). Patient #3 showed a p.G1049R missense mutation in the HER2- invasive component, which was not detected in the DCIS components, irrespective of their HER2 status. Patient #6 showed a p.Q546E *PIK3CA* mutation which was present in the HER2- invasive component, and absent in the admixed HER2+ invasive component. Patient #4 had a p.H1047R *PIK3CA* mutation in each tumour component, but the HER2- invasive component harboured an additional p.W1057X mutation, which was not detected in the other tumour components. Somatic *ARID1A, MLL3* and *NF1* mutations were found in at least one component in three, six and four breast tumours respectively, and the presence of these mutations was unrelated to the HER2 status (**Figure S4.2**, **Table 4.2**). For instance, patient #1 showed a p.C327F *MLL3* mutation in the HER2+ carcinoma component, which was absent in both DCIS components. Patient #3 had a nonsense mutation in the HER2- invasive component, which was lacking in both DCIS components. Patient #8 had a missense MLL3 mutation in the HER2+ DCIS and HER2- invasive component, whereas the HER2- DCIS component presented with a different nonsense *MLL3* mutation.

Less common somatic variants were observed in *AKAP9, ATM, BRCA1, CBFB, CDH1, EGFR, ESR1, FBXW7, GATA3, MAP3K1, MED12, MLL2, MLLT4, NFATC2, PTEN, RB1, RNF213, RUNX1, SF3B1, SPEN*, and *TBX3* (**Table S4.4**). Somatic mutations in these genes were often heterogeneously present throughout the different tumour components, and their presence seemed unrelated to the *HER2* amplification status, except for *GATA3* mutations. Somatic *GATA3* mutations occurred in HER2+ tumour components. Patient #2 had a *GATA3* mutation in the HER2+ in

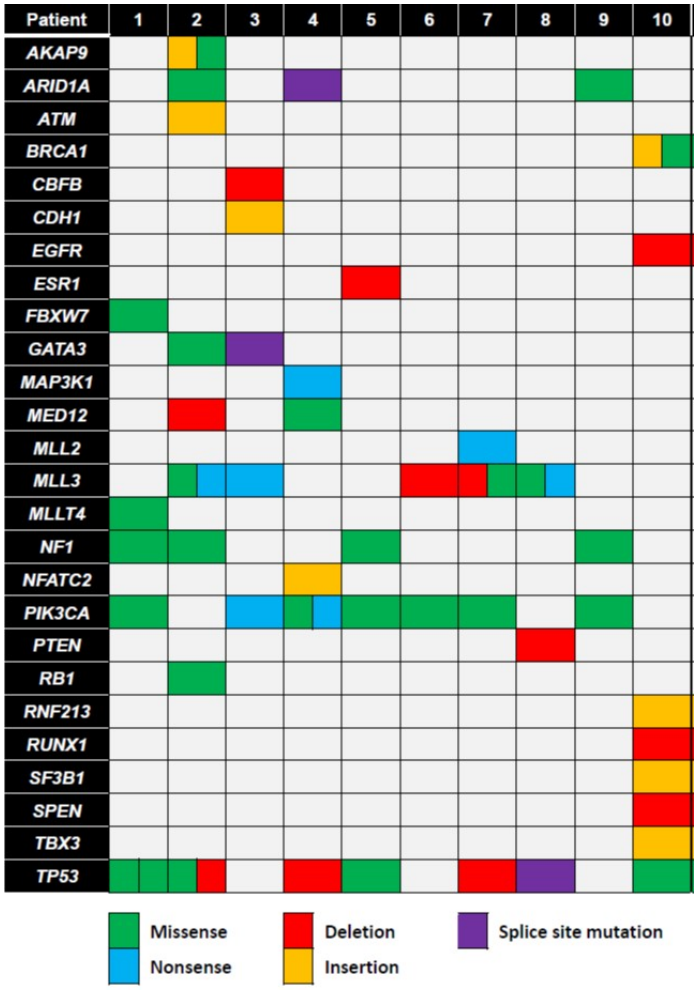

Figure 4.3: Overview of detected pathogenic and likely pathogenic variants in 26 genes per patient. Green and blue squares indicate missense and nonsense mutations, respectively. Red and orange squares indicate deletions and insertions, respectively. Splice site mutations are indicated by purple squares.

Table 4.2: Detailed information on the presence of somatic mutations in the more frequently mutated genes per patient and per tumour component.

Pt	Gene	Chr	Exon	Genbank Transcript ID	Mutation	Protein change	COSMIC ID	Mutation consequence	FS	Presence in in situ component		Presence in invasive component	
										HER2-	HER2+	HER2-	HER2+
1	*MLL3*	7	7	NM_170606	c.980G>T	p.C327F	COSM340281	Missense	No	Absent	Absent	-	Present
1	*PIK3CA*	3	10	NM_006218	c.1624G>A	p.E542K	COSM760	Missense	No	Present	Present	-	Present
1	*TP53*	17	7	NM_000546	c.700T>C	p.Y234H	COSM2744649	Missense	No	Absent	Present	-	Absent
1	*TP53*	17	7	NM_000546	c.742C>T	p.R248W	COSM10656	Missense	No	Present	Absent	-	Present
2	*ARID1A*	1	3	NM_006015	c.1778C>T	p.S593F	-	Missense	No	-	Absent[$]	Absent [$]	Present
2	*MLL3*	7	7	NM_170606	c.851G>A	p.R284Q	COSM1179107	Missense	No	-	Present	Absent	Present
2	*MLL3*	7	36	NM_170606	c.6439C>T	p.Q2147X	-	Nonsense	Yes	-	Absent	Present	Absent
2	*RB1*	13	8	NM_000321	c.748C>T	p.P250S	COSM5730807	Missense	No	-	Absent [$]	Present	Absent
2	*RB1*	13	8	NM_000321	c.784C>T	p.R262W	COSM5047280	Missense	No	-	Absent [$]	Present	Absent
2	*TP53*	17	7	NM_000546	c.723delC	p.S241fs	COSM45831	Deletion	Yes	-	Present	Absent	Present
2	*TP53*	17	8	NM_000546	c.817C>T	p.R273C	COSM10659	Missense	No	-	Absent	Present	Absent
3	*MLL3*	7	14	NM_170606	c.2447dupA	p.Y816_I817delinsX	-	Nonsense	Yes	Absent	Absent	Present	-
3	*PIK3CA*	3	21	NM_006218	c.3145G>C	p.G1049R	COSM12597	Missense	No	Absent	Absent	Present	-
4	*ARID1A*	1	12	NM_006015	c.3425_3430AGGGC	p.?	-	Splice site change	?	-	Present	Present	Present
4	*PIK3CA*	3	21	NM_006218	c.3140A>G	p.H1047R	COSM775	Missense	No	-	Present	Present	Present
4	*PIK3CA*	3	21	NM_006218	c.3170G>A	p.W1057X	-	Nonsense	Yes	-	Absent	Present	Absent
4	*TP53*	17	6	NM_000546	c.578delA	p.H193fs	COSM45856	Deletion	Yes	-	Present	Present	Present
5	*PIK3CA*	3	21	NM_006218	c.3140A>G	p.H1047R	COSM775	Missense	No	Present	-	Present	Present
5	*TP53*	17	8	NM_000546	c.824G>A	p.C275Y	COSM10893	Missense	No	Present	-	Present	Present
6	*MLL3*	7	43	NM_170606	c.10224_10225del	p.Q3408fs	-	Deletion	Yes	-	-	Absent	Present
6	*PIK3CA*	3	10	NM_006218	c.1636C>G	p.Q546E	COSM6147	Missense	No	-	-	Present	Absent
7	*MLL3*	7	20	NM_170606	c.3258delC	p.S1086fs	-	Deletion	Yes	-	Absent	Present	-
7	*MLL3*	7	14	NM_170606	c.2468T>C	p.I823T	COSM6506545	Missense	No	-	Present	Present	-
7	*PIK3CA*	3	10	NM_006218	c.1633G>A	p.E545K	COSM760	Missense	No	-	Present	Present	-
7	*TP53*	17	6	NM_000546	c.626_627del	p.R209fs	COSM45817	Deletion	Yes	-	Present	Absent	-
8	*MLL3*	7	7	NM_170606	c.851G>A	p.R284Q	COSM1179107	Missense	No	Absent	Present	Present	Absent
8	*MLL3*	7	14	NM_170606	c.2447dupA	p.Y816_I817delinsX	-	Nonsense	Yes	Present	Absent	Absent	Absent
8	*TP53*	17	(intron)	NM_000546	c.782+1G>T	p.?	COSM473431	Splice site change		Present	Present	Present	Present
9	*ARID1A*	1	3	NM_006015	c.1778C>T	p.S593F	-	Missense	No	-	Absent	Present	-
9	*PIK3CA*	3	21	NM_006218	c.3140A>G	p.H1047R	COSM775	Missense	No	-	Present	Present	-
10	*TP53*	17	7	NM_000546	c.776A>T	p.D259V	COSM43724	Missense	No	-	Absent	Present [°]	-

Chr: chromosome; FS: frameshift; ID: identity; Pt: patient

[°] The mutation was not detected in the HER2- axillary metastasis.

[$] Low coverage of the corresponding amplicon (i.e. <100 reads)

situ and invasive components, which was absent in the HER2- invasive carcinoma component. Patient #3 had a *GATA3* splice site mutation in the HER2+ LCIS component, which was not observed in the HER2- in situ and invasive components.

4.3.3 Copy Number Variation Analysis

The presence of high level CNVs was investigated, and confirmed the presence of *HER2* amplification in all HER2+ carcinoma samples, which served as an internal quality control (**Figures 4.1,4.2, S4.1 and S4.2**). Additionally, we observed an *EGFR* copy number gain in the HER2- DCIS component of patient #1 (**Figure 4.1**). Patients #3 and #5 had an *FGFR1* copy number gain in at least one tumour component. In patient #3, the HER2- invasive lobular carcinoma harboured this *FGFR1* amplification (**Figure 4.2**), which was absent in the HER2- and HER2+ LCIS components. In patient #5, all tumour components displayed the *FGFR1* copy number gain. Patients #9 and #10 both had a HER2- tumour component with a gain of 8q24, which comprised a copy number gain of both *MYC* and the adjacent long non-coding RNA (lncRNA) plasmocytoma variant translocation 1 (*PVT1*). Patient #9 also had a *CCND1* copy number gain in the HER2- tumour component. The potential presence of copy number losses was difficult to interpret with certainty, as some amplicons showed a consistently lower coverage throughout this series. The use of FFPE samples caused a relatively high background, which further hampered the assessment of any potentially relevant deletions.

4.3.4 Complementary Immunohistochemical Analysis

Immunohistochemistry for EGFR was performed on all tumour tissue samples. In patient #1, the identified EGFR amplification in the HER2- DCIS component was associated with EGFR protein overexpression (**Figures 4.1F-4.1H**). No EGFR protein overexpression was noted in the other tumours (data not shown). Immunohistochemistry for FGFR1 was performed on tumour tissue samples of patients #3 and #5, which revealed no apparent positivity in either of the tumour components (data not shown).

4.4 Discussion

Carcinogenesis is an evolutionary process governed by the principles of Darwinian dynamics[29]. Tumours are clonal proliferations, originating from a single cell that acquired genomic instability through an accumulation of somatic mutations. Early genomic anomalies, including crucial oncogenic drivers, will therefore be present in all tumour cells and constitute clonal molecular aberrations. Acquisition of additional oncogenic drivers and passenger mutations will result in subpopulations of cancer cells with different genotypes and phenotypes, and these subclonal aberrations contribute to intra-tumour heterogeneity[30]. This heterogeneity is caused by somatic mutations and CNVs, as well as differences in epigenetics[31,32]. Somatic evolution is driven by a combination of genetic instability and a selective tumour microenvironment, including acidosis, hypoxia, and cytotoxic stress imposed by chemotherapy, hormonal therapy and/or targeted therapies[29]. HER2-targeted therapies impose an evolutionary selection pressure on HER2+ cancer cells. Those cancer cell populations that are not exclusively dependent on the overexpression of the *HER2* oncogene will be able to constitute an anti-HER2 therapy-resistant subclone, regard-

less of their HER2 status. These subclones harbour alternative and/or collaborative drivers of carcinogenesis, which circumvent the blockade of the HER2-driven pathways. The high prevalence of both intrinsic and acquired resistance to single-agent treatment regimens already caused a shift towards dual HER2-targeted therapy, such as pertuzumab or T-DM1[33,34].

Interestingly, 5-41% of HER2+ breast cancers present with regional heterogeneous *HER2* amplification[11,12], although this percentage depends on the applied definition. In this study, we subjected ten breast cancers with spatially heterogeneous *HER2* amplification and corresponding HER2 overexpression to targeted NGS. We investigated the potential heterogeneity in the somatic mutational landscape of each individual tumour component. Some mutations were, if present, homogeneously found in each component. For instance, four of seven tumours with a *PIK3CA* mutation presented this mutation in each component. Somatic *TP53* mutations seemed more often heterogeneously distributed, and their presence seemed generally unrelated to the *HER2* amplification status.

Two breast cancers in this series harboured a gain of the 8q24 region, comprising both *MYC* and the adjacent lncRNA *PVT1*, which stabilizes the MYC protein and enhances its activity[35]. Co-amplified *MYC* and *PVT1* genes have been identified as candidate oncogenes in ER-positive HER2+ breast cancers[36]. A recent meta-analysis concluded that increased PVT1 expression was associated with lower overall survival in a wide variety of solid tumours, including breast cancer[37]. High PVT1 expression was associated with clinicopathological markers of poor prognosis, such as larger tumour size, higher TNM stage, and the presence of both lymph node and distant metastases[37]. In vitro studies demonstrated that PVT1 expression drives cancer cell proliferation through promotion of the KLF5/BAP1/beta-catenin signalling pathway[38].

One patient had a *CCND1* copy number gain in a HER2- invasive tumour component. *CCND1* amplification is associated with a particular gene expression profile and decreased survival in ER-positive, HER2- node-negative breast cancer patients[39] (Lundberg et al., 2019), indicating that CCND1 amplification might act as an alternative driver of carcinogenesis. Similar observations have been reported for *FGFR1* amplification within breast cancer and other types of carcinoma[40].

By using targeted NGS with a 53 gene panel, we identified a plethora of somatic mutations and CNVs within the HER2- components in this series of ten HER2 heterogeneous breast cancers. The genetic heterogeneity within both the HER2- and HER2+ components of a single tumour suggest that a wide range of different somatic mutations and/or CNVs may act as potential alternative drivers. These genetic aberrations might counterbalance the absence of *HER2* amplification in the HER2- components. Of note, this targeted NGS-driven study focussed only on a subset of 53 breast-cancer related genes in a limited series of ten breast cancer patients. Since we did not apply whole-genome sequencing on a large patient series, it is impossible to exclude the existence of a more commonly present alternative driver in HER2- tumour components. Due to the use of FFPE material, we were confronted with high levels of background in some tumour tissue samples, which precluded an in-depth analysis of potentially important copy number losses. Nevertheless, our findings are in accordance with the observations of the TCGA network, who described a high frequency of *TP53* (55%) and *PIK3CA* (31%) mutations, and an low frequency of mutations in *RUNX1* (1%), *PTEN* (0%), *NCOR* (0%) and *CDH1* (3%) in 75 clinically HER2+ breast cancers[23]. The TCGA network identified a high frequency of TP53 mutations in ER-negative HER2+ breast cancers, whereas ER-positive HER2+ breast cancers displayed more often a GATA3 mutation[23]. This hormone receptor-dependent duality was not observed in our series, which might be due to its small size.

The limited gene panel precludes strong statements regarding the clonal relationship of all components within a single tumour. However, the integration of histopathological and immunohistochemical features, together with the uniform presence of some well-defined pathogenic mutations (such as *TP53* or *PIK3CA* mutations), suggests a common progenitor for most heterogeneous lesions in this series. Based on the frequent homogeneous presence of the observed variants, it was estimated that the tumours of patients #1, #3, #4, #5, #7, and #9 were likely to have a common progenitor. The tumours of patients #2, #6 and #10 were considered to be less likely related to one another (i.e. a collision tumour of two independent neoplastic lesions), or to have a common progenitor with very early divergence of the subclones. Despite its limited size, this series of ten breast cancers demonstrates that regional heterogeneity in HER2 status is associated with further heterogeneity at the molecular level, and sometimes also at the protein level, since some tumour components presented with different hormone receptor status and/or EGFR protein expression status. Although regional HER2 heterogeneity is uncommon, this series illustrates that not all cells within one tumour depend exclusively on *HER2* amplification and overexpression.

Due to its relatively high prevalence in invasive breast cancer and its association with worse prognosis, HER2 overexpression is suspected to play a major role as a driver of mammary carcinogenesis. HER2+ invasive breast cancer more often presents with an associated in situ component and, if present, this DCIS component is substantially larger than in HER2- tumours[41]. The prevalence of HER2 overexpression amounts 35% in pure ductal carcinoma in situ (DCIS), which is paradoxically higher than its prevalence in invasive breast cancer[42]. Overall, HER2 expression profiles are highly concordant between admixed in situ and invasive breast cancer, but overexpression/amplification is less common in the DCIS component of admixed lesions than in pure DCIS[43–46]. One in three women with a HER2+ pure DCIS lesion develops a subsequent HER2- invasive breast cancer[47], although the clonal relationship between primary and recurrent lesions was not investigated in that study. Taken together, these observations indicate that HER2 overexpression is more likely to play a role as an instigator of tumour cell proliferation, rather than being a crucial driver of cancer cell invasion[48]. The series of pathogenic and likely pathogenic somatic variants that we describe here yields a wide range of potential alternative drivers of cancer cell proliferation and invasion. Moreover, some genetic anomalies (such as *PIK3CA* and *GATA3* mutations, or *FGFR1* copy number gain) might drive resistance to treatment[34,49].

In conclusion, the HER2- components of HER2 heterogeneous breast cancers display a variety of somatic mutations and CNVs within 53 breast-cancer related genes. Although these somatic mutations and CNVs were often present in the HER2+ component as well, they might act as potential alternative drivers to counterbalance the absence of *HER2* amplification. Since these potential alternative drivers may have the capacity to circumvent HER2 pathway blockade, their widespread presence throughout these HER2 heterogeneous cancers might explain the high level of innate and acquired resistance to HER2-targeted therapies in breast cancer. Our findings indirectly imply that a targeted monotherapy is unlikely to have high efficacy in the long term, since it causes cytotoxic distress and selection of those resistant clones that already harbour alternative drivers of carcinogenesis. Future translational breast cancer research should focus on how to handle this molecular heterogeneity in the clinical setting.

4.5 References

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Supplementary information

Table S4.1: Materials and methods for immunohistochemistry.

Antigen	Antibody clone	Host species primary antibody	Manufacturer	Dilution	HIER procedure	Incubation time primary antibody	Secondary antibody
Estrogen receptor	SP1	Rabbit	Ventana/Roche	RTU	CC1 – 64 minutes	32 minutes	Ultraview
Progesterone receptor	1E2	Rabbit	Ventana/Roche	RTU	CC1 – 36 minutes	12 minutes	Ultraview
HER2	4B5	Rabbit	Ventana/Roche	RTU	CC1 – 36 minutes	32 minutes	Ultraview
p63	4A4	Mouse	Ventana/Roche	RTU	CC1 – 64 minutes	32 minutes	Optiview
E-cadherin	36	Mouse	Ventana/Roche	RTU	CC1 – 32 minutes	32 minutes	Optiview
EGFR	L8A4	Mouse	Absolute Antibody	1:200	CC1 – 64 minutes	32 minutes	Ultraview
FGFR1	M2F12	Mouse	Abcam	1:100	CC1 – 64 minutes	32 minutes	Optiview

CC1: Cell Conditioning 1; EGFR: epidermal growth factor receptor; FGFR1: fibroblast growth factor receptor 1; HIER: heat-induced epitope retrieval; RTU: ready-to-use

Table S4.2: Detailed information on base coverage and number of reads for targeted next-generation sequencing of normal and tumour tissue samples of ten breast cancers with regional HER2 heterogeneity.

Patient	Tissue component*	Uniformity of base coverage (%)	Percent reads on target (%)	Amplicons with at least 100 reads (%)	Amplicons with at least 500 reads (%)	Number of mapped reads	Average base coverage depth
1	Normal	88,28	94,85	95,82	86,11	6078194	2179
1	DCISneg	87,75	94,48	95,03	85,10	5824110	2072
1	DCISpos	78,31	94,34	90,78	74,15	5481058	1910
1	INVpos	64,74	94,36	80,27	58,35	5074948	1732
2	Normal	82,68	91,78	96,47	86,39	10025501	3242
2	DCISpos	69,62	93,70	84,05	60,48	4409961	1444
2	INVneg	59,18	91,52	67,64	49,93	4016225	1248
2	INVpos	71,78	94,75	86,00	63,32	4748795	1601
3	Normal	89,66	93,43	98,67	95,68	15458763	5433
3	LCISneg	87,26	93,63	97,44	87,08	7720501	2746
3	LCISpos	90,18	93,60	97,34	92,19	8504719	3040
3	INVneg	94,93	93,06	98,56	95,82	8084802	2827
4	Normal	94,52	92,68	97,26	88,80	4186999	1387
4	DCISpos	93,71	93,44	98,52	93,45	6970194	2373
4	INVneg	89,50	94,32	94,20	65,84	2596273	901
4	INVpos	90,55	94,02	93,92	52,77	2113011	731
5	Normal	90,16	92,47	94,74	59,47	2440388	830
5	DCISneg	78,93	94,73	89,99	57,34	2681654	971
5	INVneg	87,40	94,18	98,31	87,54	7446156	2648
5	INVpos	81,75	95,34	98,27	82,87	7845897	2872
6	Normal	88,48	92,22	97,05	90,42	8691226	2746
6	INVneg	90,53	93,92	83,59	18,72	949324	324
6	INVpos	93,30	93,18	97,25	86,14	4605543	1536
7	Normal	97,11	92,69	99,24	97,95	12247741	4127
7	DCISpos	88,84	91,45	96,15	85,96	8262244	2765
7	INVneg	88,31	90,67	95,63	84,72	7723536	2542
8	Normal	95,27	92,24	98,74	96,72	11672278	3896
8	DCISneg	95,61	93,28	98,92	96,18	8194519	2808
8	DCISpos	94,65	93,54	98,31	96,00	10322507	3585
8	INVneg	95,34	93,15	97,95	95,72	8479271	2926
8	INVpos	95,53	94,59	99,06	97,12	10235938	3629
9	Normal	95,09	92,55	98,49	95,32	8124384	2736
9	DCISpos	94,74	92,45	98,63	95,18	7408619	2510
9	INVneg	93,63	92,40	98,24	94,46	8349903	2827
10	Normal	95,08	91,88	97,88	77,83	3162727	1026
10	DCISpos	85,35	90,29	87,01	34,81	1631621	502
10	INVneg	91,64	91,05	95,50	57,70	2401653	775
10	mINVneg	93,71	90,07	97,16	57,52	2385315	746

*DCIS: ductal carcinoma in situ; INV: invasive carcinoma; LCIS: lobular carcinoma in situ; mINV: metastasis of invasive carcinoma; neg: HER2-negative; pos: HER2-positive.

Table S4.3: Detailed information on the presence of somatic mutations in the less frequently mutated genes per patient and per tumour component.

Pt	Gene	Chr	Exon	Genbank Transcript ID	Mutation	Protein change	COSMIC ID	Mutation consequence	FS	Presence in in situ component		Presence in invasive component	
										HER2-	HER2+	HER2-	HER2+
1	FBXW7	4	4	NM_033632	c.629G>T	p.G210V	-	Missense	No	Absent	Present	-	Present
1	MLLT4	6	25	NM_001207008	c.3338A>G	p.E1113G	-	Missense	No	Absent	Absent	-	Present
1	NF1	17	15	NM_000267	c.1658A>G	p.H553R	COSM98377	Missense	No	Present	Absent	-	Present
2	AKAP9	7	41	NM_005751	c.10129_10130TG	R3377Lfs	-	Insertion	Yes	-	Absent	Present	Absent
2	AKAP9	7	41	NM_005751	c.10118C>T	p.S3373F	-	Missense	No	-	Absent	Present	Absent
2	ATM	11	11	NM_000051	c.1681_1682TA	Q561Lfs	-	Insertion	Yes	-	Absent $	Present	Absent
2	GATA3	10	6	NM_001002295	c.1198C>T	p.H400Y	-	Missense	No	-	Present	Absent $	Present
2	MED12	X	42	NM_005120	c.6237delC	I2079Nfs	-	Insertion	Yes	-	Absent	Present	Absent
2	NF1	17	18	NM_000267	c.2224G>A	p.A742T	-	Missense	No	-	Absent	Present	Absent
2	NF1	17	38	NM_000267	c.5627G>A	p.G1876D	-	Missense	No	-	Absent $	Present	Absent $
2	NF1	17	38	NM_000267	c.5633G>A	p.C1878Y	-	Missense	No	-	Absent $	Present	Absent $
2	NF1	17	44	NM_000267	c.6665C>T	p.S2222F	-	Missense	No	-	Present	Absent	Absent
3	CBFB	16	3	NM_022845	c.170_180del	p.F57fs	-	Deletion	Yes	Present	Present	Present	-
3	CDH1	16	3	NM_004360	c.204dupT	p.Y68fs	-	Insertion	Yes	Present	Present	Present	-
3	GATA3	10	(intron)	NM_001002295	c.925-3delCA	p.?	COSM166053	Splice site change	Yes	Absent	Present	Absent	-
4	MAP3K1	5	14	NM_005921	c.3352G>T	p.E1118X	-	Nonsense	Yes	-	Present	Present	Present
4	MED12	X	26	NM_005120	c.3592G>A	p.G1198R	-	Missense	No	-	Present	Absent	Absent $
4	NFATC2	20	4	NM_012340	c.1503_1504CC	p.L503Pfs	-	Insertion	Yes	-	Present	Present	Absent
5	ESR1	6	5	NM_001122741	c.975delG	p.P325fs		Deletion	Yes	Absent	-	Present	Present
5	NF1	17	15	NM_000267	c.1658A>G	p.H553R	COSM98377	Missense	No	Absent	-	Absent	Present
7	MLL2	12	48	NM_003482	c.15061C>T	p.R5021X	COSM5704469	Nonsense	Yes	-	Present	Present	-
8	PTEN	10	8	NM_000314	c.950_953del	p.V317fs	COSM5347162	Deletion	Yes	Present	Present	Absent	Absent
8	PTEN	10	8	NM_000314	c.955_958del	p.T319fs	-	Deletion	Yes	Absent	Present	Present	Present
9	NF1	17	32	NM_000267	c.4312G>A	p.E1438K	-	Missense	No	-	Absent	Present	-
10	BRCA1	17	10	NM_007300	c.1071dupA	p.L358fs	-	Insertion	Yes	-	Present	Absent °	-
10	BRCA1	17	10	NM_007300	c.3113A>G	p.E1038G	COSM3755562	Missense	No	-	Present	Present*	-
10	EGFR	7	13	NM_005228	c.1539delG	p.E513fs	-	Deletion	Yes	-	Absent	Present °	-
10	RNF213	17	26	NM_001256071	c.5552dupC	p.T1851fs	-	Insertion	Yes	-	Present	Absent °	-
10	RUNX1	21	4	NM_001754	c.178_183GCCCG	p.P61fs	-	Deletion	Yes	-	Absent	Present °	-
10	SF3B1	2	20	NM_012433	c.2931_2932AGT	p.L978fs	-	Insertion	Yes	-	Present	Absent °	-
10	SPEN	1	11	NM_015001	c.5849delG	p.G1950fs	-	Deletion	Yes	-	Absent	Present °	-
10	TBX3	12	7	NM_005996	c.2168dupC	p.P723fs	-	Insertion	Yes	-	Present	Absent °	-

Chr: chromosome; FS: frameshift; ID: identity; Pt: patient
* The mutation was detected in the HER2-negative axillary metastasis
$ Low coverage of the corresponding amplicon (i.e. <100 reads)
° The mutation was not detected in the HER2-negative axillary metastasis.

Table S4.4: Detailed information on the presence of somatic mutations in the less frequently mutated genes per patient and per tumour component.

Pt	Gene	Chr	Exon	Genbank Transcript ID	Mutation	Protein change	COSMIC ID	Mutation consequence	FS	Presence in in situ component		Presence in invasive component	
										HER2-	HER2+	HER2-	HER2+
1	FBXW7	4	4	NM_033632	c.629G>T	p.G210V	-	Missense	No	Absent	Present	-	Present
1	MLLT4	6	25	NM_001207008	c.3338A>G	p.E1113G	-	Missense	No	Absent	Absent	-	Present
1	NF1	17	15	NM_000267	c.1658A>G	p.H553R	COSM98377	Missense	No	Present	Absent	-	Present
2	AKAP9	7	41	NM_005751	c.10129_10130TG	R3377Lfs	-	Insertion	Yes	-	Absent	Present	Absent
2	AKAP9	7	41	NM_005751	c.10118C>T	p.S3373F	-	Missense	No	-	Absent	Present	Absent
2	ATM	11	11	NM_000051	c.1681_1682TA	Q561Lfs	-	Insertion	Yes	-	Absent $	Present	Absent
2	GATA3	10	6	NM_001002295	c.1198C>T	p.H400Y	-	Missense	No	-	Present	Absent $	Present
2	MED12	X	42	NM_005120	c.6237delC	I2079Nfs	-	Insertion	Yes	-	Absent	Present	Absent
2	NF1	17	18	NM_000267	c.2224G>A	p.A742T	-	Missense	No	-	Absent	Present	Absent
2	NF1	17	38	NM_000267	c.5627G>A	p.G1876D	-	Missense	No	-	Absent $	Present	Absent $
2	NF1	17	38	NM_000267	c.5633G>A	p.C1878Y	-	Missense	No	-	Absent $	Present	Absent $
2	NF1	17	44	NM_000267	c.6665C>T	p.S2222F	-	Missense	No	-	Present	Absent	Absent
3	CBFB	16	3	NM_022845	c.170_180del	p.F57fs	-	Deletion	Yes	Present	Present	Present	-
3	CDH1	16	3	NM_004360	c.204dupT	p.Y68fs	-	Insertion	Yes	Present	Present	Present	-
3	GATA3	10	(intron)	NM_001002295	c.925-3delCA	p.?	COSM166053	Splice site change	Yes	Absent	Present	Absent	-
4	MAP3K1	5	14	NM_005921	c.3352G>T	p.E1118X	-	Nonsense	Yes	-	Present	Present	Present
4	MED12	X	26	NM_005120	c.3592G>A	p.G1198R	-	Missense	No	-	Present	Absent	Absent $
4	NFATC2	20	4	NM_012340	c.1503_1504CC	p.L503Pfs	-	Insertion	Yes	-	Present	Present	Absent
5	ESR1	6	5	NM_001122741	c.975delG	p.P325fs		Deletion	Yes	Absent	-	Present	Present
5	NF1	17	15	NM_000267	c.1658A>G	p.H553R	COSM98377	Missense	No	Absent	-	Absent	Present
7	MLL2	12	48	NM_003482	c.15061C>T	p.R5021X	COSM5704469	Nonsense	Yes	-	Present	Present	-
8	PTEN	10	8	NM_000314	c.950_953del	p.V317fs	COSM5347162	Deletion	Yes	Present	Present	Absent	Absent
8	PTEN	10	8	NM_000314	c.955_958del	p.T319fs	-	Deletion	Yes	Absent	Present	Present	Present
9	NF1	17	32	NM_000267	c.4312G>A	p.E1438K	-	Missense	No	-	Absent	Present	-
10	BRCA1	17	10	NM_007300	c.1071dupA	p.L358fs	-	Insertion	Yes	-	Present	Absent °	-
10	BRCA1	17	10	NM_007300	c.3113A>G	p.E1038G	COSM3755562	Missense	No	-	Present	Present*	-
10	EGFR	7	13	NM_005228	c.1539delG	p.E513fs	-	Deletion	Yes	-	Absent	Present °	-
10	RNF213	17	26	NM_001256071	c.5552dupC	p.T1851fs	-	Insertion	Yes	-	Present	Absent °	-
10	RUNX1	21	4	NM_001754	c.178_183GCCCG	p.P61fs	-	Deletion	Yes	-	Absent	Present °	-
10	SF3B1	2	20	NM_012433	c.2931_2932AGT	p.L978fs	-	Insertion	Yes	-	Present	Absent °	-
10	SPEN	1	11	NM_015001	c.5849delG	p.G1950fs	-	Deletion	Yes	-	Absent	Present °	-
10	TBX3	12	7	NM_005996	c.2168dupC	p.P723fs	-	Insertion	Yes	-	Present	Absent °	-

Chr: chromosome; FS: frameshift; ID: identity; Pt: patient

* The mutation was detected in the HER2-negative axillary metastasis

$ Low coverage of the corresponding amplicon (i.e. <100 reads)

° The mutation was not detected in the HER2-negative axillary metastasis.

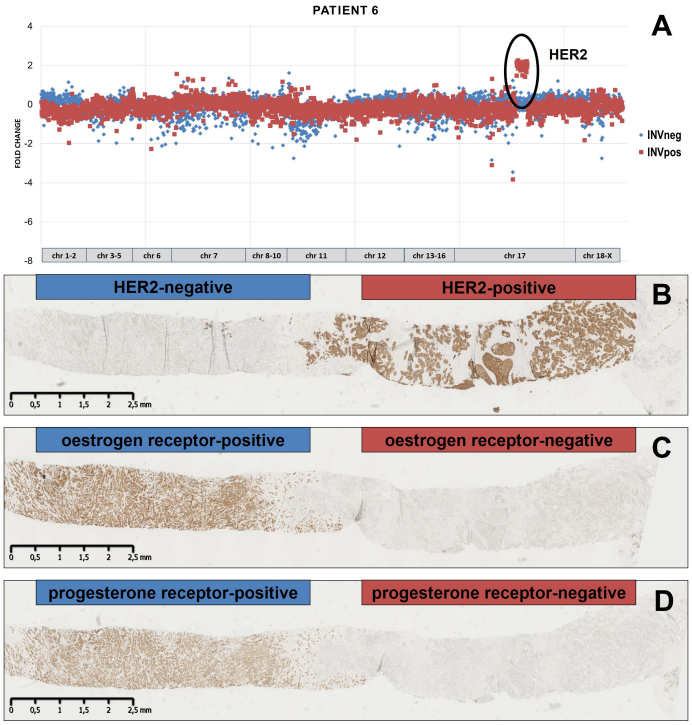

Figure S4.1: Copy number variations, HER2 status and hormone receptor status in the breast cancer of patient #6. The scatter plot confirms the presence of a HER2 copy number gain in the HER2-positive invasive carcinoma component (A; indicated by red squares). Immunohistochemistry for HER2 (B), oestrogen receptor (C) and progesterone receptor (D) illustrate opposite protein expression profiles in both invasive carcinoma components (original magnification 12.5×).

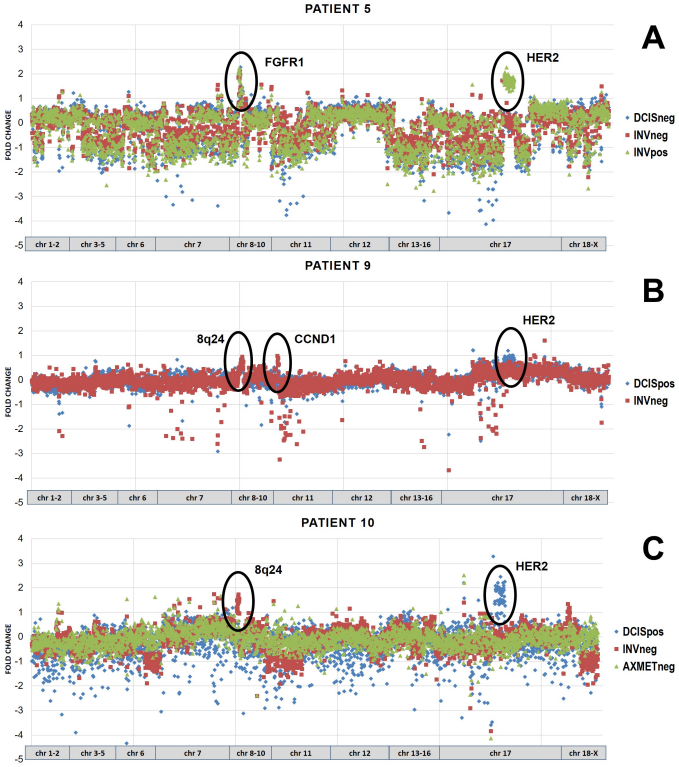

Figure S4.2: Copy number variations in the breast cancers of patients #5, #9 and #10. The scatterplot of patient #5 (A) confirms a HER2 copy number gain in the HER2-positive invasive carcinoma component (indicated by green triangles), and demonstrates an FGFR1 copy number gain (cytogenetic location: 8p11.23) in each tumour component. The tumour of patient #9 harbours a neighbouring copy number gain located at 8q24 in all carcinoma components, which comprises both the MYC and PVT1 genes, as well as a CCND1 copy number gain in the HER2-negative invasive carcinoma component (B). A similar 8q24 copy number gain was noted in the HER2-negative invasive carcinoma component (indicated by red triangles) of patient #10 (C). In patient #9, this co-amplification was present in both the HER2-positive DCIS and the HER2-negative invasive carcinoma components, indicating that this genetic aberration can occur as an early event in carcinogenesis. However, this co-amplification was not present in the HER2-positive DCIS component and the HER2-negative axillary metastasis.

Part II

A role for the tumor immune-microenvironment in DCIS behavior

Chapter 5

Tumor infiltrating lymphocytes and ductal carcinoma in situ of the breast: friends or foes?

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Abstract

In the last three decades, the detection rate of ductal carcinoma in situ (DCIS) of the breast has dramatically increased due to breast screening programs. As a consequence, about 20% of all breast cancer cases are detected in this early, in situ stage. Some DCIS cases will progress to invasive breast cancer (IBC), while other cases are likely to have an indolent biological behavior. The presence of tumor infiltrating lymphocytes (TILs) is seen as a promising prognostic and predictive marker in IBC, mainly in HER2 positive and triple negative subtypes.

Here, we summarize the current understanding regarding immune infiltrates in IBC and highlight recent observations regarding the presence and potential clinical significance of such immune infiltrates in patients with DCIS. The presence of TILs, their numbers, composition and potential relationship with genomic status will be discussed. Finally, we propose that a combination of genetic and immune markers may better stratify ductal carcinoma in situ subtypes with respect to tumor evolution.

Keywords: ductal carcinoma in situ; breast; immune response; tumor infiltrating lymphocytes; progression

5.1 Introduction

Ductal carcinoma in situ is the most common breast cancer precursor lesion[1]. The implementation of mammography screening in women >40-50 years old has exponentially increased the number of patients diagnosed with DCIS in the last three decades[1–3]. The majority of patients with DCIS are treated by surgery, followed by radiotherapy in case of breast conserving surgery[1]. However, it has been estimated that around 40-50% of cases with DCIS will remain in situ when left untreated[4–6] and some cases even show signs of regression[7,8]. Consequently, a substantial proportion of patients with DCIS are currently being overtreated, which results in unnecessary morbidity and costs. Therefore, finding DCIS progression markers is of clinical importance since this could result in reduction of overtreatment in low-risk patients, while still providing effective treatment for patients with a high progression risk.

In recent years, it has generally been accepted that tumor infiltrating lymphocytes (TILs) play a role as prognostic and predictive markers in invasive breast cancer (IBC)[9–12]. However, with regard to DCIS, data on the presence, composition and clinical significance of immune infiltrate is limited. Since DCIS is an early stage of disease, increased knowledge of the role of the immune response in DCIS progression could have major clinical consequences, as it might form the basis for future immune-modulation and potential prevention of progression to IBC. This review summarizes current knowledge regarding the role of the immune response in IBC and highlights current studies concerning the presence and potential clinical significance of the immune infiltrate in patients with DCIS.

The interplay between cancer and the immune system is seen as one of the most promising areas with respect to the development of novel anti-cancer treatments[13–16]. Emerging therapies, such as immune checkpoint inhibitors have provided encouraging results, especially in the treatment of immunogenic tumor types, such as advanced melanoma and non-small cell lung cancer[17,18]. For several immune cell subsets, a potential role in tumor evolution has been described. **Table 5.1** provides a summary of these immune cell subpopulations, including their general proposed effect on tumor progression or control.

The interplay between cancer and the immune system was summarized by Dunn and colleagues as a process of cancer immune editing, which consists of three stages[13]. In the first stage, immune cells constantly survey the environment and suppress the outgrowth of dysplastic cells. During this process, neoplastic cells are recognized as foreign, which elicits a pro-inflammatory immune response. This anti-tumor immune response is generally characterized by infiltration of type 1 macrophages, dendritic cells, natural killer cells, CD8+ cytotoxic T-cells and CD4+ T helper 1 cells[19–23]. With the help of pro-inflammatory chemokines and cytokines, these immune cells are recruited towards the tumor. In case of CD8+ T cells, they are capable of recognizing tumor antigens presented on the surface of tumor cells, which might result in tumor cell lysis and elimination[21]. In this stage of carcinogenesis, the immune system is able to prevent the outgrowth of neoplastic cells. However, neoplastic cells can persist during the second stage, in which initially immunogenic tumor cells are maintained in a stage of latency. In the final stage of immunoediting, tumor cells are able to escape the immune control as genetic instability and tumor heterogeneity progresses. This stage is characterized by low antigenicity, low numbers of immune effector cells, high numbers of immune suppressor cells and expression of immune checkpoints[24,25]. In this stage, the immune profile has a more anti-inflammatory profile and predominantly consists of myeloid-derived suppressor cells, CD4+FOXP3+ regula-

Table 5.1: Tumor associated immune cells and their proposed mechanism of action during tumor growth

Immune cell	Immune subsets	Differentiation markers	Effector enzymes, cytokines and chemokines	Receptor - Ligand interactions	Proposed mechanism of action	Effect on tumor growth
T cell	Cytotoxic T cell	CD8	Granzyme, Perforin and Interferon gamma	FAS ligand -FAS and TNF Related Apoptosis Inducing Ligand - TNF related apoptosis indused receptor	Activates tumor cell lysis	Suppression
	T helper cell 1	CD4 CCR1	Granzyme, Perforin, Interferon gamma Tumor Necrosis Factor alpha and Nitric Oxide Synthase	Not applicable	Activates cytotoxic T cell	Suppression
	T helper cell 2	CD4 CCR3	Interleukine-4 and 10	Not applicable	Not applicable	Stimulation
	Folical T helper cell	CD4	CXCL13	Not applicable	Mediation of B-cell activation	Suppression
	Regulatory T cell	CD4 FOXP3	Adenosine and Interleukine-10	Receptor Activator of Nuclear Factor KappaB Ligand - Receptor Activator of Nuclear Factor KappaB	Stimulates tumor cells, Suppresses cytotoxic T cell, T helper 1, type 1 macrophage and dendritic cells	Stimulation
B cell	Not applicable	CD19 CD20	Tumor necrosis factor alpha, Interleukin6, lymphotoxin $\alpha 1$-$\beta 2$	Anti-tumor antibodies, development of tertiary lymphnode structures	Activation T cells through antigen presentation	Suppression
	Regulatory B cell	CD19 CD20	Interleukin-10 and 35	Complement system	Increased regulatory T cells and decreased inflammation	Stimulation
Natural killer cells	Not applicable	CD16 or CD56	Not applicable	Not applicable	Activate tumor cell lysis and enhanced T helper 1 activation	Suppression
Macrophages	Type 1 macrophage	CD68	Granzyme, Perforin, Interferon gamma Tumor Necrosis Factor alpha and Nitric Oxide Synthase	Fragment crystallizable Receptor	Enhanced T helper 1 activation and antigen presentation	Suppression
	Type 2 macrophage	CD163	Vascular Endothelial Growth Factor, Epidermal Growth Factor, Basic Fibroblast Growth Factor, Transforming Growth Factor beta, interleukin-10 and 4, Matrix Metalloproteinase 2 and 9	Programmed cell death ligand 1 – Programmed cell death receptor 1, and B7-1/2 - Cytotoxic T-lymphocyte-associated antigen	Stimulates tumor cells, Suppresses CD8+ T cells, T helper 1, type 1 macrophage and dendritic cells	Stimulation
Myeloid-derived supressor cells	Not applicable	CD11b CD33	Arginase,Indoleamine 2,3-dioxygenase, reactive oxygen species and reactive nitrogen species	Not applicable	Suppresses cytotoxic T cells and natural killer cells	Stimulation
Dendritic cells	Not applicable	B7, CD1a/c	Interleukin-6	Programmed cell death ligand 1 – Programmed cell death receptor 1, and B7-1/2 - Cytotoxic T-lymphocyte-associated antigen	Activation T cells through antigen presentation	Suppression

tory T cells and type 2 macrophages[15]. The exact role of myeloid-derived suppressor cells in this context, which includes a heterogeneous cell population, is yet to be established[26]. Nevertheless, there is evidence that myeloid-derived suppressor cells down-tune an effective immune response through the production of several immune suppressive factors such as arginase and indolamina-2,3-dioxygenase[27,28]. CD4+FOXP3+ regulatory T cells and type 2 macrophages are able to inhibit CD8+ T cells and stimulate apoptosis of CD8+ T cells, partly by upregulating co-inhibitory ligands such as programmed cell death-1/2 ligand (PD-L1/2) that bind programmed cell death-1[15,19,20,22,23].

Next to the tumor phenotypes described above, there are also tumor phenotypes without a visible cancer-immune interaction[29]. These immune-deserted or immune-excluded phenotypes result from downregulated immunogenicity or a specific chemokine and cytokine profile. Altogether, tumor-associated immune cells can have both positive and negative effects on cancer progression, which has been observed in numerous types of cancer, including IBC[15,30,31].

5.2 Invasive breast cancer immune response

Breast cancer is a heterogeneous disease that can be divided in several molecular subtypes with distinct biological behavior and prognosis[32]. Each of these subtypes, which are based on gene-expression studies, has an immunohistochemical surrogate: luminal A (ER+ and PR+/HER2-, low Ki-67 index), luminal B (ER+,HER2-, PR-or low and/or high Ki-67 index or ER+ HER2+ with any PR expression and Ki-67 index), triple negative (ER-, PR- and HER2-) and HER2-overexpressed (ER-, PR- and HER2+)[33]. Several studies reported that these subtypes also differ immunologically; dense immune infiltration for instance has mainly been associated with high histological grade, triple negative and HER2+ subtypes[11,34–37]. The latter might be explained by the relatively high mutational load that is associated with these subtypes, compared to luminal subtypes[38,39]. However, within HER2+ and triple negative subtypes, a high mutational load is not associated with high levels of immune gene expression[40,41].

5.3 Immune infiltrates in invasive breast cancer: prognostic markers

There is extensive literature with respect to TILs as a prognostic marker for IBC[11,36]. One of the first large studies investigating TILs in IBC included over 1900 patients with a follow up time of 14 years. The density of TILs was scored based on hematoxylin and eosin staining. These studies reported a strong association between density of TILs and improved breast cancer specific survival in young patients (< 40 years) ($p < 0.001$)[34]. This effect was consistent with other studies, which reported an association between dense infiltrates of TILs and high grade and HER2+ IBC, irrespective of ER status[36,37]. Studies using adjuvant chemotherapy in cohorts of over 2000 and 935 patients respectively, reported that an increase in the number of TILs significantly correlated with decreased risk of both local recurrence and overall survival[41,42]. For every 10% increase in the number of TILs, a 15-17% decreased risks for local recurrence and a 17-27% reduced risk of death was reported in ER-HER2- IBC. With respect to HER2+ IBC, high densities of TILs are associated with increased response to adjuvant trastuzumab[42]. This was later confirmed by a large prospective study of over 1200 patients with HER2+ IBC, using transcriptome analysis[43].

However, some other studies did not find an association between TILs and improved outcome, or even reported the opposite effect[11,36]. A recent meta-analysis, including microarray based gene-expression data, evaluated the prognostic value of genes associated with TILs in over 1000 patients. They reported an ER-dependent association between TILs and outcome; high numbers of TILs in ER- tumors correlated with improved survival while high numbers of TILs in ER+ tumors were associated with decreased survival[44] (Calabrò et al., 2009). These conflicting results might partly be explained by different definitions and scoring methods of TILs. An international working group therefore defined a standardized methodology to evaluate TILs in IBC, in order to improve consistency and reproducibility in the measurement of TILs for future studies[45].

Tertiary lymphocyte structures have also been observed in IBC. These structures contain a T cell zone with dendritic cells, a germinal center with proliferating B cells and high endothelial venules[46]. In line with TILs, high numbers of tertiary lymphocyte structures are associated high grade, ER-/HER2+ IBC[47]. Furthermore, high numbers of tertiary lymphocyte structures are reported to be associated with improved outcome in IBC[48,49], specifically in triple negative breast cancer[50,51]. The latter study also found an association between high numbers of tertiary lymphocyte structures and high numbers of TILs[51]. The prognostic effect of TILs correlated with the presence of tertiary lymphocyte structures: patients with high levels of TILs and high levels of tertiary lymphocyte structures had a longer disease free survival compared to those with high numbers of TILs but low tertiary lymphocyte structures. Since TILs consist of different cell types, this prognostic role might also depend on the specific immune cell composition.

Generally, CD8+ T cells have been associated with favorable clinical outcome in ER- IBC[52–56]. Regarding ER+ IBC, the numbers of CD8+ T cells are generally lower, which makes the prognostic effect of these cells less evident[52,54,56]. Besides CD8+ T cells, CD4+ follicular helper T cells and B cells have also been reported to have an anti-tumorigenic effect[19]. CD4+ follicular helper T cells have been suggested to function as mediators of B cell activation and were also linked to improved survival in HER2+ IBC[19]. Concerning B cells, high numbers have been reported to be associated with improved breast cancer specific survival[57,58]. Nevertheless, no significant or the opposite effect has also been reported[36,59,60]. This could be explained by the fact that B cells can be differently activated via a T cell dependent or T cell independent pathway[19].

The presence of CD4+FOXP3+ regulatory T cells has generally been associated with poor clinical outcome, possibly by facilitating tumor growth though immune suppressing properties[11,15,19,22,23,36,61]. To our knowledge, Bates and colleagues were the first to correlate high CD4+ FOXP3+ regulatory T cell infiltration to poor prognosis[62]. This was consistent with other studies, although these studies concluded that this effect was only true for ER+ IBC[30,54,63,64]. In ER- IBC, high CD4+FOXP3+ regulatory T cell infiltration was associated with improved prognosis[65]. A recent analysis of over 7200 IBC samples reported an association between increased gene expression related to CD4+FOXP3+ regulatory T cell infiltration and impaired outcome in HER2+ IBC, regardless of ER status[30]. Tumor associated macrophages have also been associated with negative clinical outcome[36,64]. Tumor associated macrophages are believe to skew into a more type 2 macrophage phenotype, once they arrive at the tumor site, which in turn is believed to cause this negative prognostic effect[66–69]. This is consistent with the results of Ali et al., who performed gene-expression analysis of over 10.000 IBC patients and concluded that gene-expression reflecting type 2 macrophages was associated with negative prognosis in ER- IBC[70]. A more recent similar study including over 7200 IBC patients reported no association between gene-expression reflecting type 2 macrophages and prognosis, but rather found that

undifferentiated macrophages were associated with unfavorable outcome[30]. Additionally, it has been reported that myeloid-derived suppressor cells are associated with poor prognosis in IBC[71]. In this study, myeloid-derived suppressor cells were isolated from fresh frozen breast tumor tissue. High numbers of myeloid-derived suppressor cells were associated with increased numbers of FOXP3+ regulatory T cells and lymph node metastases.

5.4 Immune infiltrates in invasive breast cancer: predictive markers

Apart from the prognostic associations mentioned above, there is also evidence for a predictive value of quantity and composition of the immune infiltrate[11,19,72]. Several studies reported an association between a high density of TILs and favorable therapy response[42,43,73]. This was demonstrated in a study of over 1000 patients treated with neoadjuvant chemotherapy[74]. Patients with high numbers of TILs (50% or more of the tumor area occupied by TILs) had a higher pathologic complete response rate compared to those with limited infiltration of TILs (p=0.001). This effect was only seen in triple negative and HER2+ IBC subtypes, and was independent of other markers.

Several studies suggested that the predictive value of TILs depends on the exact composition of the infiltrate. Patients who reached a pathological complete response after neoadjuvant chemotherapy had high baseline levels of CD4+FOXP3+ regulatory T cells and CD8+ T cells in the pre-treatment needle biopsy[75–77]. Both CD8+ T cells and FOXP3+ regulatory T cells numbers were reported to be predictors of a pathological complete response in triple negative or HER2+ IBC. Interestingly, the association of CD4+FOXP3+ regulatory T cells with pathological complete response seemed to depend on the presence of CD8+ T cells. This suggests that the previously observed effect of CD4+FOXP3+ regulatory T cells may be caused by CD8+ T cells solely[76]. CD4+ follicular T helper cells have also been reported to be associated with higher levels of pathologic complete response and favorable disease free survival after neoadjuvant chemotherapy[48]. With regard to tumor associated macrophages, type 1 macrophages have been associated with higher pathological complete response rates in ER+ IBC, irrespective of HER2 status[30].

5.5 DCIS immune response

A gradual increase in the number of immune cells during the progression from normal breast tissue to IBC has been reported[62,78,79]. The highest density of immune cells was observed in IBC, however the sharpest difference in immune cell density was observed between the adjacent normal breast tissue and DCIS. In other words, the immune cells are already present in the in-situ stage of breast cancer development. In studies exclusively incorporating pure DCIS (without adjacent IBC), the presence of TILs was reported two decades ago[80]. However, this was only based on hematoxylin and eosin stainings; detailed analyses regarding the exact composition of these immune cells has not been investigated until very recently[81–84]. This area of research is growing and studies are shifting from a more descriptive perspective to correlation with clinical outcome. **Table 5.2** provides an overview of studies regarding DCIS associated immune infiltrates.

Table 5.2: Tumor associated immune cells and their proposed mechanism of action during tumor growth

Year	Author	Number of patients	Cohort	Whole section vs tissue microarray	Immune cells	Immunohistochemical markers	Additional assays	Immune cells and histopathological features	Clinical outcome
1997	Lee	41	Ductal carcinoma in situ	Whole section	Cytotoxic T cells, regulatory T cells, B cells and tumor associated macrophages	CD3, CD8, FOXP3, CD20, CD68	Not applicable	Clustered immune pattern (high numbers of tumor infiltrating lymphocytes andlow numbers of tumor associated macrophagess) is associated with high grade DCIS and HER2+ subtype	Not applicable
2010	Sharma	285	Ductal carcinoma in situ	Tissue microarray	Tumor associated macrophages	CD138, CD163, MMP11	Gene-expression analysis	Macrophage response signature is associated with high grade, ER-/PR- DCIS	Not applicable
2015	Knopfelmacher	46	Ductal carcinoma in situ	Whole section	Tumor infiltrating lymphocytes	Not applicable	Gene-expression analysis	High numbers of TILs is associated with a high Oncotype DX DCIS score	Not applicable
2016	Morita	82	Ductal carcinoma in situ	Whole section and Tissue microarray	Cytotoxic T cells	CD8	Not applicable	High numbers of TILs is associated with high numbers of CD8+T-cells , high grade, presence of comedonecrosis, HER2+, triple negative subtype and spontaneous 'healing'	Not applicable
2016	Pruneri	1488	Ductal carcinoma in situ	Whole section	Tumor infiltrating lymphocytes	Not applicable	Not applicable	High numbers of TILs is associated with high grade, presence of comedonecrosis and HER2+ subtype	TILs number is not associated with ipsilateral recurrence
2016	Thompson	27	Ductal carcinoma in situ with invasive breast cancer*	Tissue microarray	T helper cells, Cytotoxic T cells, regulatory T cells and B cells	CD3, CD4, CD8, FOXP3, CD20, PD-L1	Not applicable	Not applicable	TILs number is not associated with ipsilateral recurrence
2016	Semeraro	199	Ductal carcinoma in situ	Not applicable	Cytotoxic T cells and regulatory T cells	CD8 and FOXP3	Not applicable	High numbers of CD8+ and FOXP3+ cells and high ratio of CD8+/FOXP3+ is associated with large tumor cell diameter	Low numbers of CD8+ cells and low CD8+/FOXP3+ ratio was associated with ipsilateral recurrence
2016	Kim	177	Ductal carcinoma in situ	Whole section	Tumor infiltrating lymphocytes and tertiary lymphoid structures	Not applicable	not applicable	High numbers of TILs and tertiary lymphoid structuressis associated with HER2+ and triple negative DCIS	Not applicable
2017	Miligy	36	Ductal carcinoma in situ	Whole section	B cells, plasma cells and tertiary lymphoid structures	CD20 ,CD19 and CD138	not applicable	High numbers of B cells and tertiary lymphoid structuress is associated with larger tumor size, HER2+ and ER-/PR- ductal carcinoma in situ	High numbers of B cells are associated with shorter recurrence free survival
2017	Campbell	117	Ductal carcinoma in situ	Whole section	T helper cells, cytotoxic T cells, regulatory T cells, B cells and tumor associated macrophages	CD4, CD8, FOXP3, CD20, CD68, CD115, HLA-DR ,HLA-DP, HLA-DQ, MRC1 and PCNA	Not applicable	CD68+Mac387+ and CD115+ macrophages are associated with high VNPI, high grade, presence of comedonecrosis and ER-/PR- DCIS CD8+ or HLADR+ cells are associated with low grade DCIS and absence of comedonecrosis	Low number of CD8+HLADR+ cells is associated with high risk of ipsilateral recurrence. High number of CD8+HLADR+ cells in combination with low numbers of CD8+HLADR- or CD115+ cells is associated with low risk of ipsilateral recurrence
2017	Hendry	138	Ductal carcinoma in situ	Tissue microarray	Tumor infiltrating lymphocytes	PD-L1	Copy number variation and mutation analysis	High numbers of TILs and PD-L1 + cells is associated with high grade, HER2+ and ER- DCIS High numbers of Tumor infiltrating lymphocytes is associated with the presence of comedonecrosis. The number of TILs is higher in cases with high chromosomal copy number variation and TP53 mutation compared to cases with low chromosomal copy number variation, PIK3CA and GATA3 mutation	Not applicable

* Majority of cases are pure DCIS (24), 3 cases of DCIS and adjacent IBC.

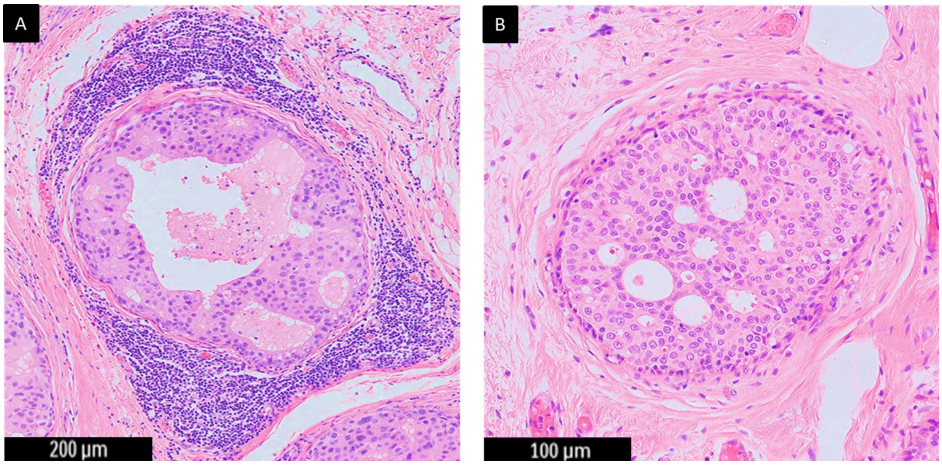

Figure 5.1: Example of a DCIS case with a high (A) and low density (B) of TILs (hematoxylin and eosin staining; original magnification x5 (A) and x10 (B))

5.6 Histopathologic and genetic features associated with DCIS immune infiltrate

In line with IBC, DCIS can also be subdivided by histological grade or surrogate subtypes based on ER, PR, HER2 status and the expression of Ki67[85]. Lee et al. reported an association between dense immune infiltrates, poor differentiation and HER2 amplification[80]. Larger studies confirmed this finding of an association between high grade, generally ER- and/or HER2+ DCIS with dense immune infiltrates[6,81,83,86–89]. In the largest study published regarding this subject (n=1400 patients with pure DCIS), Pruneri et al reported that 6.5% of all DCIS cases were associated with dense infiltrates of peri-ductal TILs. The DCIS subtype which was most frequently associated with high number of TILs was ER-HER2+ (23.6% of 254 patients), followed by ER-HER2- (11.1% of 63 patients) and ER+/HER2+ (8.9% of 258 patients)[83]. **Figure 5.1** provides an example of DCIS with a high and low density of TILs (A and B respectively).

Some studies also assessed the presence of tertiary lymphoid structures in DCIS[87,88]. Both of these studies reported that high levels of tertiary lymphoid structures were significantly associated with high grade DCIS and the presence of comedonecrosis. Regarding surrogate DCIS subtypes, the number of tertiary lymphoid structures was higher in ER-HER2+ and triple negative subtypes compared to ER+HER2+ and ER+HER2- subtypes. This might be explained by the high correlation between the presence of tertiary lymphoid structures and TILs.

The DCIS-associated immune response has recently also been linked to genetic features of the tumor cells and the expression of PD-L1[86]. Hendry et al. assessed the presence of TILs and the expression of PD-L1 in a series of 138 patients with DCIS. The presence of TILs and PD-L1 was then further associated with genetic features including copy number variation and TP53, GATA3 or PIK3CA mutation data. They reported PD-L1 expression on 11% of the tumor cells and 25% of the immune cells, while Thompson et al[84] reported no PD-L1 expression on the DCIS cells, but 81% of the DCIS-associated TILs, in a series of 23 cases. With regard to genetic features,

copy number variation was only available for 55 cases of DCIS[86]. There was a positive correlation between the number of TILs and the fraction of the genome altered by copy number variation and number of telomeric allelic imbalance. Cases with low numbers of TILs had a significantly lower fraction of the genome altered by copy number variation and a lower number of telomeric allelic imbalances. Additionally, patients with a TP53 mutation had significantly higher numbers of TILs compared to patients with a PIK3CA or a GATA3 mutation. In this analysis, ER and HER2 status was not considered. However, since these mutations are associated with ER and HER2 status of IBC, this is also likely to be the case in DCIS[40,90]. These data suggest that the DCIS-associated immune response is associated with DCIS subtype, which is in line with IBC studies[35,37,61,91]; high numbers of TILs are mainly seen in DCIS cases with high grade, ER- and/or HER2+ status and genomic imbalance.

5.7 Characterization of the composition of the DCIS-associated immune infiltrate

The first detailed description of the DCIS-associated immune infiltrate included a series of 41 patients with DCIS[80]. The authors reported two architectural patterns of DCIS-associated inflammation; a clustered (defined as patchy) or a diffuse pattern. The infiltrate density was semi-quantified as absent/minimal, mild, moderate or marked. The clustered pattern primarily consisted of B cells in the center, surrounded by CD8+ T cells. By contrast, the diffuse pattern consisted of very few numbers of B cells and high(er) numbers of tumor associated macrophages and T cells.

Sharma et al. examined the presence of a stromal induced response of tumor associated macrophages, in a set of 40 cases of DCIS, using publicly available data of 112 genes[92]. These genes were reported to be involved in a tumor associated macrophages response to colony stimulating factor in IBC. The majority of these genes, 100 out of 112, were also expressed in DCIS. The presence of this tumor associated macrophages signature gene response was associated with high grade and ER-PR- DCIS. Additional analysis were performed in a larger series, including 117 patients with pure DCIS[81]. In this study, the overall number of TILs was significantly higher in high grade DCIS compared to non-high grade DCIS. In depth analyses of the infiltrate showed higher percentages of FOXP3+, CD68+ and CD68+/-PCNA+ tumor associated macrophages (proliferating macrophages), HLA-DR+ cells, CD4+ T-cells and CD20+ B cells in high grade DCIS. CD68+ and/or Mac387+ tumor associated macrophages (reactive/infiltrating macrophages) were associated with a high Van Nuys Prognostic Index, which is an index used to estimate the DCIS progression risk. These cells were also associated with palpability, high grade, presence of comedonecrosis, ER and PR negativity. On the other hand, CD68+MRC1+ (type 2 macrophages) tumor associated macrophages did not have any correlation with clinicopathological features. CD8+ or HLA-DR+ cells were associated with low grade DCIS, absence of comedonecrosis and low risk of local recurrence[81]. The number of B cells and plasma cells have also been associated to clinicopathological features by Miligy et al[88]. In this study, whole tissue sections of 36 patients with pure DCIS were immunostained for CD19, CD20 and CD138. They reported an association of high numbers of tertiary lymphocyte structures and stromal B cells with larger tumor size, ER/PR negativity and HER2 positivity[88]. There was no association between the number of plasma cells and clinicopathological features.

Recently, Gil Del Alcazar et al analyzed the composition of immune cells in normal breast tissue, DCIS and IBC[82], using flow cytometry and RNA sequencing. In this study, DCIS-associated T cells were enriched for CD8+ and undifferentiated/naïve CD4+Th17 compared to IBC, whereas the cases of IBC had more activated CD4+ T cells and regulatory T cells compared to DCIS. The cytotoxic T lymphocyte antigen 4 (checkpoint inhibitor) pathway was upregulated in IBC compared to DCIS, while the interleukin-4 signaling pathway was downregulated in the invasive component. Besides, DCIS associated T cells appeared to be in a relatively activated state. In this study, the T cell activation state was based on the expression of granzyme B and Ki67. Overall, these results suggest that there is an immune escape during the progression from DCIS to IBC.

5.8 DCIS-associated immune infiltrate and DCIS evolution

One of the potential ways of DCIS evolution is regression. This concept of regression, also referred to as 'spontaneous healing', is a process in which changes in the stromal structure somewhat indulge the neoplastic cells[8,89]. This process has been associated with the presence of dense immune infiltrates. In a cohort study of 82 pure DCIS patients, 32 showed signs of spontaneous healing[89]. The majority of these cases with 'healing' were ER+ and HER2- (73.2%), followed by HER2+ (23.2%) and triple negative (3.6%), respectively. The presence of CD8+ T cells was associated with spontaneous healing. These CD8+ T-cells were also reported to be predictive for the risk of local recurrence[81,93]. Semeraro et al. reported an association between low numbers of CD8+ T cells or a low CD8+/FOXP3+ T cell ratio and local recurrence in a cohort of 199 patients with DCIS[93]. In depth analysis by Campbell et al. provided a series of immune cell subsets predictive for local recurrence[81]. The number of CD8+HLADR+ (activated), CD8+HLA-DR- T cells (non-activated) and CD115+ cells, expressed on monocytes/macrophages and dendritic cells, predicted the risk of local recurrence, with an accuracy of 87% (sensitivity= 76%, specificity = 89%). Patients with low numbers of CD8+HLA-DR+ cells had the highest risk of local recurrence, regardless of the numbers of CD8+HLA-DR- and CD115+ cells. The lowest risk for local recurrence was observed in cases with high numbers of CD8+HLA-DR+ cells and low numbers of CD8+HLA-DR- and CD115+ cells. Therefore, low risk for local recurrence seems to be catalyzed by CD8+HLA-DR+ T cells, which argues that CD8+ T cells need to be activated in order to be effective.

Knopfelmacher et al. reported an association between high numbers of DCIS-associated TILs and a high Oncotype DX DCIS recurrence score (a gene expression score which is clinically used to select patients with a high risk of local recurrence)[94]. However, in this study, a high oncotype DX score was also significantly associated with high grade. Recently, Miligy et al reported a significant association between the number of stromal B cells and recurrence free survival in DCIS[88]. The authors defined five locations of B cells and plasma cells in relation to the lesion. There was no significant association between the number of plasma cells and recurrence free survival, regardless of their location. However, they reported a significant association between low numbers of B cells and longer recurrence free survival. This effect was only seen for those B cells that directly surrounded the DCIS component; the presence of B cells at a somewhat larger distance (>1 mm from the DCIS component) did not affect DCIS recurrence[88]. This study suggests that not only the presence of different immune cells is important, but also their exact location.

Other support for the role of DCIS-associated TILs in tumor evolution is extracted from the difference in the frequency of HER2+ and triple negative DCIS versus HER2+ and triple negative IBC, which are both frequently associated with high numbers of TILs. In studies restricted to patients with DCIS, the frequency of HER2+ DCIS is relatively high (15-34%)[83,89,95,96] compared to IBC studies, which report HER2+ IBC in about 11-20% of cases[97–99]. In contrast, the frequency of triple negative DCIS is relatively low in DCIS studies (4-8%)[83,89,95,96] compared to IBC studies, which report that 10-13% of all IBC cases are triple negative[97–99]. In line with this, there is a high frequency of extensive DCIS adjacent to HER2+ IBC, whereas the DCIS-component in triple negative DCIS, if present, is rather limited[97]. DCIS-associated TILs might therefore play divergent roles with regard to progression of HER2+ and triple negative DCIS; they might have an anti-invasion effect in HER2+ DCIS and a pro-invasion effect in triple negative DCIS.

5.9 Prospects for immunotherapy against DCIS

In the last decade, HER2 pulsed dendritic cell vaccines have been tested in vivo as a potential treatment of HER2+ DCIS patients[100–103]. These dendritic cells are able to induce an anti-tumor immune response by stimulating and activating CD8+ T cells via HER2 antigen presentation[100]. An early clinical study in 27 HER2+ DCIS patients vaccinated with autologous HER2 peptide pulsed dendritic cells reported a major impact of this vaccine; 5 out of 27 patients showed a pathologic complete response in the surgical specimen[103]. Of the remaining 22 patients with residual DCIS, 11 had a complete loss of HER2 expression. Overall, HER2 pulsed dendritic cell vaccination appear to induce either destruction of HER2+ cells or loss of HER2 expression. This effect seemed more pronounced in ER-/HER2+ DCIS compared to ER+/HER2+ DCIS[103], despite an equivalent immune response after vaccination[101]. More recently, a randomized clinical trial with 42 HER2+ DCIS patients, reported that anti-HER2 dendritic cell vaccination is a save way to induce an anti-tumor T cell response in HER2+ patients[102]. Regardless of the administration route, a total number of 12 out of 42 patients achieved pathologic complete response, which is in line with previous studies[100–103]. These studies demonstrate that vaccination with dendritic cells might be an effective way to treat HER2+ DCIS. However, other immune modulating strategies might also be worth considering in treating HER2+ and other DCIS subtypes. Data regarding DCIS-associated PD-L1 is limited, but PD-L1 expression is reported in a subset of DCIS cases[84]. Currently, immune-modulating checkpoint inhibitors are expensive and associated with substantial adverse effects. Therefore, checkpoint blockade as treatment for DCIS patients is not anticipated at this moment. Nevertheless, the rapid development of checkpoint inhibitors with increased effectiveness, combined with decreased costs and side effects, may facilitate its use at earlier disease stages[104].

5.10 Summary

Research regarding the role of the immune system in breast cancer progression has primarily been focused on IBC. High levels of immune infiltrates, in particular effector immune cells such as CD8 T cells, are more frequently observed in HER2+ and triple negative IBC. In line with this, the prognostic and predictive value of these immune infiltrates is linked to these subtypes.

Concerning the immune infiltrate composition, there also seems to be an association between the presence of certain cell types and prognosis; the presence of CD8+ T cells has mainly been associated with favorable clinical outcome in ER- IBC[11,30,36]. A consensus on the observed effects with regard to CD4+FOXP3+ regulatory T cells and subsets of tumor associated macrophages is yet to be reached.

In the last few years, there is increased interest regarding the presence and potential clinical significance of the immune infiltrate in patients with DCIS. In line with IBC studies, dense immune infiltrates are mainly present in HER2+ and triple negative DCIS. The presence of high chromosomal copy number variation or a TP53 mutation also seems to initiate more immune response compared to limited copy number variation or a GATA3 and PIK3CA mutation, although it is unknown whether this effect is independent from ER and/or HER2 status[105,106].

In-depth analysis of DCIS-associated immune cell subsets reported that specific subsets (e.g. CD8+ cells, CD115+ cells and CD20+ B cells) were associated with local recurrence[81,88], which supports the hypothesis of an active role for TILs during the progression of DCIS. However, based on current data no definite conclusion can be drawn regarding the exact role of immune cell subsets regarding the progression of DCIS subtypes. Therefore, in our opinion, there is no indication yet for standard reporting of ER, PR, HER2 and presence of DCIS-associated TILs in daily clinical practice. However, as data with respect to the clinical significance of the DCIS-associated immune response is rapidly expanding, pathologists might be challenged to report the presence, density and composition of the immune cells in the future.

In conclusion, based on current literature, TILs are mainly present in HER2+ and triple negative DCIS, which is in line with IBC studies. This is consistent with studies that report high numbers of TILs in high grade and genetically instable DCIS. Several studies reported an association between TILs and local recurrence, although the exact role of the immune system during the progression of DCIS has to be elucidated. In depth analyses of the interaction between DCIS genetics and the immune cell composition and function are needed for a better understanding of the immune response in DCIS subtypes. This might provide targets for successful immune-intervention at an early disease stage, and thus prevention of progression to IBC.

Declaration of interest

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5.11 References

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Chapter 6

Ductal carcinoma in situ of the breast: immune cell composition according to subtype

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Abstract

Ductal carcinoma in situ (DCIS) of the breast includes several subtypes with a divergent biological behavior. Data regarding the composition of DCIS-associated immune cells and their potential role in progression is limited. We studied DCIS-associated immune response by characterizing immune cell subsets according to DCIS subtypes.

DCIS-associated tumor infiltrating lymphocyte (TIL) density was evaluated based on H&E stained sections from 473 patients. Cases were subtyped based on ER, PR and HER2. Patients were categorized as TIL-high or TIL-low. DCIS-associated immune cells of TIL-high cases were immunostained on whole slides with CD4, CD8, CD20, CD68, FOXP3 and PD-L1 (SP142 and SP263).

In total, 131/473 patients (27.7%) were considered as TIL-high. The percentage of TIL-high cases was significantly higher in HER2+ and Triple Negative (TN) DCIS (P<0.0001). Overall, no statistical difference in immune cell composition according to subtypes was found. However, individual subtype comparison showed that ER+HER2+ cases had a significantly higher proportion of CD8+ T-cells compared to TN cases (p=0.047). In TIL-high DCIS cases, PD-L1-SP142 expression on tumor cells was associated with DCIS subtype (p=0.037); the lowest number of positive cases was observed in HER2+ DCIS subtype (independent of ER). However, overall, in TIL high DCIS, PD-L1 expression by both clones was limited.

In conclusion, high numbers of TILs are mostly observed in HER+ and TN DCIS. The ER+HER2+ DCIS subtype seems to attract a higher proportion of CD8+ T-cells compared to the TN subtype. Among TIL-high DCIS, the HER2+ subgroup had the lowest PD-L1-SP142 expression on tumor cells. This suggests a more pronounced anti-tumor immunity in HER2+ DCIS, which could play a role in its biological behavior.

Keywords: breast ductal carcinoma in situ; immune cell subsets –TILs – PD-L1, CD4, CD8, CD20 and CD68; Ipsilateral recurrence

6.1 Introduction

Invasive breast cancer (IBC) is a heterogeneous disease that can be divided in several molecular subtypes with distinct clinical outcome[1]. Each molecular subtype has an immunohistochemical surrogate: luminal (ER+ with or without HER2+), basal (ER-, PR- and HER2-, known as triple negative (TN)) and HER2-driven (ER-, HER2+)[2]. IBC subtypes trigger the immune system differently; high numbers of tumor infiltrating lymphocytes (TILs) are associated with TN and HER2+ IBC[3,4]. Numerous reports illustrate a prognostic effect of TILs in IBC[5–7].

Ductal carcinoma in situ (DCIS) is a non-obligate precursor of IBC[8,9]. The DCIS detection rate has exponentially increased with the implementation of population screening by mammography[10–12]. Since the majority of patients are treated, data with respect to its treatment-naive behavior is limited. However, cases of regressive DCIS have been described and an estimated 40-50% remains in situ carcinoma when left untreated[13–18]. Consequently, a substantial proportion of patients with DCIS might be overtreated, resulting in unnecessary morbidity and health care costs. On the other hand, although a substantial proportion of breast cancer patients are detected in an early in situ stage, the incidence and morbidity of IBC remains high[11]. Novel therapeutic and preventive strategies are therefore needed in order to optimize early risk assessment and intervention. In recent years, immunotherapy is regarded as one of the most promising approaches in cancer therapy[19]. However, the non-response rate failure rate is substantial[20,21], potentially due to treatment at late stages and due to incomplete understanding of interactions between cancer cells and immune cells. Increased knowledge regarding the role of the immune response in an early disease stage as DCIS could have major clinical consequences, as it could contribute to future immune modulation and potential prevention of progression.

DCIS has a heterogeneous biological behavior[22]. In studies restricted to patients with pure DCIS, the frequency of HER2+ DCIS is relatively high (22-76%) compared to IBC studies, which report HER2 positivity in about 11-23% of cases[15,23–28]. In contrast, the frequency of TN DCIS is relatively low in pure DCIS studies (4-7.5%) compared to IBC studies, which report that 10-13% of all IBCs are TN[23,24,26–28]. In line with this, there is a high frequency of extensive DCIS adjacent to HER2+ IBC, whereas the DCIS component in TN DCIS, if present, is rather limited[24,29,30]. These data suggest a different biological behavior according to DCIS subtype: HER2+ DCIS seems to either remain in situ for a longer period of time or has a rapid in situ growth rate. TN DCIS on the other hand seems to have a relatively rapid progression to IBC. These differences could be related to characteristics of the tumor cells and/or the microenvironment, i.e. the DCIS-associated immune response.

Tumor associated immune cells are aggregated in heterogeneous infiltrates, of which TILs make up the largest group[31]. Data regarding the composition and role of TILs in DCIS is limited, although this is an emerging field of research[32]. High TIL infiltration has been associated with high grade, TN and/or HER2+ DCIS, which is in line with IBC studies[27,33–35]. Further analyses according to the composition of the immune cells showed an association between high numbers of CD8+ T-cells, DCIS regression and low ipsilateral recurrence risk[15,36,37]. On the other hand, CD19/CD20+ B-cells and CD68+Mac387+ macrophages were associated with a larger DCIS diameter, high grade, presence of comedo necrosis and shorter recurrence free survival[36,38]. In addition, two recent studies reported on PD-L1 expression in DCIS[34,39]. Hendry et al. reported PD-L1 (clone: SP263, Ventana) expression on 11% of DCIS cells and 25% of DCIS-associated TILs (n=138 cases). On the other hand, Thompson et al. reported no PD-L1 (clone: 5H1, Abcam)

expression in DCIS cells, but 81% of DCIS-associated TILs (n=23 cases).

These studies described above have some limitations, including study size and the use of tissue micro arrays for analysis of DCIS-associated immune cells. Larger series were restricted to the analysis of one immune cell subset (such as CD20+ B-cells or CD8+ T-cells) or did not include ER, PR and HER2 status[15,27,38]. Therefore, a more complete knowledge on how different immune subsets are associated with DCIS subtype is lacking. Based on the biological behavior of DCIS subtypes, as described above, we hypothesize that there is an association between DCIS subtype and the composition of the immune infiltrate. The extensive growth pattern of HER2+ cases suggests a potential protective/anti-invasion effect of the immune cells. The biological behavior of triple negative DCIS on the other hand, which is also associated with dense immune infiltrates like HER2+ DCIS, rather suggests immune evasion. The objective of this study was therefore to characterize the presence and composition of DCIS-associated TILs in a large series of patients, in order to allow subgroup analyses according to DCIS subtype.

6.2 Patients and methods

6.2.1 Patient selection and clinical data collection

For this retrospective study, we included patients with a primary diagnosis of DCIS, diagnosed at the Erasmus Medical Center (EMC) Cancer Institute Rotterdam or the Laboratory for Pathology (PAL) Dordrecht between 2000 and 2016. Clinical data collection included age, type of surgery (breast conserving surgery or mastectomy) and follow-up. Patients with bilateral DCIS were included as two cases. Patients with an ipsilateral IBC within 6 months after the initial DCIS diagnosis were excluded. Other exclusion criteria were microinvasion and insufficient tissue. Recurrences were defined as ipsilateral histologically proven DCIS, IBC or regional/distant breast cancer metastases without a contralateral IBC, occurring $\geq$6 months after the initial DCIS diagnosis. For this study, we used encoded leftover patient material, and therefore did not need an informed consent or approval from an ethical committee according to the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands[40].

6.2.2 Pathological assessment of DCIS characteristics

According to Dutch guidelines, DCIS lesions < 4 cm are embedded completely since 2005. In lesions with a diameter > 4 cm, at least 10 blocks of the lesion are embedded in order to exclude an invasive component. All Hematoxin & Eosin (H&E) stained whole sections of excision specimen were centrally reviewed by two observers to assess histological grade (based on nuclear atypia, according to the WHO classification), the predominant growth pattern, presence of comedonecrosis, micro-calcifications and TILs. In case of a disagreement, consensus was reached. TILs density was semi-quantified as minimal/absent, mild, moderate or severe. DCIS-associated stroma was defined as the stroma within 1 mm of the duct, as described by Toss and colleagues[41]. Cut-offs for TILs density were defined as 0-5%, 5-30%, 30-50% and >50% of the DCIS-associated stroma occupied by TILs, respectively, adapted from Beguinot and colleagues[33]and illustrated in **Figure 6.1**. TILs distribution was scored as focal, patchy or diffuse, defined as <10%, 10-50% and >50% of all DCIS ducts surrounded by TILs respectively. Based on density and distribution, cases were

classified as either TIL-high or TIL-low. TIL-low DCIS was defined as a low TIL density (<30% of the DCIS-associated stroma occupied by TILs) or a focal TILs distribution. TIL-high DCIS was defined as a moderate or high TIL density (>30% of the DCIS-associated stroma occupied by TILs) and a patchy or diffuse TILs distribution. DCIS-subtypes were determined by ER, PR and HER2 immunohistochemistry (**Table 6.1**). ER and PR were defined as positive when at least 10% of the tumor cell was positive, which is extracted from the Dutch guidelines for IBC scoring[42] (Dutch Institute for Clinical Auditing, 2017). HER2 was scored according to international guidelines[43]. HER2 In situ hybridization (ISH) was performed in cases with equivocal HER2 immunohistochemistry. DCIS were classified as ER+PR+/-HER2-, ER+PR+/-HER2+, ER-PR-HER2+ or TN.

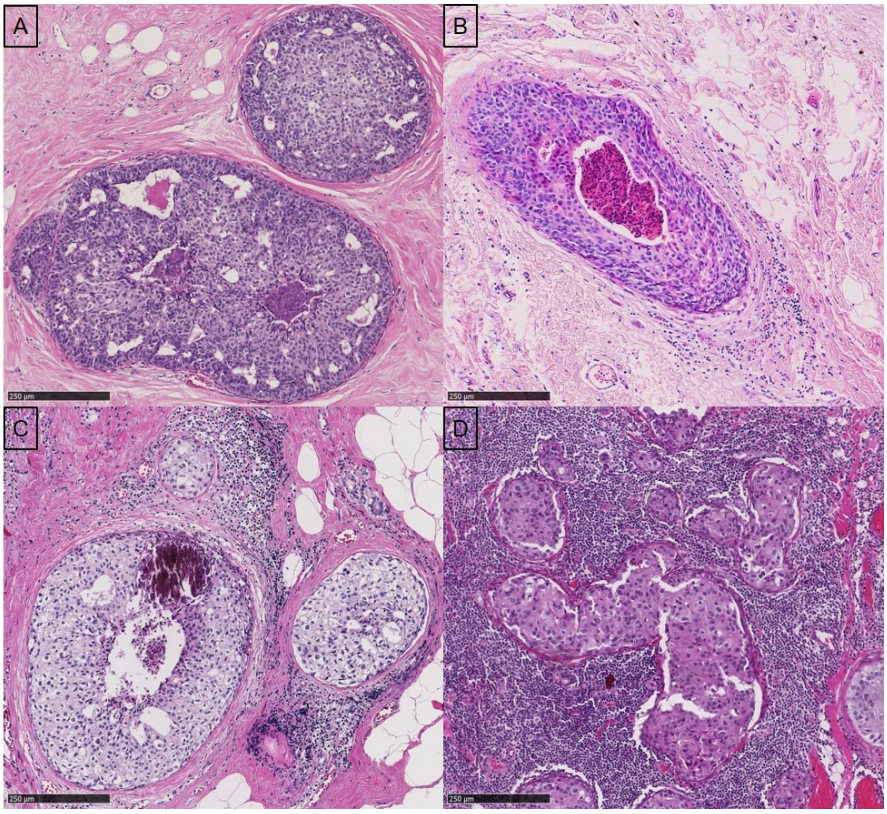

Figure 6.1: H&E images of DCIS with absent/minimal (A), mild (B), moderate (C) or severe (D) TIL density (10× magnification).

Table 6.1: Antibody characteristics and used protocol for whole tissue IHC

Antibody	Cell type	Type	Concentration	Company	Clone	Lot number	Procedure	Incubation time
ER	n.a.	Anti-Rabbit	1 µg/ml	Ventana	SP1	F02583	Ultraview CC1 64'	32 minutes
PR	n.a.	Anti-Rabbit	1 µg/ml	Ventana	1.00E+02	Y08684	Ultraview CC1 36'	12 minutes
HER2neu	n.a.	Anti-Rabbit	6 µg/ml	Ventana	4B5	E06192	Ultraview CC1 36'	32 minutes
CD4	T-helper cell	Anti-Rabbit	2.5 µg/ml	Ventana	SP35	G07304	Ultraview CC1 64'	8 minutes
CD8	Cytotoxic T-cell	Anti-Rabbit	0.35 µg/ml	Ventana	SP57	Y04591	Optiview CC1 16'	32 minutes
CD20	B-cell	Anti-Mouse	0.3 µg/ml	Ventana	L2G	Y19660	Ultraview CC1 64'	44 minutes
CD68	Macrophage	Anti-Mouse	0.4 µg/ml	Ventana	KP1	G01685	Optiview CC1 16'	8 minutes
FOXP3	Regulatory T-cell	Anti-Mouse	0.5 mg/ml	Thermofisher	236A/E7	4339062	Optiview CC1 32'	32 minutes
PDL1	n.a.	Anti-Rabbit	7 µg/ml	Ventana	SP142	Y03641	Optiview CC1 64'	16 minutes
PDL1	n.a.	Anti-Rabbit	1.61 µg/ml	Ventana	SP263	Y2898U	Optiview CC1 64'	16 minutes

* All antibodies were purchased from Ventana Roche, **PD-L1 antibodies were supplied by ROCHE

6.2.3 Pathological assessment of DCIS-associated TILs

To determine DCIS-associated immune cell composition, we immunostained the DCIS-associated TILs of TIL-high DCIS cases by automated immunohistochemistry using the Ventana Benchmark ULTRA (Ventana Medical System Inc.). Since TIL-low DCIS have little to no TILs to characterize, characterization of TILs was restricted to TIL-high cases. Sequential 4-µm thick formal fixed paraffin embedded (FFPE) whole tissue sections were stained for CD4 (T-helpers), CD8 (Cytotoxic T-cells), CD20 (B-cells), CD68 (Macrophages) and FOXP3 (Regulatory T-cells). **Table 6.1** provides an overview of used antibodies and protocols. Additionally, PD-L1 (Programmed cell death ligand) was assessed using two clones (SP263 and SP142). Briefly, following deparaffinization and heat-induced antigen retrieval, tissue samples were incubated according to their optimized time and protocol with the antibody of interest. Incubation was followed by hematoxylin II counter stain for 8 minutes and then a blue coloring reagent for 8 minutes according to the manufactures instructions (Ventana). The percentages of CD4+, CD8+, CD20+ and CD68+ immune cells were assessed manually by eyeballing, relatively to one another, with a combined score of 100%. Next, the percentage of FOXP3+ cells was determined as a proportion of all immune cells. PD-L1 expression in IBC is yet to be standardized; we therefore used two clones, which were scored individually. Membranous PD-L1 expression was scored for the DCIS cells and the immune cells, as the percentage of positive cells. The cut-off for PD-L1 positivity was set at 1%, both for DCIS cells and immune cells. Two observers, blinded for ER, PR and HER2 status, assessed all immunohistochemical staining.

6.2.4 Statistical analysis

IBM SPSS statistics 21.0 was used to perform statistical analysis. Chi-square test was used to test for associations between clinicopathological characteristics, DCIS subtypes based on immunohistochemistry, TILs and PD-L1 expression. After testing for normal distribution, the Kruskal-Wallis test was used to analyze difference in the proportion immune subset density across the DCIS IHC-subtypes. Differences in the proportion immune subsets between individual IHC-subtypes were analyzed using the Mann-Whitney U test. Differences between PD-L1 expressions of both clones were assessed using the Wilcoxon Signed Ranks Test. A Log Rank Mantel-Cox test was used to analyses the effect of TILs and PD-L1 expression on ipsilateral recurrence. Results were considered significant with a P-value <0.05.

6.3 Results

6.3.1 General clinicopathologic characteristics

In total, 501 DCIS cases were reviewed for this study. After central revision, 28 samples were excluded because of an ipsilateral IBC within 6 months (n=22) or at deeper sectioning for additional IHC staining (n=6). We therefore included 473 patients with pure DCIS for further subtyping of DCIS-associated TILs. **Table 6.1** provides an overview of clinicopathologic characteristics of this series. The median age at diagnosis was 58 years (range: 27-84), with a median follow up time of 98 months (range: 24-218). Most patients (61.9%) were treated with breast conserving surgery. The majority of patients (51.6%) were diagnosed with high-grade DCIS and most cases were associated with calcifications (71.7%). DCIS was subtyped as ER+PR+/-HER2- (n=225; 53.9%), ER+PR+/-HER2+ (n=80; 16.9%), ER-PR-HER2+ (n=85; 18.0%), TN (n=22; 4.7%) or missing (n=31).

6.3.2 Presence of TILs according to DCIS characteristics

After stratification according to TIL density and distribution, 131 out of 473 patients (27.7%) were classified as TIL-high. TIL density and distribution were strongly associated (p<0.0001, chi-square test, **Table S6.1**, see section on supplementary data given at the end of this chapter). TIL-high DCIS was associated with high grade (p<0.0001), a predominantly solid growth pattern (p=0.006), presence of comedonecrosis (p<0.0001), larger DCIS diameter (p<0.0001) and IHC-subtype (p<0.0001). In multivariate analysis, only DCIS diameter, grade and IHC subtype remained significantly associated with TILs (**Table 6.3**).

Overall, the majority of the ER-PR-HER2+ and TN DCIS subtype was TIL-high (61.2% and 63.6% respectively), whereas the opposite was true for the ER+PR+HER2- and ER+PR+HER2+ subtype (11.4% and 38.8% respectively) (**Figure 6.2**). Within the ER+HER2- and ER-PR-HER2+ DCIS subtypes, there was a significant association between grade and presence of TILs; high grade cases had significantly more TILs compared to low grade cases (p=0.001 for ER+HER2- DCIS and p=0.008 for ER-PR-HER2+ DCIS).

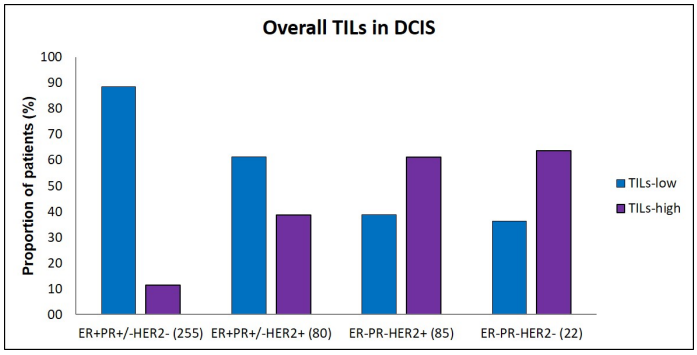

Figure 6.2: The proportion of TIL low versus TIL high DCIS according to IHC-subtype. X-axis shows the DCIS IHC-subtypes and the Y-axis illustrates the proportion of patients.

Table 6.2: Clinicopathological characteristics (n= 473)

Age at diagnosis (in years)	
- Median (range)	58 (27 - 84)
Surgery (%)	
- Breast conserving surgery	293 (62)
- Mastectomy	180 (38)
Diameter (in cm)	
- Median (range)	2.00 (0.1 - 23.0)
- Missing (n)	65
Growth pattern (%)	
- Solid	239 (51)
- Cribriform	190 (40)
- Micropapillary	37 (8)
- Papillary	7 (1)
Grade (%)	
- Low	63 (13)
- Intermediare	166 (35)
- High	244 (52)
Calcification (%)	
- Absent	134 (28)
- Present	339 (72)
Comedonecrosis (%)	
- Absent	234 (49)
- Present	239 (51)
TILs density (%)	
- Minimal	212 (45)
- Mild	110 (23)
- Moderate	113 (24)
- Severe	38 (8)
TILs distribution (%)	
- Focal	71 (15)
- Patchy	133 (28)
- Diffuse	57 (12)
- N.A.	212 (45)
Immunohistochemical subtype (%)	
- ER+PR+/-HER2-	255 (54)
- ER+PR+/-HER2+	80 (17)
- ER-PR-HER2+	85 (18)
- ER-PR-HER2-	22 (5)
- Missing	31 (7)
Ipsilateral recurrence (%)	
- None	450 (95)
- Ductal carcinoma in situ	4 (1)
- Invasive breast cancer	5 (1)
- Invasive breast cancer and metastasis	5 (1)
- Metastasis	2 (0)
- Missing	7 (2)

Table 6.3: Ductal carcinoma in situ characteristis according to TILs

	Overall TILs in ductal carcinoma in situ		p-value Univariate analysis	P-value Multivariate analysis
	TILs Low (n=342)	TILs High (n=131)		
Age at diagnosis (years)				
- Median (range)	58.00 (29.00 - 84.00)	57.00 (25.00 - 82.00)	0.366	0.660
Surgery				
- Breast conserving therapy	222 (76)	71 (24)	**0.032**	0.513
- Mastectomy	120 (67)	60 (33)		
Diameter (missing n=53)(in cm)				
- Median (range)	1.65 (0.1 - 23.0)	2.90 (0.1 - 16.0)	**<0.0001**	**0.044**
Growth pattern (%)				
- Solid	160 (67)	79 (33)		
- Cribriform	153 (81)	37 (19)	**0.006**	0.350
- Micropapillary	23 (62)	14 (38)		
- Papillary	6 (86)	1 (14)		
Grade (%)				
- Low (G1)	60 (90)	3 (5)	**<0.0001**	**<0.0001**
- Intermediate (G2)	149 (90)	17 (10)		
- High (G3)	133 (55)	111 (45)		
Calcification (%)				
- Absent	104 (78)	30 (22)	0.105	0.796
- Present	238 (70)	101 (30)		
Comedonecrosis (%)				
- Absent	191 (82)	43 (18)	**<0.0001**	0.208
- Present	151 (63)	88 (37)		
Immunohistochemical subtype (missing n=31)(%)				
- ER+PR+/-HER2-	226 (89)	29 (11)		
- ER+PR+/-HER2+	49 (61)	31 (39)	**<0.0001**	**<0.0001**
- ER-PR-HER2+	33 (39)	52 (61)		
- ER-PR-HER2-	8 (36)	14 (64)		
Ipsilateral recurence				
- No	330 (73)	120 (27)	0.338	0.175
- Yes	10 (63)	6 (38)		

6.3.3 TIL composition

Due to insufficient remaining tissue, 13 high-TILs DCIS were excluded for TIL composition analysis. We therefore included 118 TIL-high DCIS cases to study CD4, CD8, CD20, CD68, FOXP3 and PD-L1 expression. Overall, the majority of the DCIS-associated immune cells were CD4+, followed by CD20+, CD8+ and CD68+ with a median of 46.0% (range 4-80), 34.5% (range 2-90), 10.0% (range 2-40) and 5.0% (range 1-74) respectively (**Figure 6.3**). The median proportion of FOXP3 expression was 3% (range 0-20).

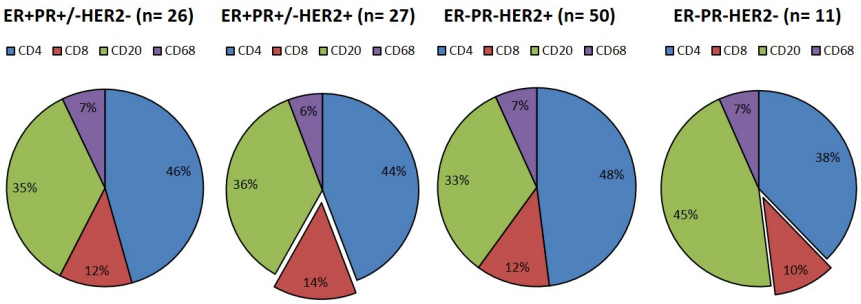

Figure 6.3: The immune cell distribution according to IHC subtype. The proportion of CD4 (blue), CD8 (red), CD20 (green) and CD68 (purple) expression is displayed as a percentage (%) according to the IHC subtype.

In these TIL-high DCIS, PD-L1 expression was observed in both DCIS cells and immune cells (**Figure 6.4**). In total, 9 out of 119 cases (7.6%) were PD-L1-SP142 positive in DCIS cells and 55 out of 119 cases (46.2%) were PD-L1-SP263 positive in DCIS cells. Regarding TILs, 97 out of 119 cases (81.5%) were PD-L1-SP142 positive and 112 out of 119 cases (94.1%) were PD-L1-SP263 positive. However, the overall PD-L1 expression was limited in both components. The median % of PD-L1 positive DCIS cells was 0 for both SP142 and SP263 (SP142 range 0-6, SP263 range 0-52). The median % of PD-L1+ immune cells was 1% for both SP142 and SP263 (SP142 range 0-7, SP263 range 0-10). However, the expression of the PD-L1-SP263 clone was higher compared to the PD-L1-SP142 clone, for both DCIS and immune cells (P<0.0001).

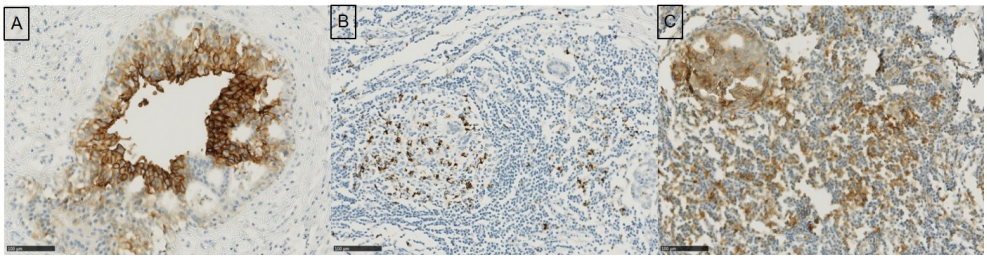

Figure 6.4: IHC images of PD-L1 expression in DCIS cells (clone SP263) (A), DCIS-associated immune cells (clone SP142) (B) and in both (clone SP263)

6.3.4 TIL composition according to DCIS characteristics

Overall, there was no statistical difference in the composition of DCIS immune cell subsets according to DCIS subtypes. However, since HER2+ DCIS and TN DCIS have a distinct biological behavior, we performed subgroup analyses restricted to these subtypes. This showed a significantly higher proportion of CD8+ T-cells in the ER+HER2+ subtype compared to the TN subtype, although the absolute difference was limited (14% versus 10%; P-value=0.047). The ER-HER2+ subtype was associated with a higher proportion of CD4+ T cells compared to the TN group, although significance was not reached (P-value=0.061). Additionally, among TIL-high DCIS, PD-L1-SP142 expression on tumor cells was associated with DCIS subtype (p=0.037); the lowest number of positive cases was observed in the HER2+ DCIS subgroups (both ER+HER2+ and ER-HER2+). There was no significant association between PD-L1 status (positive versus negative) and other features of neither the DCIS component nor other immune cell parameters.

6.3.5 TILs and ipsilateral recurrence

In total, follow-up was available for 466 cases. The median follow-up time was 98 months (range, 24- 218 months). We observed an ipsilateral recurrence in 16 patients (**Table 6.2**). The majority of these recurrences were invasive (10 out of 16); the remaining cases had a DCIS recurrence. Half of the patients with an invasive recurrence (5 out of 10) were associated with regional or distant metastasis. Two of these patients had a histologically proven distant breast cancer metastasis (liver and bone respectively), in the absence of a proven primary IBC. Overall, there were no significant associations between general DCIS features and ipsilateral recurrence. The majority of patients with an ipsilateral recurrence had a primary diagnosis of ER+PR+HER2- DCIS (n=10), remaining cases were HER2+. No recurrences were observed in those patients with a TN DCIS. In total, 6 out of 16 patients with a recurrence had TIL-high DCIS in the original specimen; the remaining 10 cases were TIL-low. There was no significant association between TILs (TIL- high versus TIL- low cases) and ipsilateral recurrence.

We compared the proportion of immune cell subsets and PD-L1 expression of TIL-high cases with ipsilateral recurrences to those without ipsilateral recurrence. There was no association between immune cell subset composition and ipsilateral recurrence. However, cases with an ipsilateral recurrence were associated with PD-L1-SP263 expression in DCIS cells (p=0.007), whereas there was no association between expression in TILs and recurrence (p=0.522) by comparing PD-L1 positive versus negative cases (**Table S6.2**). Regarding PD-L1 expression as a continuous variable however, expression levels in both DCIS and TILs were significantly higher in the recurrent group versus the non-recurrent group (p=0.002 and p=0.022 respectively).

There was no significant association between PD-L1-SP142 expression and ipsilateral recurrence (p=0.408 for DCIS and p=0.221 for TILs) when comparing PD-L1 positive versus negative cases. However, the level of PD-L1-SP142 expression as a continuous variable in TILs was higher in the recurrent group compared to the non-recurrent group (p=0.045).

6.4 Discussion

This is the largest study evaluating the presence, including the composition, of DCIS associated immune cells in relation to DCIS IHC-subtype. In our series, we found an association between

DCIS IHC-subtype and the presence of TILs, whereby ER-PR-HER2+ and TN cases had the highest numbers of TILs, which is in line with previous studies[27,34,35]. The majority of DCIS-associated immune cells were CD4+ T-cells, of which 3% were likely to be regulatory T-cells (FOXP3 positive). Overall PD-L1 expression was rather low in both DCIS cells and immune cells. Nonetheless, high CD4+ FOXP3+ T-cell infiltrations, which partly express PD-L1, suggest a suppressed tumor immune microenvironment. This is in line with our finding, whereby PD-L1-SP263 expression was associated with an ipsilateral recurrence. Besides, this was also previously suggested by other studies demonstrating that CD4+FOXP3+ regulatory T-cell infiltration was associated with large, highly proliferative DCIS and micro invasion[33,36,39,44].

Our data regarding DCIS IHC-subtype and immune cell composition of TIL-high cases shows that the HER2+ IHC-subtype (independent of ER) had significantly less PD-L1 expression as detected by SP142 in DCIS cells compared to the other DCIS subtypes, which suggests less immune suppression. This is in line with a previous study of Thompson and colleagues, reporting that HER2+ DCIS did not have PD-L1-high TILs whereas all included TN DCIS had PD-L1-high TILs[39]. Additionally, the ER+PR+/-HER2+ subtype was associated with a significantly higher proportion of CD8+T-cells compared to the TN subtype. This association was also suggested by Morita and colleagues, who reported an association between HER2+ DCIS and high numbers of CD8+ T-cells[15]. Previous studies also reported an association between high infiltration of DCIS-associated CD8+T-cells and a low recurrence risk[36,37], which is in line with IBC studies[45–47]. These findings suggest a more pronounced anti-tumorigenic immune response in HER2+ DCIS cases, which could play a role in its biological behavior.

To our knowledge, our study is the first to characterize DCIS-associated immune cells and their association with pure DCIS IHC-subtype at this scale. Previous cohorts either evaluated immune cells, included DCIS with micro-invasion, small cohorts, did not consider the ER, PR and HER2 status or they only evaluated TILs based on H&E in relation to ER/PR/HER2 status[15,27,33,34,36,38,39,41,44]. Moreover, we are the first to have used two sets of PD-L1 clones (SP142 and SP263, Ventana) to assess PD-L1 expression in TIL-high DCIS. Several studies reported about the clinical results of immunotherapy in IBC, but the non-response rate is still high[20,21]. There is currently no generally accepted PD-L1 clone or scoring system for IBC or DCIS. Increased knowledge regarding PD-L1 expression during breast carcinogenesis could contribute to future development of immune modulating therapies. Currently, immune modulation is mainly used for late stages of disease. However, if side effects and costs decrease during future drug development, it is likely that immune modulation will also be considered for earlier disease stages. The limited expression of PD-L1 expression in our DCIS cohort does however not support the use of immunotherapy in the DCIS stage.

Nevertheless, our study also has some limitations. First, consensus for TIL evaluation in DCIS is yet to be reached, which results in different quantification methods, which restricts the comparison of results from several studies[41]. Besides, the quantification of TILs and immune cell subset composition is partly subjective, due to tissue selection bias and/or quantification methods, which could affect reproducibility between observers. We only analyzed PD-L1 expression of TIL-high cases, which limited our knowledge with respect to PD-L1 expression on tumor cells of TIL-low DCIS. Besides, there are several limitations of the PD-L1 clones. The use of the SP142 antibody using the Ventana assay to assess PD-L1 expression was reported to be less sensitive than other commercially available antibodies, even though this antibody itself is equally sensitive[48]. This difference in sensitivity due to the assay could have impacted our results. In

addition, we started with a large series, but the numbers of ipsilateral recurrences were low during a relatively limited follow-up time, which restricted the correlation of several pathological features with outcome.

In conclusion, high numbers of TILs are mainly observed in HER+ and TN DCIS and the majority of these are CD4+ T-cells. The ER+HER2+ subtype seems to attract a higher proportion of CD8+ T-cells compared to the TN subgroup. Additionally, the TIL-high HER2+ subgroup (independent of ER) had the lowest PD-L1-SP142 expression on tumor cells. This suggests a more pronounced anti-tumorigenic immune response in HER2 positive DCIS, which might play a role in its distinct biological behavior.

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Supplementary information

Table S6.1: Association between TIL density and TIL distribution

	TILs distribution				
TIL density	**Focal**	**Patchy**	**Diffuse**	**N.A.***	**Chi-sqare p-value**
Minimal	0	0	0	212	
Mild	51	54	5	0	
Modorate	18	68	27	0	<0.0001
Severe	2	11	25	0	

Table S6.2: Association between PD-L1 expression and ipsilateral recurrence (n=119)

	Recurence			
	Yes (n=6)	**No (n=109)**	**Unkown (n=4)***	**p-value**
PD-L1 SP263 ductal carcinoma in situ cells (%)				
- positive (n= 55)	6 (11)	47 (89)	2	**0.007**
- negative (n=64)	0 (0)	62 (100)	2	
PD-L1 SP263 immune cells (%)				
- positive (n=112)	6 (6)	102 (94)	4	0.522
- negative (n=7)	0 (0)	7 (100)	0	
PD-L1 SP142 ductal carcinoma in situ cells(%)				
- positive (n=9)	1 (11)	8 (89)	0	0.408
- negative (n=110)	5 (5)	101 (95)	4	
PD-L1 SP142 immune cells (%)				
- positive (n=97)	6 (7.0)	87 (94)	4	0.221
- negative (n=22)	0 (0)	22 (100)	0	

* In total 4 cases were missing due to lack of follow-up data, these were excluded from analysis

Chapter 7

Transcriptomic properties of HER2+ ductal carcinoma in situ of the breast associate with absence of immune cells

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Abstract

The identification of transcriptomic alterations of HER2+ ductal carcinoma in situ (DCIS) that are associated with the density of tumor infiltrating lymphocytes (TILs) could contribute to optimize choices regarding the potential benefit of immune therapy. We compared the gene-expression profile of TIL-poor HER2+ DCIS to that of TIL-rich HER2+ DCIS.

Tumor cells from 11 TIL-rich and 12 TIL-poor DCIS cases were microdissected for RNA isolation. The Ion AmpliSeq Transcriptome Human Gene Expression Kit was used for RNA sequencing. After normalization, a Mann-Whitney Ranksum test was used to analyze differentially expressed genes between TIL-poor and TIL-rich HER2+ DCIS. Whole tissue sections were immunostained for validation of protein expression.

We identified a 29 gene-expression profile that differentiated TIL-rich from TIL-poor HER2+ DCIS. These genes included CCND3, DUSP10 and RAP1GAP, which were previously described in breast cancer and cancer immunity and were higher expressed in TIL-rich DCIS. Using immunohistochemistry, we found lower protein expression in TIL-rich DCIS. This suggests regulation of protein expression at the posttranslational level.

We identified a gene-expression profile of HER2+ DCIS cells that was associated with the density of TILs. This classifier may guide towards more rationalized choices regarding immune mediated therapy in HER2+ DCIS, such as targeted vaccine therapy.

Keywords: Breast ductal carcinoma in situ; HER2 amplification; Gene expression analysis; Next Generation sequencing; RNA; Protein; Immunohistochemistry

7.1 Introduction

Ductal carcinoma in situ (DCIS) of the breast is characterized by a proliferation of neoplastic cells confined within the duct[1,2] . When left untreated, DCIS can progress into invasive breast cancer (IBC)[3,4]. Several markers for progression have been proposed, including high nuclear grade, large DCIS size and the overexpression of the human epidermal growth factor receptor 2 (HER2)[4–8] . HER2+ DCIS accounts for 23-37% of all DCIS cases and is associated with high nuclear grade, large diameter and comedonecrosis[9,10]. HER2 overexpression in DCIS has also been reported as a biomarker for local recurrence and upstaging to IBC in the final excision specimen after a biopsy diagnosis[10–13]. Since HER2+ IBC has an aggressive biological behavior, optimal early treatment is needed to minimize the risk of DCIS progression to IBC. Previous studies reported the potential of targeted treatment of HER2-enriched DCIS including vaccine therapy to prevent invasive disease[14–18].

The first HER2 vaccine study in HER2+ DCIS was conducted over a decade ago, using dendritic cells pulsed with HER2 peptides[15]. This study included 13 patients, who were treated with this vaccine after a biopsy diagnosis of HER2+ DCIS. After surgical resection, partial pathologic response was reported in 7 patients and complete tumor regression in 1 patient[15]. Other studies demonstrated the feasibility and safety of the vaccine, whereby complete tumor regression was seen in up to 30% of the patients[14,16,18]. Although these results are promising, identifying patients that benefit from the vaccine remains challenging. The vaccine gives rise to an increased number of anti-HER2 CD4+ T-cells in peripheral blood, but this does not always result in a local immune response[18]. Lowenfeld et al. demonstrated that all DCIS patients with pathologic complete response had elevated levels of anti-HER2 CD4+Th1 cells in their sentinel lymph node[18]. This emphasizes the importance of a loco-regional immune response. However, the pre-vaccination local immune response could also be important with respect to the efficacy of immune modulating therapies.

HER2+ breast cancer is associated with a pronounced local immune response, marked by high numbers of tumor infiltrating lymphocytes (TILs), both in DCIS as well as IBC lesions[19,20]. With regard to IBC, numerous studies reported an association between high numbers of TILs and improved prognosis in triple negative and HER2+ cases[21–24]. Additionally, patients with high numbers of TILs seem to have a better response to immune modulating therapies such as immune checkpoint inhibitors[25]. With respect to the prognostic role of DCIS-associated TILs, data remains limited. In general, high numbers of TILs have been associated with invasive recurrence. This could be related to the distribution of TILs across DCIS subtypes, since TILs are mainly seen in high grade, HER2+ or triple negative cases[20,26–28]. The prognostic role of DCIS-associated TILs within DCIS subtypes (i.e. HER2+ DCIS with high numbers of TILS versus HER2+ DCIS with low numbers of TILs) is not well known, since the majority of current studies did not correct for DCIS subtype. Nonetheless, HER2+ DCIS presents with a high number of CD8+ T cells, compared to HER2- DCIS[29–31].

HER2 overexpression in DCIS is a potential immune antigen. This might contribute to the relatively high frequency of TILs observed in HER2+ DCIS compared to HER2+ IBC[7,32–35]. However, not all HER2+ DCIS cases present with increased numbers of TILs. This suggests that other molecular alterations of the tumor cells could also contribute to the density of TILs. Several methods to manipulate the tumor in order to convert TIL-low tumors in TIL-high tumors have been reported in breast cancer[36–38]. The identification of (potentially targetable) transcriptomic

alterations of tumor cells that are associated with the density of TILs could guide further development of immune mediated therapy in HER2+ DCIS, such as targeted vaccine therapy. The aim of this study was therefore to compare the gene-expression profile of TIL-poor HER2+ DCIS to that of TIL-rich HER2+ DCIS.

7.2 Patients and methods

7.2.1 Study cohort and histopathological assessment

We included treatment-naïve patients diagnosed with DCIS (without an invasive component) at the Erasmus Medical Center in Rotterdam or the Laboratory for Pathology in Dordrecht, between 2004 and 2016. Patients for this study were selected from a previously described cohort of pure DCIS cases. All included patients were estrogen receptor (ER) and progesterone receptor (PR) negative and HER2 positive (ER-PR-HER2+). ER (SP1; Ventana) and PR (1E2; Ventana) were previously scored according to Dutch guidelines for IBC and HER2 (4B5; Ventana) was scored according to international guidelines[39,40]. Additionally, Ki67 (30-9; Ventana) and P53 (Bp53011; Ventana) were determined by automated immunohistochemistry using the Ventana Benchmark Ultra (Ventana Medical System Inc.). Ki67 was scored as the percentage of positive cells and P53 was scored as wild type, absent or overexpressed. Detailed staining procedure is listed in **Table S7.1**.

Using all diagnostic hematoxylin & eosin stained whole sections of excision specimens, TIL-density of these HER2+ DCIS cases was semi-quantitatively scored as previously described by Agahozo et al.[30].

Using these scores, cases with minimal/mild TIL-density and matched cases with severe TIL-density were included. Cases with 0-30% of the DCIS-associated stroma occupied by TILs were classified as TIL-poor and cases with >50% of the DCIS-associated stroma occupied by TILs were classified as TIL-rich. These cases were matched based on age, histologic grade, presence of comedonecrosis and tumor diameter. Patients with IBC within the first 6 months after diagnosis were excluded. According to the code of conduct of the Federation of Medical Scientific Societies in The Netherlands, there was no need for an informed consent, since only encoded left-over patient material was used for this study[41]. This work was approved by the Medical Ethics Committee (MEC 02.953).

7.2.2 Microdissection and RNA isolation

Tumor cells from both TIL-rich and TIL-poor cases were micro-dissected **Figure 7.1**. DCIS cells were micro-dissected separately from the TILs, to ensure pure tumor cell samples. Prior to micro-dissection, the areas for micro-dissection were marked using the last 4μm thick hematoxylin & eosin stained slide of 11 sequential slides. The remaining 10 slides with 10μm thick sections of formalin-fixed paraffin-embedded tissues of DCIS were dewaxed and rehydrated, followed by a hematoxylin staining. Following hematoxylin staining, DCIS cells were micro-dissected manually using a sterile scalpel under a stereomicroscope (Zeiss, Oberkochen, Germany) and stored into RNAse/DNase free tubes containing RNALater (Thermo Fisher). Samples were stored at -80°C until further RNA isolation.

Prior to isolation, samples were spun down, RNALater was removed and samples were washed with ethanol. Next, RNA was isolated using the AllPrep RNA/DNA formalin-fixed paraffin-embedded isolation kit (Qiagen, Germany) according to the manufacturer's instructions. After isolation, RNA concentrations were measured by Nanodrop (Thermo Fisher Scientific) and picogreen by Qubit (Thermo Fisher Scientific). Additionally, we performed a quality control on all RNA samples using RT-qPCR (Bioline) to validate the amplification and MultiNA (Shimadzu) to quantify the fragment size and concentration, as previously described by Siewerts et al.[42]. Samples were then stored at -80°C.

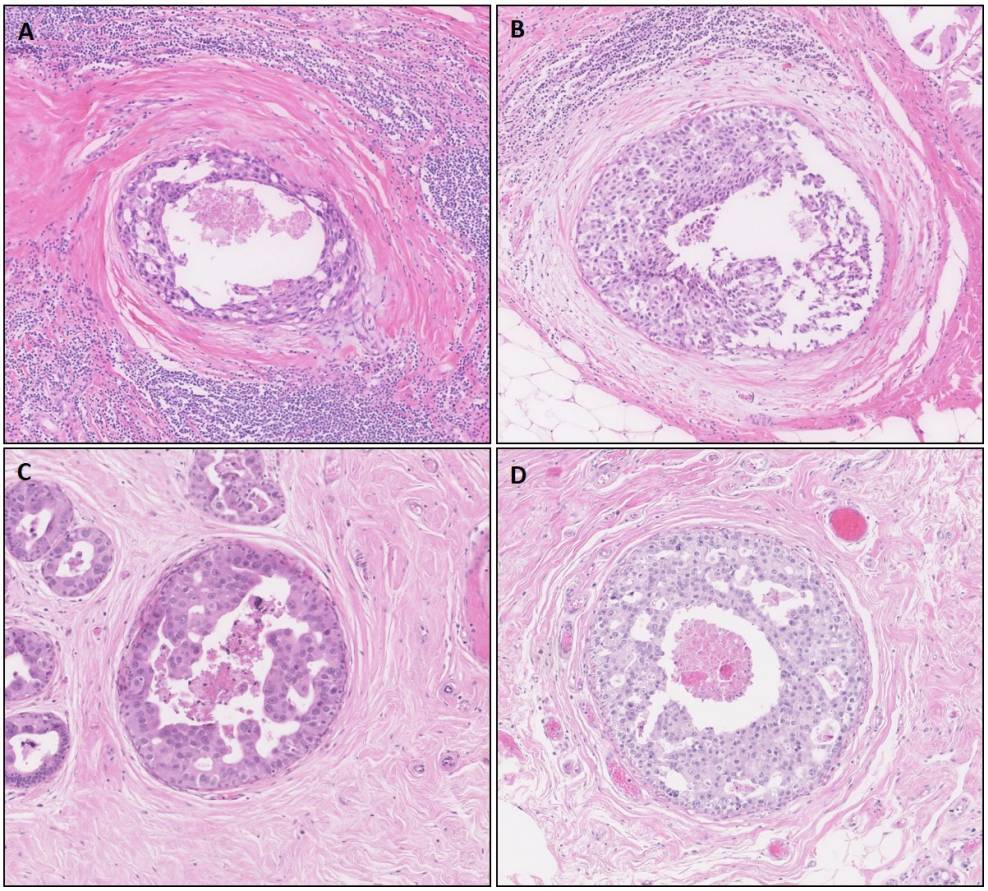

Figure 7.1: Histological representation of TIL-rich (A and B) DCIS. Neoplastic cells are surrounded by a TIL-poor area of reactive stroma and subsequently, a zone of many TILs. Figure C and D represent TIL-poor DCIS cases.

7.2.3 Targeted RNA sequencing

The Ion AmpliSeq Transcriptome Human Gene Expression Kit was used for targeted RNA sequencing. Using 5 to 43 ng of formalin fixed paraffin-embedded RNA, cDNA was generated

using the SuperScript® VILO™ cDNA Synthesis Kit, followed by target regions amplification using the Ion AmpliSeq Transcriptome Human Gene Expression core panel. After partial digestion of the primers, adapters were ligated to amplicons and purified. The generated library was quantified by qPCR with the Ion Library TaqMan Quantitation kit. Pooled libraries, with 6 to 8 samples per pool, were templated on the Ion Chef and sequenced using a 540 chip on the Ion GeneStudio S5 Prime system. Finally, transcription data were generated as raw read counts using the ampliSeqRNA(target region:hg19_AmpliSeq_Transcriptome_21K_v1).

7.2.4 Data processing and analysis

R v3.6 was used for data analysis. First, raw read counts were normalized using EdgeR[43]. After normalization, a Principle Component Analysis (PCA) showed large differences in overall expression distribution according to the batch of the samples. To correct for this, genes were removed if not expressed in all samples from a single batch. The remaining missing data were imputed per gene by using the median expression level of the gene. These data (8753 genes) were used for input in ComBat to correct for the batch-effects[43]. Finally, a PCA was used to confirm correction of the batch-effect. The PCA after batch-correction did not separate the samples based on TIL-rich or TIL-poor cases.

In order to measure the purity of our tumor RNA samples, we assessed the potential admixture of TILs using a previously described TIL-signature[44]. This TIL-signature was generated using breast cancer samples with a high and low TIL count, combined with gene-expression data from these samples (GEO54219). This analysis resulted in a 152-probe signature that highly correlated with the percentage of TILs in the specimens. Recently, this signature was validated in an independent set and associated with subtype specific prognosis in breast cancer, which included 109 genes[45]. A Mann-Whitney Ranksum test was used to analyze differentially expressed genes between TIL-poor and TIL-rich HER2+ DCIS. We used genes with a P-value of <0.05 to generate an s-curve of the fold-change to determine a cut-off. The top and bottom tail of the s-curve included genes with a fold-change of >4 or $\leq$4. This included the genes from which we selected the ones suitable for immunohistochemistry. Next, we searched for the protein function in breast cancer and immune regulation via PubMed database and Uniprot.org. We selected three genes for immunohistochemistry based on their association with breast cancer, immune regulation and finally, antibody availability.

7.2.5 Immunohistochemistry

From these 29 differentially expressed genes, three genes, including CCND3, DUSP10 and RAP1GAP were selected for further analysis of protein expression. CCND3 and DUSP10 were previously associated with survival in IBC and RAP1GAP was associated with breast cancer invasiveness[46–50]. Finally, these genes were also described in relation to immune regulation[46,50,51].

To determine protein expression, we stained 4-μm thick formalin-fixed paraffin-embedded whole tissue sections by automated immunohistochemistry using the Ventana Benchmark ULTRA (Ventana Medical System Inc.). We also included breast cancer cell lines in triplicate, with known microarray gene-expression data[52]. Detailed staining procedures are listed in **Table S7.1**. Briefly, following deparaffinization and heat-induced antigen retrieval, tissue samples were incubated with DUSP10 (polyclonal; Abcam), RAP1GAP (Y134; Abcam) or CCND3 (DCS2.2; Ab-

cam) (**Table S7.1**). After incubation, hematoxylin II counter stain was incubated for 8 minutes and followed by a blue coloring reagent for 8 minutes according to the manufacturer's instructions (Ventana). The protein expression of DUSP10 and RAP1GAP was semi-quantitively scored according to the H-score, whereby the staining intensity (0-3) was multiplied by the total percentage of positive epithelial cells (0-100)[53]. CCND3 was scored as the percentage of positive epithelial cells.

IBC cell lines were scored independently by two observers. The average score was used for analyses.

7.2.6 Statistical analysis

All clinical and protein data was statistically analyzed using SPSS Statistics 21 (IMB). A chi-square test was used to test for associations between the TIL status and categorical variables. For continuous variables, a Mann-Whitney U test was used to test for differences in case these were not normally distributed. A student's T test was used to compare means of continuous variables. Correlations were tested using the Spearman's Rho. Results were considered significant with a P-value <0.05.

7.3 Results

7.3.1 Clinicopathologic patient characteristics

A total of 23 patients were included, of which 11 TIL-rich and 12 TIL-poor cases, with a median age at diagnosis of 56, ranging from 37.0 to 73.0 years. General patient and DCIS characteristics are depicted in **Table 7.1**. The majority of DCIS cases were high grade, with comedonecrosis and calcification. The median Ki67-expression was 14.2% and the majority of the patients (69.5%) had a mutated p53 protein expression, whereby overexpression was detected in 47.8% of the cases. These clinicopathological characteristics were similar between TIL-rich and TIL-poor patients (**Table S7.2**).

Table 7.1: Patient and DCIS characteristics

Characteristic	n/median	%/range
Age	**56**	**37.0 - 73.0**
- DCIS size (cm)	3.8	0.9 - 9.0
- Grade		
- Low	0	0
- Intermediate	1	4.3
- High	22	95.7
Comedonecrosis		
- Absent	3	13
- Present	20	87
Growth Pattern		
- Solid	15	65.2
- Cribriform	7	30.4
- Papillary	1	4.3
p53		
- Wild type	7	30.4
- Aberrant	11	47.8
- NULL	5	21.7
- Ki67 (%)	14.2	3.0 - 25.0

7.3.2 Differentially expressed genes on RNA level

In order to validate the purity of our micro-dissected tumor cells, we determined the TIL score of each sample using normalized (log2 scale), imputed and batch corrected data, using the TIL-signature (109 genes)[45]. Then, we compared the TIL score from TIL-rich DCIS samples to that of TIL-poor samples. There was no difference in the mean TIL score between TIL-rich and TIL-poor samples (P=0.294, score=2.1 vs 2.5 respectively).

Besides analyzing the TIL-signature as a whole, we also investigated individual genes of the signature, but we did not identify any TIL-signature gene that was significantly differentially expressed between the micro-dissected tumor cells of TIL-rich and TIL-poor samples. Thus, these data are suitable to identify differentially expressed genes between TIL-rich and TIL-poor samples that are indeed derived from transcriptomic differences of the tumor cells and not related to the potential admixture of TILs.

After data processing and analysis, 29 differentially expressed genes were selected (**Table 7.2**). Many of these genes (14 out of 29) were involved in the cell cycle or protein transportation. A hierarchical clustering was performed on these genes (**Figure 7.2**). This hierarchical clustering demonstrates two clear groups, whereby TIL-rich DCIS are clustered together and TIL-poor DCIS are clustered together, with an exception of three samples. Two samples were TIL-poor (A6751 and A6793) that clustered with TIL-rich samples and one was a TIL-rich sample (A6700) that clustered with TIL-poor samples.

7.3.3 Differentially expressed genes on protein level

The mRNA expression levels of CCND3, DUSP10 and RAP1GAP were higher in TIL-rich DCIS compared to TIL-poor DCIS, with a median Log2 value of 5.60 vs 3.50 for CCND3, 7.17 vs 4.44 for DUSP10 and 6.20 vs 3.58 for RAP1GAP, respectively. To validate this differential expression on the protein level, immunohistochemical analysis was performed.

Protein expression of CCND3, DUSP10 and RAP1GAP is depicted in **Figure 7.3**, including representative images (**Figure 7.3** D, E and F). Nuclear CCND3 protein expression was scored as the percentage of positive DCIS cells. CCND3 protein expression was detected in 10 out of 23 patients, the majority (n=8) was TIL-poor. TIL-rich DCIS showed a lower CCND3 protein expression compared to TIL-poor DCIS, P=0.029, with a median percentage of positive cells of 0.0 vs 3.0%. This remained after dichotomization, using presence or absence of CCDN3 expression (cut-off at 1%) (P=0.036, chi-square test). The presence of CCND3 protein expression was associated with TIL-poor DCIS. Cytoplasmic DUSP10 and RAP1GAP protein expression was detected in all patients and scored as an H-score.DUSP10 protein expression was lower in TIL-rich DCIS compared to TIL-poor DCIS, P=0.008, with a median H-score of 30.0 vs 110.0 respectively. We also observed lower RAP1GAP protein expression in TIL-rich DCIS compared to TIL-poor DCIS, median H-score of 90.0 vs 135.0, respectively. However, this difference was not significant(P=0.064).

RNA and protein expression of CCND3, DUSP10 and RAP1GAP was evaluated on 52 breast cancer cell lines with known gene-expression, to assess the correlation between gene and protein expression of these genes. There was no correlation between the mRNA and protein expression. Spearman's Rho were -0.150, 0.073, 0.198 for CCND3, DUSP10 and RAP1GAP respectively (P=0.292, 0.613 and 0.164 respectively). This was also true for ER-HER2+ cell lines (n=11).

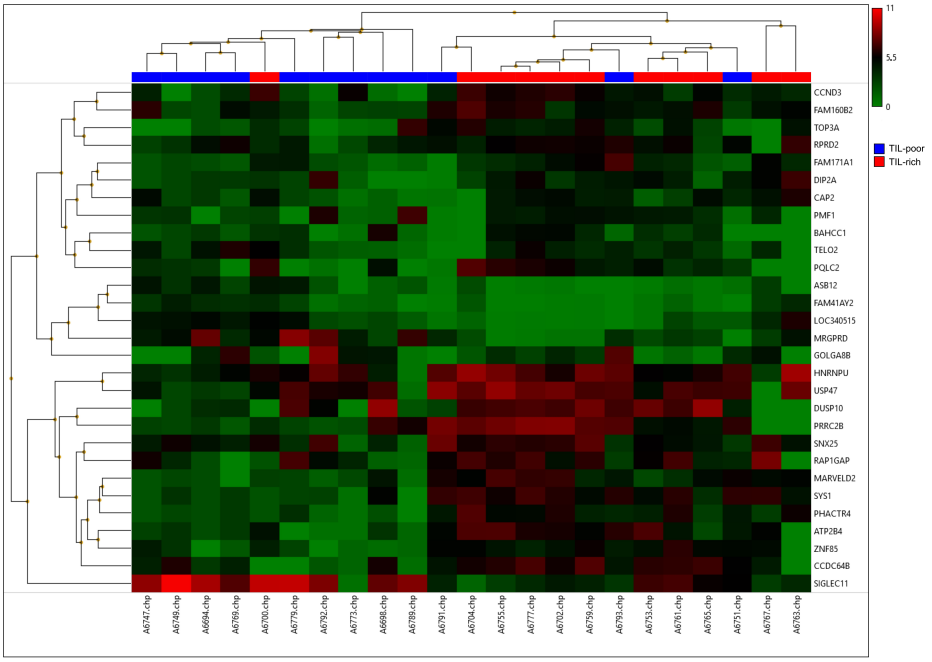

Figure 7.2: A hierarchical cluster of the top 29 differentially expressed genes. High expression is depicted in red and low expression is depicted in green. TIL-poor DCIS are depicted in blue and TIL-rich DCIS are depicted in red.

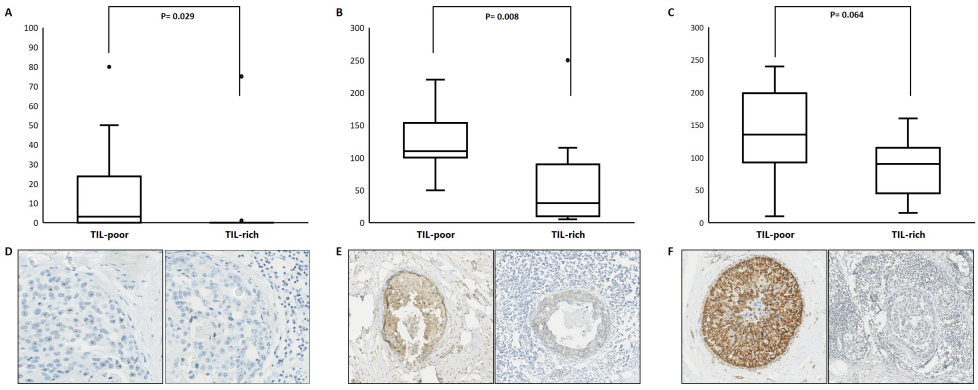

Figure 7.3: Protein expression of DUSP10 and RAP1GAP according to TIL-poor and TIL-rich DCIS. The upper panels depict the protein expression level as H-scores of DUSP10 (A) and RAP1GAP (B). The lower panels depict representative immunohisctohemical images of DUSP10 (C) and RAP1GAP (D).

Table 7.2: 29 gene signature.

Gene	p-Value	FDR Hochberg	Median TIL-high (log2)	Median TIL-low (log2)	Fold Change (high-low)	Role in (breast) cancer	Reference	General Function (uniprot.org)
ASB12	0.05	1.00	-0.18	1.92	-4.28	unknown	n.a.	E3 ubiquitin protein, translation
ATP2B4	0.01	1.00	5.75	2.59	8.89	metastasis surpressor of BRAF mutated melanoma cells, overexpression plays a role in Chronic lymphocytic leukemia pathogenesis, lower ATP2B4 mRNA expression in invasive breast cancer tissue samples compared to normal breast tissue	Hegudus et al., Int J Cancer 2017, Johnston et al., Mol Cell Proteomics 2018, Varga et al., BMC Cancer 2028	Enzyme/catalyzes calcium transport
BAHCC1	0.01	1.00	4.14	1.98	4.47	Predicts survival in Melanoma, upregulated in hepatocellular carcinoma	Gao et al., Biomed Res Int 2020, Nalesnik et al., Am J Pathol 2012	Chromatine binding protein, Cell cycle
CAP2	0.02	1.00	3.81	1.58	4.70	Associated with PR expression and decreased overall survival in brest cancer, suggested prognostic marker in gastric cancer	Xu et al., Oncol Rep 2016, Li et al., Pathol Oncol Res. 2020	regulatory protein, Unknown
CCDC64B	0.01	1.00	6.26	4.21	4.15	unknown	n.a.	Rab GTPase binding, Transport
CCND3	0.02	1.00	5.60	3.50	4.29	Amplified in basal like breast cancer, correlates with reduced overall suvival in breast cancer, discriminates inflammatory breast cancer from non-inflammatory breast cancer	Smid et al., Nat Comm 2016, Ding et al., Cancer Medicine 2019, Keyomarsi et al., N Engl J Med 2002, Lerebours et al., BMC 2008,	regulatory protein, Cell cycle
DIP2A	0.05	1.00	4.65	2.42	4.68	promotes FSTL1 immune resistance and correlates with poor prognosis in Non small cell lung cancer patiens	Kudo-Saito et al., Cell Rep 2018	regulatory protein, developmental protein
DUSP10	0.01	1.00	7.17	4.44	6.62	Mediates immune response, increased DUSP10 dwonregulates inflamation and overexpressed in HER2+ breast cancer, High expression associated with reduced relapse free survival in ER+ wt P53 breast cancer	Jiménez-Martínez et al., Int J Mol Sci. 2019, Hrstka et al., Mol Onc 2015	Enzyme, proliferation and differentiation
FAM160B2	0.03	1.00	5.12	3.03	4.26	Enhances tumorigenesis in hapatocellular carcinoma (RAI16)	Wang et al., Carcinogenesis 2012	Unknown
FAM171A1	0.00	1.00	3.69	1.32	5.16	Increased expression in invasive vs in situ breast carcinoma, correlates with loss of ER and formation of mammospheres (in cell lines) (suggested to increase metastatic potential) in triple negative breast cancer, Suggested prognostic marker in triple negatrieve breast cancer (cell lines), ref.	Rsila et al., The American Joural of Pathology 2019, Sanawar et al., Ongegenesis 2019, Bao et al., Cell Death Dis. 2019	**Cell shape/mortality**
FAM41AY2	0.02	1.00	-0.79	1.35	-4.38	unknown	n.a.	Unknown
GOLGA8B	0.04	1.00	2.39	4.48	-4.27	associated with shorter overall survival in patients with renal cell carcinoma, associated with tumor progression and prognosis in prostate cancer	Wang et al., Journal of Cellular Biochemistry 2018, Cheng et al., J Cell Mol Med. 2020	**Protein transport**
HNRNPU	0.02	1.00	7.04	5.02	4.07	Upregulated in pancreatic ductal adenocarcinoma, mediates invasion and migration in pancreatic ductal adenocarcinoma cell lines	Shen et al., Med Sci Monit 2018, Sutaria et al., Noncoding RNA 2017	DNA/RNA binding protein, cell cycle/transcription
LOC340515	0.04	1.00	0.56	2.56	-4.02	Unkown	n.a.	
MARVELD2	0.02	1.00	4.44	1.98	5.49	Generally described in pancreas and liver carcinoma. Overexpression (tricellulin) associated with unfavorable pronnosis in primary liver carcinomas, decreased expression correlates with poor prognosis in pancreatic adenocarcionma	Somoracz et al., Pathol Oncol Res. 2014	**Cell-cell junction**
MRGPRD	0.01	1.00	2.33	4.67	-5.09	Unkown	n.a.	**Transcription**
PHACTR4	0.02	1.00	4.76	2.27	5.61	Suggested tumorsupressor in various cancers including breast cancer, overexpression inhibits cell proliferation and invasion in hepatocellular carcinoma by inhibiting IL6/Stat3 pathway	Solimini et al., PNAS 2012, Cao et al., Eur Rev Med Pharmacol Sci. 2016	regulatory protein, Cell cycle
PMF1	0.04	1.00	5.00	2.51	5.65	Regulates the expression of SSAT in breast cancer cell lines, Methilation associated with bladder cancer progression	Husbeck et al., Biochem Biophys Res Commun 2003, Aleman et al., Clin Cancer Res. 2008	involved in Cell cycle
PQLC2	0.00	1.00	5.78	3.73	4.13	Overexpression promotes cel growth and tumor formation of gastric cancer in nude mice. Supression/inhibition causes cell death of cancer cells and supressed growth.	Jeung et al., Cancer Sci 2019	**Protein transport**

Table S7.3 *Continued* 29 gene signature.

Gene	p-Value	FDR Hochberg	Median TIL-high (log2)	Median TIL-low (log2)	Fold Change (high-low)	Role in (breast) cancer	Reference	General Function (uniprot.org)
PRRC2B	0.02	1.00	6.47	2.89	11.98	Unknown, somatic variant found in T-cell lymphoma	Donner et al., Fam Cancer 2019	RNA binding, cell differentiation
RAP1GAP	0.03	1.00	6.20	3.58	6.18	Tumorsupressive in several cancers: inhibits progression in endometrial cancer, Increased in ductal carcinoma in situ compared to invasive breast cancer, reduced expression enhances invasion.	Tamate et al., Biochem Biophys Res Commun. 2017, Shah et al., Neoplasia 2018,	regulatory protein, differentiation and proliferation
RPRD2	0.04	1.00	5.92	3.90	4.05	Mutated in burkitt lymphoma	Kaymaz et al., Mol Cancer Res. 2017	regulatory protein, transcription
SIGLEC11	0.03	1.00	4.29	7.40	-8.59	Unknown	n.a.	regulatory protein
SNX25	0.01	1.00	5.97	3.82	4.44	Unknown	n.a.	**Protein transport**
SYS1	0.04	1.00	5.81	2.75	8.32	Overexpressed in cervical cancer	Wu et al., Mol Med Rep 2018	**Protein transport**
TELO2	0.05	1.00	4.38	2.21	4.52	Associated with oncogenic profile in breast cancer cell line	Morais-Rodrigues et al., Gene 2020	**Cell cycle**
TOP3A	0.02	1.00	4.55	2.26	4.89	Unknown	n.a.	DNA/RNA binding protein, cell cycle/transcription
USP47	0.03	1.00	7.75	5.64	4.33	Promotes EMT (mortality and disasociation) in breast cancer cells	Silvestrini et al., J Proteomics 2020	Ubiquitin specific protease, negative regulator of cell cycle
ZNF85	0.02	1.00	4.97	2.93	4.12	unknown, overexperssed in SCLC cell lines	Loiselle et al. Heliyon 2016	DNA/RNA binding protein, transcription

7.4 Discussion

HER2+ IBC is associated with increased numbers of TILs, which is associated with better prognosis. With regard to DCIS, data regarding the clinical relevance of TILs remains limited, but previous data suggested a potential role of DCIS-associated TILs with respect to its biological behavior[20,27,30]. Additionally, not all HER2+ DCIS cases present with increased numbers of TILs. Differently expressed genes of HER2+ DCIS cells might play a role in the density of TILs and could therefore contribute to DCIS progression. In this study, we compared the gene-expression profile of TIL-poor HER2+ DCIS to that of TIL-rich HER2+ DCIS.

Clinicopathological characteristics did not differ between TIL-poor and TIL-rich DCIS cases. This included Ki67 and P53 protein expression, which are generally linked to higher number of TILs in IBC[54–56]. Since DCIS cells and DCIS-associated TILs are anatomically separated in the majority of cases, as illustrated in Figure 1, manual microdissection under a stereomicroscope has no substantial effect on the purity of tumor cells. Based on the gene expression profile of micro-dissected DCIS cells, there was indeed no indication that the purity of tumor cells in our samples could have affected the results. Overall, we identified 29 differentially expressed genes potentially playing a role in the density of TILS in HER2+ DCIS, of which many were involved in the cell cycle or protein transportation.

Out of these 29 differentially expressed genes, we analyzed three genes of interest at the protein level. These genes are CCND3, DUSP10 and RAP1GAP and are involved in cell proliferation, differentiation and migration. Additionally, they were previously described in breast cancer and cancer immunity. CCND3 belongs to the cyclin D family and functions as a regulator of the CDK kinases, which are involved in the differentiation and proliferation of tumor cells[57]. It is predominantly amplified in basal like breast cancer, associated with breast cancer progression and reduced overall and disease free survival[47,57–59]. In our study, TIL-rich DCIS cases were associated with higher CCND3 gene expression compared to TIL-poor DCIS. On the contrary, protein expression of CCND3 was predominantly found in TIL-poor DCIS. The discordance between mRNA and protein expression might indicate posttranslational regulation of CCND3 expression. Indeed ubiquitin mediated degradation of CCND3 by FBXL2 has been demonstrated in lung cancer, while in breast cancer cells the RNA-binding protein IMP-3 can regulate the CCND3 protein expression by directly binding to its mRNAs[60,61]. Further corroborating with our finding is the fact that the presence of CDK4/6, which are regulated by CCND's, has previously been associated with absence of TILs in IBC[62]. Additionally, inhibiting these kinases increased T cell infiltration and activation of effector T cells in (murine) tumors[63,64]. We therefore suggest a potential involvement of TILs in the regulation of CCND3 expression. However, we cannot exclude it to be the regulation to be the other way around. Nonetheless, the actual causal role of TILs in this regulation has yet to be investigated.

DUSP10 is a phosphatase that is upregulated in HER2+ IBC and reduces the inflammatory response[65–67]. Similar to CCND3, we found higher DUSP10 gene-expression levels in TIL-rich DCIS compared to TIL-poor cases, while the opposite was found at the protein level. Our data suggest that DUSP10 protein expression, despite lower transcript level, might be linked to suppressing TIL density in HER2+ DCIS. This is also in line with data demonstrating that reduced DUSP10 expression in airway epithelial cells potentiated the release of CXCL8, CXCL1 and increased IL-1β levels, which promote immune infiltration[68].

RAP1GAP is a GTPase-deactivating protein, which controls the activity of Rap1. It has been

reported as a tumor suppressor gene in various solid tumors, including IBC[69,70]. Specifically, downregulation of RAP1GAP has been demonstrated to occur at the switch from DCIS to IBC[70]. Its expression is increased in the DCIS stage and drops in the IBC stage. However, this study did not report the presence of TILs. We demonstrated increased RAP1GAP gene expression in TIL-rich HER2+ DCIS compared to TIL-poor DCIS, but observed the opposite regarding the protein expression. While these results might seem opposing, RAP1GAP is regulated at the posttranslational level. The RAP1GAP protein can be degraded though the PLK1-mediated ubiquitination pathway[69]. On its turn, PLK1 expression is positively associated with increased number of TILs in IBC[71–73]. This suggests that TIL-rich HER2+ DCIS could have increased PLK1 expression, which may lead to more RAP1GAP degradation on the protein level. However, the mechanism on how RAP1GAP and PLK1 expression are linked to TILs remains unanswered.

In summary, we identified 29 genes in HER2+ DCIS that are associated with the density of TILs, which supports a potential role of these genes in the local immune response and consequently, the biological behavior of DCIS. Considering our small cohort and lack of significant FDR P-values, these genes need to be further validated and evaluated. However, our data support that the density of TILs in HER2+ DCIS is a consequence of mechanisms whereby genetic modifications might need to be altered at the protein level. The identified gene-classifier may guide towards more rationalized choices with respect to immune mediated therapy in HER2+ DCIS, such as targeted vaccine therapy.

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Supplementary information

Table S7.1: Antibody characteristics and used protocol for whole tissue IHC

Antibody	Type	Concentration/dilution	Company	Clone	Lot number	Procedure	Incubation time
ER	Anti-Rabbit	1 µg/ml	Ventana	SP1	F02583	Ultraview CC1 64'	32 minutes
PR	Anti-Rabbit	1 µg/ml	Ventana	1E2	Y08684	Ultraview CC1 36'	12 minutes
HER2neu	Anti-Rabbit	6 µg/ml	Ventana	4B5	E06192	Ultraview CC1 36'	32 minutes
KI67	Anti-Rabbit	2 µg/ml	Ventana	30-9	F01342	Ultraview CC1 36'	28 minutes
P53	Anti-Mouse	2.5 µg/ml	Ventana	Bp53-11	F27098	Ultraview CC1 64'	4 minutes
CCND3	*Anti-Mouse*	*1:100*	*Abcam*	*DCS2.2*	*GR3223822-11*	*Optiview CC1 32'*	*32 minutes*
DUSP10	Anti-Rabbit	1:100	Abcam	polyclonal	GR3213653-10	Ultraview CC1 36'	32 minutes
RAP1GAP	Anti-Rabbit	1:3000	Abcam	Y134	GR257921-12	Ultraview CC1 36'	32 minutes

Table S7.2: Patient and DCIS characteristics according to TIL denisty

Age (in years; mean - range)	57.3 (38.0-72.0)	54.5 (37.0-73.0)	0.689
DCIS size (in cm; mean - range)	3.2 (0.9-6.50)	4.38 (1.5-9.0)	0.341
Grade (%)			
- Low	0 (na)	0 (na)	1
- Intermediate	1 (100)	0 (0)	
- High	11 (50)	11 (50)	
Comedonecrosis (%)			
- Absent	2 (67)	1 (33)	1
- Present	10 (50)	10 (50)	
Growth Pattern (%)			
- Solid	9 (60)	6 (40)	0.427
- Cribriform	3 (43)	4 (57)	
- Papillary	0 (0)	1 (100)	
p53 expression (%)			
- Wild type	4 (57)	3 (43)	0.822
- Abarrent	6 (55)	5 (45)	
- NULL	2 (40)	3 (60)	
Ki67 (mean - range)	14.8 (3.0-25.0)	13.5 (5.0-25.0)	0.507
Immune cell composition (n; mean - range)			
- CD4+	n.a.	30.0 (0.0-110.0)	n.a.
- CD8+	n.a.	50.5 (14.0-105.0)	
- CD20+	n.a.	5.1 (0.0-45.0)	
- CD68+	n.a.	96.8 (5.0-288.0)	
- FOXP3+	n.a.	7.1 (0.0-20.0)	

descriptives: n.a. = not applicable

Chapter 8

Immune response and stromal changes in ductal carcinoma in situ of the breast are subtype-dependent

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Abstract

Ductal carcinoma in situ (DCIS) associated stromal changes and influx of immune cells might be mediators of progression to invasive breast cancer. We studied the interaction between DCIS-associated stromal changes and immune cell distribution and composition in a well-characterized patient cohort.

We included 472 patients with DCIS. The presence of stromal changes, signs of regression and DCIS-associated immune cell position were determined on hematoxylin and eosin-stained slides. Immune cell composition was characterized by immunohistochemistry (CD4, CD8, CD20, CD68 and FOXP3). The number of intraductal immune cells was quantified per mm2. The interaction between stromal changes, signs of DCIS regression, immune cell composition and location was explored.

Stromal changes and signs of DCIS regression were identified in 30% and 7% of the patients, respectively. Intraductal immune cells mainly comprised CD68+ macrophages and CD8+ T cells. Patients with stromal changes had significantly less influx of immune cells within the duct. DCIS regression was associated with an increased number of intraductal FOXP3+ T cells. The highest number of intraductal CD8+ T cells was seen in the ER+HER2+ subtype.

We suggest that DCIS-associated stromal changes prevent the interaction between immune cells and DCIS cells. However, in case of DCIS regression, we surmise a direct interaction between DCIS cells and immune cells, in particular FOXP3+ cells. Furthermore, the increased number of intraductal CD8+ T cells in the ER+HER2+ DCIS subtype suggests a subtype-specific immune response, which is likely to play a role in the distinct biological behavior of different DCIS subtypes.

Keywords: Breast ductal carcinoma in situ; Periductal stromal changes; Regressive changes; Immune cell subsets; Ipsilateral recurrence

8.1 Introduction

Ductal carcinoma in situ (DCIS) is considered to be a non-obligate precursor of invasive breast cancer and it is treated as such, with respect to local therapy[1,2]. DCIS is a heterogeneous disease and, similar to invasive breast cancer, it can be classified into several surrogate subtypes based on Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor (HER2) expression as determined by immunohistochemistry[3]. Data are limited regarding the treatment-naive behavior of DCIS, but DCIS subtypes have been reported to be associated with distinct biological behavior[4,5]. A substantial proportion of DCIS cases are associated with changes in their microenvironment, such as stromal changes and influx of immune cells[6,7]. These changes could be important mediators of the propensity of DCIS to evolve to invasive breast cancer.

Generally, high numbers of tumor-infiltrating lymphocytes (TILs) are observed in high grade, triple negative or HER2+ DCIS[4,8–10]. These DCIS-associated TILs are generally located in the stroma and mainly consist of CD4+ T cells[4,11–13]. Specific immune cell subsets are reported in association with outcome. High amounts of CD19/CD20+ B cells were associated with shorter recurrence-free survival in patients with DCIS, while high levels of CD8+ T cells were associated with a low risk for ipsilateral recurrence[11,14,15].

Stromal changes have also been associated with outcome and can be described as myxoid stroma or sclerotic stroma[7,13,15]. The presence of myxoid stroma has been associated with an increased ipsilateral recurrence risk[7]. Sclerotic stroma is regarded as part of DCIS regression[13,15]. DCIS regression is a process whereby stromal changes indulge neoplastic cells, which are replaced by fibrosis[13,15–17]. Signs of DCIS regression are associated with high grade DCIS and ER/PR-negative and HER2+ subtypes[13,15–17].

Co-occurrence of DCIS-associated stromal changes, including DCIS regression, and influx of TILs has been described[13,15]. Intraductal influx of CD8+ T cells in particular has been linked to DCIS regression[15]. This observation suggests that DCIS regression could be the result of a targeted immune response. However, data are limited with respect to the interaction of DCIS with the surrounding stroma and immune cells. The aim of this study was therefore to evaluate the interaction between DCIS histopathological characteristics, DCIS-associated stromal changes (including signs of regression) and immune cells (composition and location) in a large, well-documented cohort of patients.

8.2 Patients and methods

8.2.1 Study cohort

This retrospective study included 472 patients with a primary diagnosis of pure DCIS from a previously described cohort[4]. Briefly, these patients were diagnosed at the Erasmus Medical Center Cancer Institute Rotterdam or at the Laboratory for Pathology Dordrecht between 2000 and 2016. Clinical data and a central pathology review, including assessment of DCIS characteristics and DCIS-associated TILs, was performed as previously described by Agahozo et al[4]. DCIS surrogate molecular subtypes were based on immunohistochemistry: ER+PR+/-HER2-, ER+PR+/-HER2+, ER-PR-HER2+ or ER-PR-HER2- (triple negative). A 10% cut-off for ER and PR was

used, according to the Dutch guidelines for hormone receptor assessment in invasive breast cancer[18]. HER2 was scored as 0, 1+, 2+ or 3+ and in situ hybridization (ISH) was performed in cases with an equivocal HER2 expression, according to the ASCO/CAP guidelines[19]. Ipsilateral recurrence was defined as a histologically confirmed in situ or invasive carcinoma $\geq$6 months after the initial diagnosis and treatment. The median follow-up time was 98 months, ranging from 24 to 218 months. Encoded leftover patient material was used, in accordance with the Dutch Code of Conduct of the Federation of Medical Scientific Societies[20].

8.2.2 DCIS-associated stromal changes and signs of regression

All hematoxylin and eosin stained slides from this cohort (n=472) were reviewed to determine the presence of DCIS-associated stromal changes, which was defined as stroma within 1.0 mm of the duct. Stromal changes were classified as absent, sclerotic or myxoid, as illustrated in **Figure 8.1**. Sclerotic stroma was defined as a dense, eosinophilic ring surrounding the duct and myxoid stroma was defined as 'loose' basophilic stroma. DCIS regression was assessed in 450 patients. The remaining 22 patients were excluded because of tissue inaccessibility. DCIS regression was defined as a combination of stromal changes, mostly sclerotic, and a reduction or absence of DCIS cells whereby microcalcification or comedonecrosis might still be present. Some examples of DCIS regression are shown in **Figure 8.2**. This definition is comparable to stage a and b from Horii et al, stage 2 and 3 from Wassermann et al, or phase B, C and D adapted from Morita et al.[13,15,17]. All cases were reviewed by two observers at the same time, using a multiheaded microscope, whereby consensus was reached in case of disagreement.

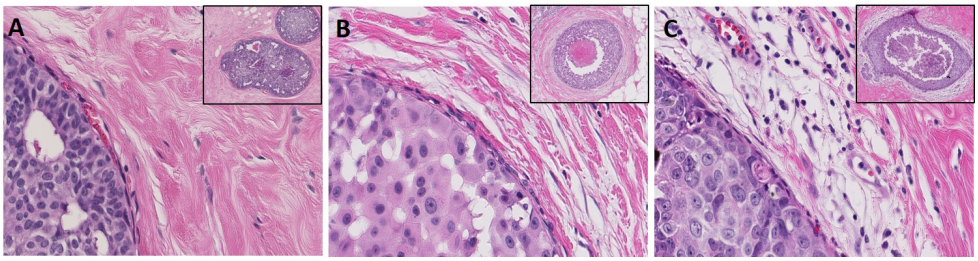

Figure 8.1: Representative images of DCIS-associated stromal changes. DCIS without stromal changes (A), DCIS with sclerotic stroma (B) and DCIS with myxoid stroma (C).

8.2.3 The position and composition of DCIS-associated immune cells

All cases in this cohort were previously classified as either TIL-high (n=131) or TIL-low (n=341) based on hematoxylin and eosin stained slides. The composition of the immune cells in the DCIS-associated stroma was described previously[4]. Briefly, CD4 (T-helpers), CD8 (cytotoxic T-cell), CD20 (B-cells), CD68 (macrophages) and FOXP3 (regulatory T-cells) expressing immune cells were assessed on consecutive 4-µm thick formalin fixed tissue slides, using the automated Ventana Benchmark ULTRA stainer (Ventana Medical System Inc.) according to the manufacturer's instructions.

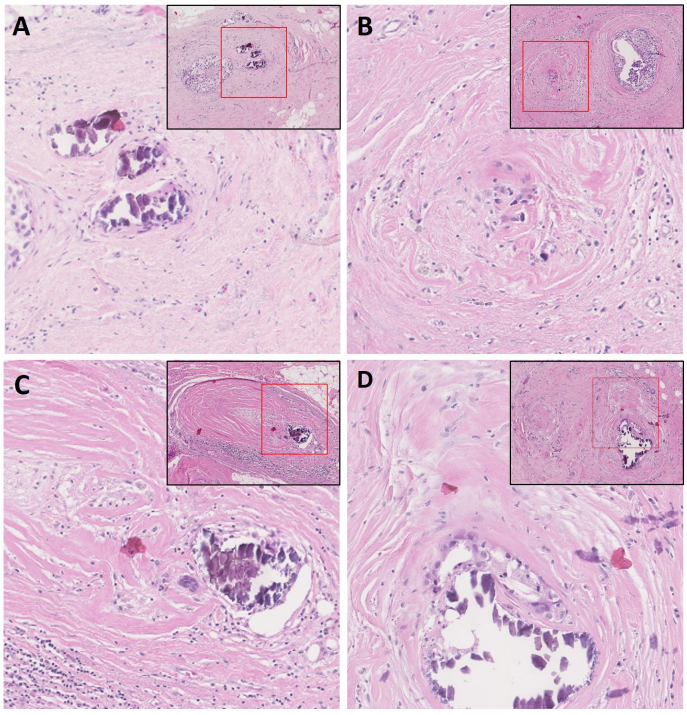

Figure 8.2: Several cases of regression, depicting 'normal' DCIS (A and B) in combination with residual calcification (A, C and D) and/or scare-like structures (B and D). Images are depicted at a 15× magnification and overview images are depicted as inserts at a 5x magnification. Red boxes indicate zoomed in areas.

For the current study, the position of the DCIS-associated immune cells was assessed for TIL-high cases based on central pathology review of hematoxylin and eosin stained slides. The following categories were defined: intraductal (influx of immune cells within the duct), touching (immune cells in direct contact with the duct, as described by Toss et al.) and periductal (area of stroma between the duct and the immune cells)[10]. **Figure 8.3** depicts representative images of these categories. In addition, the composition of the intraductal immune cells was determined, based on immunohistochemically stained slides. Intraductal immune cells were quantified manually by counting the number of positive cells within the duct, either within the epithelium or within the lumen, per mm^2, further described as intraepithelial or intraluminal.

8.3 Statistical analysis

IBM SPSS statistics 21.0 was used to perform the statistical analysis. The Chi-square test was used to test univariate and multivariate associations. In case of a 2×2 table, the Fisher exact test was used. A stepwise backward conditional regression model was used for multivariate testing. For variables with more than two outcome measures, we used a multinominal regression model

for multivariate testing. Unpaired analysis of non-parametric continuous data was performed using the Mann-Whitney U test or the Kruskal-Wallis test for two groups and more than two groups, respectively. Analysis of paired data was performed using the Wilcoxon Signed Ranks Test.

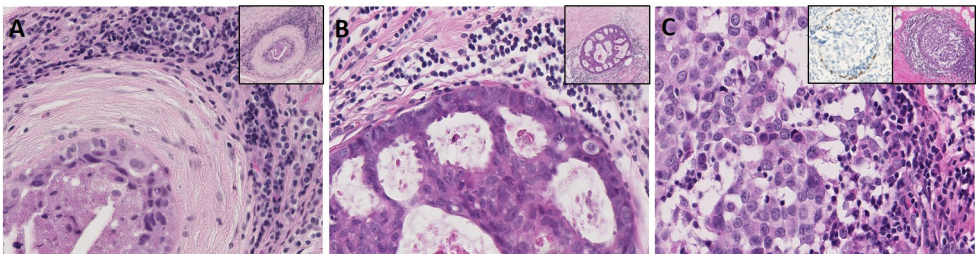

Figure 8.3: Representative images of periductal (A), touching (B) and intraductal (C) DCIS-associated TILs.

8.4 Results

8.4.1 Stromal changes according to clinicopathological characteristics

In this cohort of 472 patients with DCIS, we observed periductal stromal changes in 143 patients (30.3%). The majority of these cases (114 out of 143; 79.7%) was classified as sclerotic and the remainder was classified as myxoid (29 out of 143; 20.3%). Baseline characteristics did not significantly differ between patients with sclerotic and myxoid stroma (**Table S8.1**). We therefore compared patients with stromal changes to those without stromal changes in the remaining analysis.

The presence of stromal changes (either sclerotic or myxoid) was associated with several other DCIS characteristics, as depicted in **Table 8.1**. In univariate analysis, DCIS with stromal changes showed significantly more often a large size (P<0.0001), high grade (P<0.0001), the presence of comedonecrosis (P<0.0001), ER-PR-HER2+ subtype (P<0.001) and TIL-high DCIS (P<0.0001). In multivariate analysis, all of these variables remained significantly associated with stromal changes, except for DCIS size and grade.

8.4.2 DCIS regression according to clinicopathological characteristics

We identified signs of DCIS regression in 30 out of 450 (6.7%) patients. The association between DCIS regression and DCIS characteristics is reported in **Table 8.2**. Overall, DCIS regression was associated with a larger size (P=0.001), high grade (P<0.0001), presence of comedonecrosis (P=0.013), ER-PR-HER2+ IHC subtype (P<0.0001) and TIL-high DCIS (P=0.006). After multivariate analysis, only the association between DCIS regression and ER-PR-HER2+ IHC subtype remained significant (P=0.001).

Table 8.1: Clinicopathological characteristics according to DCIS-associated stromal changes

	Periductal stromal changes (n=472)			
	Present n (%)	**Absent** n (%)	**Univariate P-value**	**Multivariate P-value**
Age at diagnosis (years)				
- Median (range)	58.0 (32.0 - 84.0)	57.0 (27.0 - 84.0)	0.175	-
Size (missing n=65)(cm)				
- Median (range)	2.90 (0.30 - 13.50)	1.70 (0.05 - 17.0)	**<0.0001**	0.054
Growth pattern				
- Solid	81 (57)	157 (48)		
- Cribriform	52 (36)	138 (42)	0.282	-
- Micropapillary	9 (6)	28 (9)		
- Papillary	1 (1)	6 (2)		
Grade				
- Low	3 (2)	60 (18)		
- Intermediate	27 (19)	138 (42)	**<0.0001**	0.07
- High	113 (79)	131 (40)		
Calcification				
- Absent	34 (34)	99 (30)	0.98	-
- Present	109 (76)	230 (70)		
Comedonecrosis				
- Absent	38 (27)	195 (59)	**<0.0001**	**<0.0001**
- Present	105 (73)	134 (41)		
IHC DCIS subtype (missing n=19)				
- ER+PR+/-HER2-	36 (25)	223 (68)		
- ER+PR+/-HER2+	30 (21)	55 (17)	**<0.0001**	**<0.0001**
- ER-PR-HER2+	55 (38)	30 (9)		
- ER-PR-HER2-	15 (10)	9 (3)		
Density of TILs				
- Low	70 (49)	271 (82)	**<0.0001**	**0.011**
- High	73 (51)	58 (18)		
Ipsilateral recurence (missing n=6)				
- No	131 (92)	319 (97)	0.162	-
- Yes	7 (5)	9 (3)		

Table 8.2: The association between DCIS regression and clinicopathological characteristics

	DCIS regression (n=450)			
	Yes n (%)	**No** n (%)	**Univariate P-value**	**Multivariate P-value**
Age at diagnosis (years)				
- Median (range)	60.5 (32.0-81.0)	58.0 (27.0-84.0)	0.469	-
Size (missing n=61)(cm)				
- Median (range)	2.9 (2.80-3.00)	2.0 (0.10-13.5)	**0.001**	0.064
Growth pattern				
- Solid	20 (67)	206 (49)		
- Cribriform	8 (27)	176 (42)	0.292	-
- Micropapillary	2 (7)	33 (8)		
- Papillary	0 (0)	5 (1)		
Grade				
- Low	0 (0)	58 (14)	**<0.0001**	0.174
- Intermediate	4 (13)	152 (36)		
- High	26 (87)	210 (50)		
Calcification				
- Absent	9 (30)	118 (28)	0.835	-
- Present	21 (70)	302 (72)		
Comedonecrosis				
- Absent	8 (27)	215 (51)	**0.013**	0.637
- Present	22 (73)	205 (49)		
IHC DCIS subtype (missing n=18)				
- ER+PR+/-HER2-	5 (17)	241 (60)		
- ER+PR+/-HER2+	6 (20)	75 (18)	**<0.0001**	**0.001**
- ER-PR-HER2+	13 (43)	70 (17)		
- ER-PR-HER2-	6 (20)	16 (4)		
Density of TILs				
- Low	15 (50)	313 (75)	**0.006**	0.818
- High	15 (50)	107 (25)		
Ipsilateral recurence (missing n=6)				
- No	28 (97)	400 (96)	1.000	-
- Yes	1 (3)	15 (4)		

Table 8.3: The association between the immune cell position and clinicopathological characteristics

	Immune cell position (n=131)			
	Periductal n (%)	**Touching** n (%)	**Intraductal** n (%)	**Univariate P-value**
Age at diagnosis (years)				
- Median (range)	60 (32-81)	56 (27-73)	53 (35-85)	0.073
Size (missing n=17)(cm)				
- Median (range)	3.00 (0.10-9.00)	2.55 (0.1-9.20)	3.00 (0.30-16.00)	0.339
Growth pattern				
- Solid	25 (46)	34 (72)	20 (67)	
- Cribriform	22 (41)	9 (19)	6 (20)	0.081
- Micropapillary	7 (13)	3 (6)	4 (13)	
- Papillary	0 (0)	1 (2)	0 (0)	
Grade				
- Low	0 (0)	1 (2)	2 (7)	**0.028**
- Intermediate	5 (9)	4 (9)	8 (27)	
- High	49 (91)	42 (89)	20 (67)	
Calcification				
- Absent	9 (17)	12 (26)	9 (30)	0.328
- Present	45 (83)	35 (74)	21 (70)	
Comedonecrosis				
- Absent	12 (22)	18 (38)	13 (43)	0.087
- Present	42 (78)	29 (62)	17 (57)	
IHC DCIS subtype (missing n=3)				
- ER+PR+/-HER2-	12 (23)	9 (19)	8 (28)	
- ER+PR+/-HER2+	13 (25)	11 (23)	10 (34)	0.786
- ER-PR-HER2+	21 (40)	22 (47)	8 (28)	
- ER-PR-HER2-	6 (12)	5 (10)	3 (10)	
Ipsilateral recurence (missing n=5)				
- No	50 (96)	44 (96)	26 (93)	0.793
- Yes	2 (4)	2 (5)	2 (7)	

8.4.3 Immune cell position according to clinicopathological characteristics

Based on hematoxylin and eosin stained slides, the overall TIL distribution in TIL-high cases was determined. The majority of these DCIS-associated TILs were classified as periductal (41.2%), followed by touching (35.9%) and intraductal (22.9%). Additionally, TIL position was associated with DCIS characteristics (**Table 8.3**). High grade DCIS presented more often with periductal or touching TILs. Contrariwise, intermediate grade DCIS presented more often with influx of TILs within the ducts (P=0.028). No other associations between TILs distribution and histopathological features of DCIS were observed.

For multivariate analysis, periductal TILs were set as a reference variable (**Table 8.4**). Patients with touching TILs were younger (P=0.013), more likely to have a solid growth pattern (P=0.020) and comedonecrosis (P=0.001). Patients with intraductal TILs did not significantly differ from patients with periductal TILs.

Table 8.4: Multivariate analysis

	Immune cell position (n=131)					
	Touching*			Intraductal*		
	Exp(B)	95% CI	p-value	Exp(B)	95% CI	p-value
Age	0.959	0.927 – 0.991	**0.013**	0.971	0.928 – 1.016	0.207
Size (missing n=17) (cm)	1.051	0.918 – 1.202	0.472	1.173	0.989 – 1.392	0.067
Growth pattern	1.841	1.100 – 3.079	**0.020**	1.566	0.769 – 3.188	0.216
Grade	1.406	0.751 – 2.634	0.287	0.565	0.255 – 1.255	0.161
Calcification	0.869	0.400 – 1.888	0.723	0.741	0.258 – 2.125	0.577
Comedonecrosis	0.319	0.158 – 0.641	**0.001**	0.577	0.213 – 1.564	0.280
IHC DCIS subtype (missing n=3)	1.050	0.750 – 1.471	0.776	1.291	0.796 – 2.094	0.300
Ipsilateral recurence (missing n=5)	0.639	0.109 – 3.743	0.620	0.938	0.097 – 9.092	0.956

* Periductal immune cell position set as reference variable

8.4.4 Intraductal immune cell composition

The composition of stromal DCIS-associated immune cells in this cohort was previously described as the percentage of positive immune cells[4]. In the present study, the composition of intraductal immune cells was assessed as the number of immune cells per mm2. Overall, the median number of intraductal immune cells per mm2 was 11.0 (range: 0-559). The majority of these intraductal immune cells were CD68+ macrophages and CD8+ T cells followed by CD4+ T cells and CD20+ B cells, with a median (range) of 5.0 (0-63), 3.0 (0-285), 1.0 (0-83) and 0.0 (0-100) cell per mm2 respectively. The remaining immune cells comprised FOXP3+ T cells. Intraductal immune cells were further classified as either intraepithelial or intraluminal. With regard to intraepithelial immune cells, the majority of the cells comprised CD8+ T cells. With respect to intraluminal immune cells, the majority of the cells comprised CD68+ macrophages.

8.4.5 Immune cell composition according to stromal changes and regression

We compared the immune cell composition according to stromal changes and signs of regression, in order to evaluate whether these changes are immune cell specific. There was no association between the periductal immune cell composition and presence of stromal changes. On the other hand, the number of intraductal CD4+, CD8+, CD20+ and CD68+ immune cells was higher in patients without stromal changes (P=0.011, P=0.048, P=0.014, and P=0.003 respectively). Specif-

ically, a higher number of intraepithelial CD4+ , CD8+ , CD20+ and CD68+ immune cells were found in patients without stromal changes (P=0.014, P=048, P=0.002 and P=0.013 respectively). With respect to intraluminal immune cells, we observed higher numbers of CD68+ macrophages in patients without stromal changes (P=0.006). Intraductal immune cell composition according to stromal changes is depicted in **Figure 8.4A**. Next, we compared the immune cell composition according to the presence of signs of DCIS regression. The proportion of periductal FOXP3+ T cells was higher in cases with DCIS regression compared to those without DCIS regression. We found a similar association with regard to intraductal immune cells. The number of intraductal FOXP3+ T cells was higher in case of DCIS regression (P=0.046). This effect was mainly based on intraepithelial FOXP3+ T cells (P=0.006). **Figure 8.4B** depicts the number of intraductal immune cells according to the presence of signs of DCIS regression.

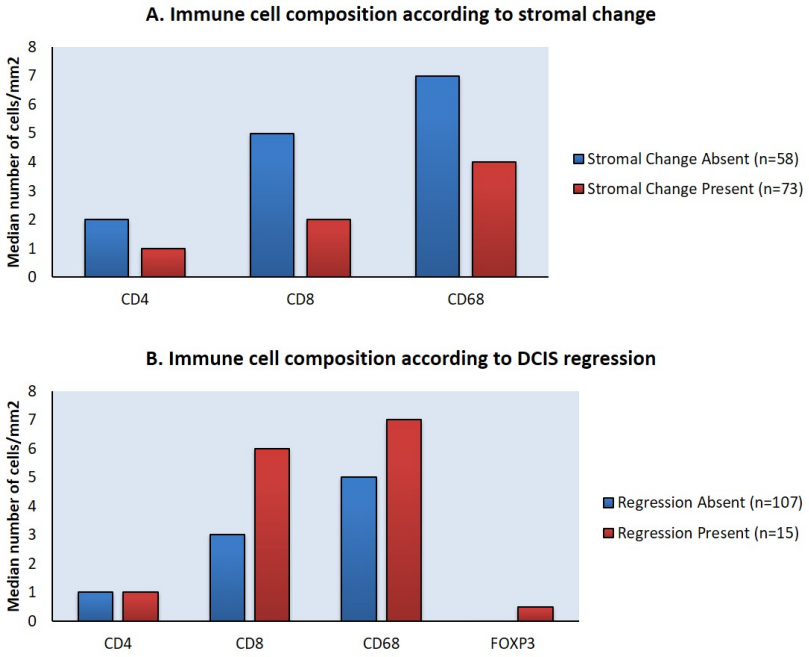

Figure 8.4: Immune cell composition according to stromal changes (A) and DCIS regression (B). Blue bars represent the absence of stromal change or regression and the red bars represent the presence of stromal change or regression. The y-axis depicts the median number of immune cells per mm2; the x-axis depicts the immune cells subset. A Mann-Whitney U test was used to test for differences according to stromal changes (A) or DCIS regression (B).

The composition of the intraductal immune cells correlated with the DCIS surrogate molecular subtype. The ER+PR+/-HER2+ subtype had the highest number of intraductal CD8+ T-cells (P=0.020), either intraepithelial (P=0.008) or intraluminal (P=0.019). The ER+PR+/-HER2- subtype was associated with the highest numbers of intraepithelial CD68+ macrophages (P=0.047). The number of intraductal immune cells according to DCIS subtype is depicted in **Figure 8.5**.

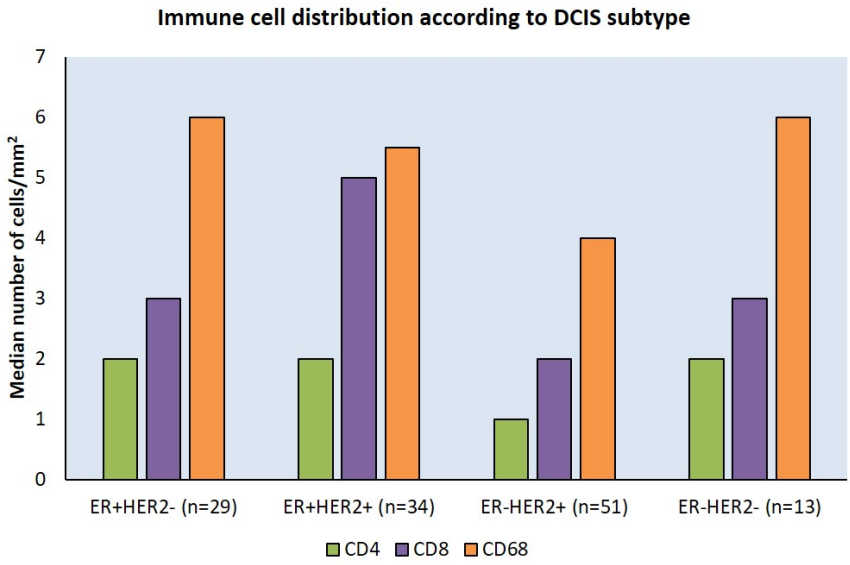

Figure 8.5: Immune cell composition according to DCIS surrogate subtype. The y-axis depicts the median number of immune cells per mm^2; the x-axis depicts the DCIS surrogate subtype. A Kruskal-Wallis test was used to test for differences within DCIS groups.

8.5 Discussion

It is well known that DCIS can induce changes in its microenvironment, including periductal stromal changes and influx of immune cells, which could be mediators of DCIS behavior. However, an in-depth analysis of this interaction is still lacking. In our previous study, we described the density and composition of stromal immune cells according to DCIS subtypes[4]. In this current study, we focused on DCIS-associated stromal changes in relation to immune cell position/composition.

Overall, stromal changes were observed in 30% of all cases, which predominantly consisted of sclerotic stroma (80%). These cases were generally high grade, ER-HER2+ DCIS and associated with a high number of immune cells. Sclerotic stroma has previously only been described as part of DCIS regression in high grade DCIS 13. The presence of myxoid stroma has been reported to correlate with a more aggressive DCIS phenotype,7 which is in line with our data. In our study, signs of DCIS regression were detected in 7% of all patients. This proportion is consistent with an earlier study, which used a similar definition for regression[17]. Several others studies described higher rates of DCIS regression, ranging between 39 and 59%[13,15,16]. However, these studies included smaller cohorts and/or had a different, less stringent definition of DCIS regression. Nevertheless, we observed an association between DCIS regression and HER2+ cases, which is consistent with these previously published findings.

The composition of the immune cell infiltrate differs according to its location. As previously described, DCIS-associated stromal immune cells mainly consist of CD4+ T cells[4,11–13]. Here,

we observed that intraductal immune cells predominantly comprised CD68+ macrophages and CD8+ T cells. When stratified into intraepithelial and intraluminal cells, we observed that the majority of intraepithelial cells were CD8+ T cells and that intraluminal cells mainly comprised CD68+ macrophages. These data illustrate the different biological roles of these cells, since CD8+ T cells are cytotoxic T-lymphocytes that are required to be in close proximity of the neoplastic cells, whereas CD68+ macrophages clean up necrotic cell debris and can rather be found in the lumen of the affected ducts.

Next, we observed that patients with stromal changes had significantly less influx of immune cells within the duct. These results suggest that the presence of stromal changes might act as a physical barrier for immune cells, preventing them from penetrating the basement membrane and subsequently infiltrating the ductal epithelium. This finding has been previously described by Gil Del Alcazar et al., who reported limited interaction between T cells and cancer cells during the DCIS stage. More recently, Mitchell et al., demonstrated that focal loss of myoepithelial cells was associated with an increased number of stromal PD1+CD8+ T cells, which suggests a link between myoepithelial cells and immune surveillance[21].

Counterintuitively, we observed that DCIS regression was associated with high numbers of FOXP3+ T cells. These immune suppressive cells are generally associated with poor outcome in advanced breast cancer and ipsilateral recurrence in DCIS[22–24]. However, in HER2+ breast cancer, they were associated with improved outcome[25,26]. Furthermore, a recent study reported that FOXP3+ regulatory T cells opposed breast cancer progression at early stages in murine mammary tumors[27]. Additionally, a recently identified CAF-S1 subtype, which was enriched in HER2+ breast cancer, was suggested to attract and enhance T cell differentiation of C25+FOXP3+ lymphocytes[28]. We therefore hypothesize that at some stages of regression, the stroma might be more permeable, making it accessible for T cells, specifically the FOXP3+ T cells, which might suppress progression of HER2+ DCIS.

With regard to DCIS IHC subtype, HER2-enriched DCIS is generally associated with a marked immune response[4,9,15]. This response could be triggered by HER2, whereby HER2 overexpression functions as an immune antigen[29]. On the other hand, HER2 overexpression increases the proliferation and growth rate of DCIS, resulting in hypoxia and increased necrosis, which might also trigger the immune response[30]. In our study, ER+HER2+ DCIS was associated with the highest numbers of intraductal CD8+ T cells. These immune cells are generally associated with improved outcome in invasive breast cancer and low risk for recurrence in DCIS[11,25,31]. In pure DCIS, intraductal CD8+ T cells were also associated with lower DCIS grade, absence of comedonecrosis and a low DCIS density score based on the number of DCIS foci[11]. Overall, these results suggest that in ER+HER2+ DCIS, CD8+ T cells might mediate their indolent behavior. Our study included a large, well-characterized DCIS cohort, enabling us to study the DCIS microenvironment in detail. However, our study also had several limitations. First, treatment modalities were unknown, the number of ipsilateral recurrences was too low and the median follow-up time too short to study the association between stromal changes and immune cell composition/position and ipsilateral recurrence risk. Secondly, we used a limited number of markers to determine immune cell composition. The use of additional markers could provide additional information with respect to immune activation. It would therefore be interesting to compare our findings with the results of functional ex-vivo assays. Thirdly, the number of triple negative DCIS cases was limited in our cohort, which is a well-known phenomenon in DCIS series[9]. Lastly, we determined stromal changes based on hematoxylin and eosin staining, while

the use of immunohistochemical markers could provide additional information regarding subtypes of cancer associated fibroblasts. Besides, stromal architecture is a feature with substantial interobserver variability, which could have affected our findings[32].

In conclusion, our study demonstrated the association between DCIS-associated stromal changes, including signs of regression in a particular subset of DCIS, and high numbers of immune cells. We demonstrated a site-specific immune cell composition: stromal immune cells include a high proportion of CD4+ T cells, while the influx of immune cells into the duct is mainly based on CD8+ T-cells and CD68+ macrophages. The influx of immune cells was significantly lower in patients with DCIS-associated stromal changes compared to those without stromal changes. This suggests that the stroma might function as a physical barrier, preventing the interaction of immune cells with DCIS cells. Signs of DCIS regression on the other hand were associated with an increased numbers of FOXP3+ T cells within the duct, suggesting interaction with DCIS cells and an active role for FOXP3+ cells in DCIS regression. Furthermore, the increased number of CD8+ T cells in the HER2+ DCIS subtype suggests that the immune response is subtype-specific. Overall, this site- and subtype-specific immune response is likely to play a role in the different biological behavior of DCIS subtypes.

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Supplementary information

Table S8.1: Clinicopathological characteristics and DCIS-associated TILs according to stromal changes

	Stromal changes (n=143)		
	Sclerotic n (%)	**Myxoid** n (%)	**Univariate p-value**
Age at diagnosis (years)			
- Median (range)	59.0 (35.0 - 84.0)	54.0 (32.0 - 79.0)	0.058
Size (missing n=53)(cm)			
- Median (range)	3.00 (0.10 - 9.20)	2.65 (0.40 - 13.50)	0.494
Growth pattern			
- Solid	60 (53)	21 (72)	
- Cribriform	44 (39)	8 (28)	0.178
- Micropapillary	9 (8)	0 (0)	
- Papillary	1 (1)	0 (0)	
Grade			
- Low	2 (2)	1 (3)	0.592
- Intermediate	20 (18)	7 (24)	
- High	92 (81)	21 (72)	
Calcification			
- Absent	26 (23)	8 (28)	0.375
- Present	88 (77)	21 (72)	
Comedonecrosis			
- **Absent**	29 (25)	9 (31)	0.348
- Present	85 (75)	20 (69)	
IHC DCIS subtype (missing n=11)			
- ER+PR+/-HER2-	29 (27)	6 (23)	
- ER+PR+/-HER2+	25 (24)	4 (15)	0.702
- ER-PR-HER2+	42 (40)	13 (50)	
- ER-PR-HER2-	10 (9)	3 (12)	
Density of TILs			
- **Low**	59 (52)	11 (38)	0.131
- **High**	55 (48)	18 (62)	
Ipsilateral recurence (missing n=5)			
- No	105 (96)	26 (90)	0.161
- Yes	4 (4)	3 (10)	

Part III

DCIS treatment and an alternative for surveillance trials

Chapter 9

Radioactive seed versus wire-guided localization for ductal carcinoma in situ of the breast: comparable resection margins

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Abstract

There are currently two widely used methods for pre-operative localization of ductal carcinoma in situ (DCIS) of the breast: wire-guided localization (WGL) and radioactive seed localization (RSL). Several studies compared these localization techniques in small cohorts. The aim of this study was to compare the surgical resection margin status between RSL and WGL in a large national cohort of patients with DCIS.

We included patients from PALGA (Dutch Pathology Registry) who underwent breast-conserving surgery for DCIS by either RSL (n=1851) or WGL (n=2187) between 2009 and 2019. Several clinicopathological characteristics were compared between these two groups, including the resection margin status and the number of re-excisions.

Patients undergoing RSL were younger (p=0.014) and were more often diagnosed with a large DCIS (p=0.013), high grade DCIS (p<0.001) and comedonecrosis (p<0.001) compared to patients undergoing WGL. There was no significant difference in resection margin status between both groups (P=0.089) and the number of re-excisions (p=0.429). However, in case of re-excision, patients in the RSL group were more often treated with breast conserving surgery (p=0.029).

In this large national cohort study of patients with DCIS, we demonstrated that there was no difference in the resection margin status between both procedures or in the number of re-excisions, but patients in the RSL group were more often treated with breast-conserving therapy in case of a re-excision.

Keywords: In situ carcinoma; breast; DCIS; pre-operative localization; radioactive seed; wire-guided; RSL; WGL

9.1 Introduction

Since the introduction and improvement of breast screening by mammography, the detection rate of ductal carcinoma in situ (DCIS) of the breast has increased[1–3]. Nowadays, DCIS accounts for approximately 20% of all breast cancers in the screened population[2]. The majority of patients with DCIS is treated with surgery, either breast conserving surgery in combination with radiotherapy, or a mastectomy depending on the DCIS size and patients preference[3–5]. Clinically, DCIS is generally non-palpable and has a diffuse growth pattern, which makes it challenging to estimate the correct size using imaging modalities.

In literature, there has been a lack of consensus regarding the most optimal resection margin for patients with DCIS. Several authors used different definitions ranging from 'no ink on the tumor' up to a 10 mm free margin[6,7]. Obviously, extensively positive resection margins are associated with higher risk for local recurrence, but wide margins (>10 mm) are more likely to compromise cosmetics, so this has to be balanced. In 2016, the Society of Surgical Oncology, the American Society for Radiation Oncology and the American Society of Clinical Oncology published a consensus guideline for patients with DCIS undergoing breast conserving surgery with whole breast irradiation. In this guidline, a margin $\geq$2 mm is proposed as being most optimal for patients with DCIS[8,9].

In line with international data, there has been a lack of consensus in the Netherlands regarding the optimal resection margin for DCIS. In the Netherlands, resection margin status for patients with invasive breast cancer (IBC)is defined as free (no ink on tumor), focally positive ($\leq$ 4 mm ink on tumor) or more than focally positive (> 4 mm ink on tumor)[10,11]. According to this guideline, invasive breast cancer patients with a focally positive margin undergo a boost of radiation instead of re-excision based on the low local recurrence rate in this group[12]. A re-excision is advised in patients with more than focally positive margins based on the increased local recurrence risk. For patients with DCIS, a re-excision was previously advised for patients with positive margins (ink on tumor), although regional differences were present[10]. Currently, the Dutch treatment guideline recommends to consider a re-excision for DCIS with a margin < 2mm, which is consistent with international recommendations[13].

In order to achieve optimal resection margins at first surgery, proper preoperative DCIS localization is crucial. There are two widely used techniques for DCIS localization: wire-guided localization (WGL) and radioactive seeds localization (RSL)[4]. In case of a WGL procedure, single or multiple wire hooks are placed into the lesion and used as a guide to the lesion[14]. In case of RSL, single or multiple radioactive iodine-I25 seeds are used to mark the lesion[15]. While WGL was historically seen as the gold standard, it has several disadvantages[14–16]. The timing of this procedure is dependent on the availability of the radiologist and the surgeon, since the wire hook has to be placed just before surgery. Besides, it causes more discomfort for the patient and it may dislocate before or during surgery[14,16]. RSL is therefore seen as an attractive alternative[15,16]. It allows more flexibility scheduling the surgery, since it can be placed during a longer period before surgery and it causes less patients discomfort[14,15]. However, misplaced seeds can only be surgically removed and due to its radioactivity, the introduction of RSL in an institution is a logistic challenge since it requires strict regulation and training of all staff involved[15].

In recent years, several studies have compared both localization techniques but the derived results are inconsistent[14,16–18]. On the one hand, studies have found comparable results between RSL and WGL in terms of excision margins, resection weight and the number of re-excisions[16,18].

On the other hand, a lower rate of positive margins and re-operations has been reported for RSL[14,17]. However, these studies included small cohorts, usually from one institute, and were not able to demonstrate highly significant differences. In order to present more conclusive results, a large multicenter study including high numbers of patients is required. The aim of this study was to perform a nationwide study, using real-world data, in order to compare the efficacy of WGL versus RSL for patient treated with breast conserving surgery for DCIS.

9.2 Patients and Methods

9.2.1 Data collection and patient cohort

All data was obtained from the Dutch Pathology Registry (PALGA), which is the nationwide registry of pathology reports. Since 2009, Dutch pathologists can use synoptic reporting modules to report the pathological test results for several common tumor types, including breast cancer[19]. In these modules, parameters are captured in numerous standardized variables instead of free text fields, which facilitates the analyses of all reports simultaneously.

In this study, synoptic reports of all patients diagnosed with primary DCIS who underwent breast-conserving surgery by either RSL of WSL between 2009 and 2019 in the Netherlands were included. Patients with an invasive component in the initial surgical specimen were excluded from further analysis. Data collection included age at diagnosis, several DCIS characteristics (size, grade, presence of comedonecrosis and microcalcifications), type of surgery, resection margins and number of re-excisions. If available, the estrogen receptor (ER), progesterone receptor (PR) and Human Epidermal growth factor Receptor 2 (HER2) status were also included.

9.2.2 Statistical analysis

Variables that were described in the data collection were analyzed using the 'tableone' package in R (version 3.5.1). To evaluate potential differences between RSL and WGL, statistical analyses were performed. To evaluate whether categorical variables were significantly different between RSL and WGL or between the different numbers of seeds injected in the RSL group, the Chi-squared test were performed. To evaluate whether the means of the numerical variables were significantly different, ANOVA tests were performed. P-values <0.05 were considered to be significantly different.

9.3 Results

9.3.1 Baseline characteristics

A total number of 4038 DCIS patients were included of which 2187 patients were treated by WGL and 1851 were treated by RSL. Overall, the mean age was 60.0 years (SD 9.25). The mean DCIS size was 1.78 cm (SD 1.45) and the majority of DCIS cases were either intermediate or high grade, accounting for 40.8% and 44.6% respectively. ER, PR and HER2 status was not available for the majority of patients. Baseline patient- and tumor characteristics of the WGL and RSL groups are described and compared in **Table 9.1**. Compared to patients treated with WGL,

patients with RSL were slightly younger at the time of treatment (59.6 vs 60.3 years, p=0.014). Regarding histopathological aspects of DCIS, patients treated with RSL were more often diagnosed with a larger DCIS size (mean diameter 1.84 cm versus 1.72 cm; p=0.013), grade 3 DCIS (47.8% vs 42.0%, p<0.001) and presence of comedonecrosis (92.1% vs 79.9%, p<0.001) compared to the WGL group.

Table 9.1: Baseline patient and tumor characteristics (n=4038)

	RSL (n=1851) n (%)	**WGL (n=2187)** n (%)	**p-value**
Age			
- Mean (SD)	59.63 (9.48)	60.35 (9.03)	0.014
DCIS size (cm)			
- Mean (SD)	1.84 (1.52)	1.72 (1.38)	0.013
Number of seeds (%)			
- 1	920 (49.7)		
- 2	101 (5.5)		
- 3	10 (0.5)		
- Unknown*	820 (44.3)	2187 (100)	
Grade DCIS (%)			
- Grade 1	252 (13.6)	295 (13.5)	
- Grade 2	698 (37.7)	951 (43.5)	**<0.001**
- Grade 3	885 (47.8)	919 (42.0)	
- Unknown*	16 (0.9)	22 (1.0)	
Comedonecrosis (%)			
- Absent	68 (3.7)	252 (11.5)	**<0.001**
- Present	799 (43.2)	1002 (45.8)	
- Unknown*	984 (53.2)	933 (42.7)	
Microcalcification (%)			
- Absent	255 (13.8)	302 (13.8)	0.214
- Present	1012 (54.7)	1354 (61.9)	
- Unknown*	584 (31.6)	531 (24.3)	
Estrogen Receptor (%)			
- Negative	18 (1.0)	15 (0.7)	0.356
- Positive	37 (2.0)	49 (2.2)	
- Unknown*	1796 (97.0)	2123 (97.1)	
Progesteron receptor(%)			
- Negative	26 (1.4)	23 (1.1)	0.292
- Positive	22 (1.2)	32 (1.5)	
- Unknown	1803 (97.4)	2132 (97.5)	
HER2 status (%)			
- Not amplified	3 (0.2)	1 (<0.1)	1
- Amplified	5 (0.3)	2 (0.1)	
- Unknown*	1843 (99.6)	2184 (99.9)	

* Excluded from analysis

9.3.2 WGL and RSL have comparable surgical resection margins

The resection margins are described in **Table 9.2**. Overall, around half of patients had a surgical resection margin $\geq$ 2 mm, which was not statistically different between the WGL and RSL group; p=0.505). In line with this, there was no significant difference in the number of re-excisions between both groups (p=0.429). In the WGL group, 13.6% of patients underwent a second surgery and 8.8% underwent a third surgery. In the RSL group, 12.6% of patients underwent a second surgery and 7.7% underwent a third surgery. However, patients with a re-excision after RSL more often underwent a breast conserving surgery, compared to the WGL group (69.2% vs 59.7%, p=0.029). This resulted in the detection of additional DCIS in 62.8% of patients in both groups. In 1.7% of the patients in the WGL group and 2.6% of the patients in the RSL group, an invasive component was present in the re-excision material.

Table 9.2: Surgical resection margins status and additional surgery (n=4038)

	RSL (n=1851) n (%)	**WGL (n=2187)** n (%)	**p-value**
Resection margins (cm)			
- Mean (SD)	0.33 (0.29)	0.35 (0.30)	0.089
Resection margins cat			
- 2 mm	984 (53.2)	1142 (52.2)	0.505
- $\geq$ 2 mm	856 (46.2)	1038 (47.5)	
- Unknown	11 (0.6)	7 (0.3)	
Radicality			
- Free	1451 (78.4)	1699 (77.7)	
- Focally not radical	253 (13.7)	314 (14.4)	0.531
- More than focally not radical	136 (7.3)	167 (7.6)	
- Unknown*	11 (0.6)	7 (0.3)	
First re-excision			
- Yes	234 (12.6)	296 (13.5)	0.429
- No	1617 (87.4)	1891 (86.5)	
Type of first re-excision			
- Breast Conserving Surgery	162 (69.2)	176 (59.5)	0.029
- Mastectomy	72 (30.8)	119 (40.2)	
- Unknown*	0	1 (0.3)	
Findings in first re-excision specimen			
- Not rest	80 (34.2)	103 (34.8)	
- DCIS	147 (62.8)	186 (62.8)	0.888
- LCIS	1 (0.4)	2 (0.7)	
- Invasive carcinoma	6 (2.6)	5 (1.7)	
Second re-excision			
- Yes	18 (7.7)	26 (8.8)	0.769
- No	216 (92.3)	270 (91.2)	
Type of second re-excision			
- Breast Conserving Surgary	2 (11.1)	2 (7.7)	1
- Mastectomy	16 (88.9)	24 (92.3)	
Findings in second re-excision specimen			
- DCIS	17 (94.4)	26 (100.0)	0.852
- No rest	1 (5.6)	0 (0.0)	

*Excluded from analysis

9.3.3 The number of seeds and clinicopathological characteristics

The association between the number of seeds and clinicopathological characteristics is depicted in **Table 9.3**. Overall, the majority of cases (89.2%) were localized by one seed. There was a significant association between the number of seeds and age at diagnosis: single seed was associated with older age at diagnosis (p=0.017). Besides, single seed localization was associated with a smaller DCIS diameter (p<0.001) and the highest percentage of patients with a resection margin $\geq$2.0mm (p=0.001) compared to multiple seed localization. However, there was no significant difference in the number of patients undergoing a re-excision (p=0.161). Subgroup analysis was performed by comparing the use of 1 seed versus more than one seeds in patients with a large DCIS (> 3 cm) (**Table 9.4**). The use of multiple seeds was associated with a larger DCIS diameter (p=0.002) and higher rate of radicality (free versus focally irradical versus more than focally irradical; p=0.025). However, there was no association between the number of seeds and the categorical resection margins (<2 mm versus $\geq$2; p=1)

Table 9.3: Clinicopathological characteristics according to number of seeds (n=1031)

	Single seed (n=920) n (%)	**Multiple seeds (2 or 3) (n=111)** n (%)	**p-value**
Age (mean (SD)			
- Mean (SD)	59.44 (9.33)	57.16 (10.39)	0.017
DCIS size (cm, mean (SD)			
- Mean (SD)	1.70 (1.37)	3.03 (2.14)	<0.001
Resection margins (cm)			
- Mean (SD)	0.36 (0.30)	0.25 (0.24)	0.002
Resection margin cat (%)			
- <2 mm	448 (48.7)	73 (65.8)	0.001
- $\geq$ 2 mm	469 (51.0)	37 (33.3)	
- Unknown	3 (0.3)	1 (0.1)	
Radicality (%)			
- Free	731 (79.5)	88 (79.3)	
- Focally not radical	123 (13.4)	12 (10.8)	0.574
- More than focally not radical	63 (6.8)	10 (9.0)	
- Unknown*	3 (0.3)	1 (0.9)	
First re-excision (%)			
- Yes	110 (12.0)	19 (17.1)	0.161
- No	810 (88.0)	92 (82.9)	

Only patients with a known number of seeds were included. *Excluded from analysis

9.4 Discussion

The WGL method has historically been used as the gold standard for preoperative localization of non-palpable breast tumors. However, in recent years, RSL has been considered to be an attractive alternative for WGL since it offers more flexibility in scheduling the surgery and is more patient friendly[15]. Several, generally small, previous studies compared both methods regarding to their surgical outcome, but the derived results were non-consistent[14,16–18]. In this large, population-based cohort study we compared the efficacy of WGL and RSL in patients with DCIS undergoing breast conserving surgery.

We demonstrated that preoperative localization with RSL was more frequently performed in patients who were younger, were diagnosed with a large or high grade DCIS, or had a DCIS with comedonecrosis. We hypothesize that these differences may be related to an unequal use of these techniques within different hospitals, since the localization technique is an institutional policy and not a patient dependent choice. Academic hospitals and specialized cancer centers are potentially more likely to use RSL compared to general hospitals, and these centers attract younger patients, whom generally have a higher rate of high grade lesions compared to older patients. However, the clinical relevance of these differences, which are relatively limited but statistically significant in this large series, is unclear.

When comparing both methods, there was no difference in the surgical resection margins or the number of re-excisions. This is in line with the majority of the studies, which demonstrated equally successful excision rates between RSL and WGL[14,16,18]. However, one study suggested significant superiority of RSL over WGL based on 169 patients (p=0.048)[17]. In our study, we observed that, in case of a re-excision, patients in the RSL group were more often treated with breast conserving surgery compared to the WGL group. Potential explanations for this difference could be that these patients are slightly younger. Another hypothesis is that the volume of the initial surgical specimen in patients treated with RSL could be smaller compared to WGL, due to a more accurate localization.

Table 9.4: Clinicopathological characteristics of patients with large DCIS according to number of seeds (n=210)

	Single Seed (n=156) n (%)	**Multiple seeds (2 or 3) (n=54)** n (%)	**P-value**
Age			
- mean (SD)	60.17 (9.60)	58.07 (10.71)	0.18
DCIS size			
- mean (SD)	4.05 (1.24)	4.73 (1.62)	0.002
Resection margins			
- mean (SD)	0.19 (0.20)	0.17 (0.17)	0.561
Resection margins categories (%)			
- <2mm	130 (83.3)	44 (81.5)	1
- ≥2 mm	25 (29.1)	9 (22.0)	
- Unknown*	1 (0.6)	1 (1.9)	
Radicality (%)			
- Free	86 (55.1)	41 (75.9)	
- Focally not radical	41 (26.3)	5 (9.3)	0.025
- More than focally	28 (17.9)	7 (13.0)	
- Unknown*	1 (0.6)	1 (1.9)	
First reexcision (%)			
- Yes	44 (28.2)	11 (20.4)	0.343
- No	112 (71.8)	43 (79.6)	

Only patients with large DCIS (>3cm) with known number of seeds were included. * Excluded from analysis

Lastly, we also showed that in terms of RSL, the use of a single seed is associated with a smaller DCIS diameter, which consequently results into a larger resection margin. In our subgroup analysis restricted to patients with extensive DCIS (≥3 cm), we demonstrated that the use of multiple seeds was associated with a higher rate of radicality. This is in line with a recent study which also suggested superiority of multiple seeds over a single seed in patients with extensive DCIS (lesions ≥3 cm)[20].

Our study included a large, national cohort of patients receiving breast conserving surgery for DCIS with either WGL or RSL, using real world data. However, several data was lacking including:the weight or size of the excision specimen,the method of marker-placement (ultrasound guided or guided by mammography), the number of wires, the use of intraoperative examination of the specimen, oncoplastic surgery and the location of the (involved) margin. Removal of large excision specimens obviously decreases the rate of positive margins[14]. The weight of the specimen would provide more information regarding the interpretation of the resection margins and post-operative cosmetics. The location of the (involved) margin could affect the decision to perform re-excision, i.e. dorsal involved margins can be less suitable for re-excision, since there might not be any more breast tissue to remove. In addition, we included patients over a period of 10 years, in which the treatment guidelines have changed. WGL was used frequently in the beginning of this period and RSL more towards the end of this period, this could have influenced the re-excision rates. Another limitation is the lack of clinical follow-up data regarding local recurrence, due to the fact that synoptic reporting only began in 2009. Finally, as mentioned above, the localization technique is not a patient driven choice, but an institutional policy, which could have biased our findings.

Previous studies mainly focused on IBC, this study is the first to include a large cohort using real-world data on patients with DCIS. We have shown that RSL and WGL have comparable resection margins and number of re-excisions. However, in case of a re-excision, patients in the RSL group were more often treated with breast conserving surgery compared to the WGL group.

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Chapter 10

A retrospective alternative for active surveillance trials for ductal carcinoma in situ of the breast.

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Abstract

Ductal carcinoma in situ (DCIS) of the breast is a non-obligate precursor of invasive breast cancer, accounting for twenty percent of screen-detected breast cancers. Little is known about the natural progression of DCIS, because most patients undergo surgery upon diagnosis. Many DCIS patients are likely being over-treated, as it is believed that only around fifty percent of DCIS will progress to invasive carcinoma. Robust prognostic markers for progression to invasive carcinoma are lacking.

In the past, studies have investigated women who developed a recurrence after breast-conserving surgery (BCS) and compared them with those who did not. However, where there is no recurrence, the patient has probably been adequately treated. The present narrative review advocates a new research strategy, wherein only those patients with a recurrence are studied. Approximately half of the recurrences are invasive cancers, and half are DCIS. So-called "recurrences" are probably most often the result of residual disease. The new approach allows us to ask: why did some residual DCIS evolve to invasive cancers, and others not?

This novel strategy compares the group of patients that developed in situ recurrence with the group of patients that developed invasive recurrence after BCS. The differences between these groups could then be used to develop a robust risk stratification tool. This tool should estimate the risk of synchronous and metachronous invasive carcinoma when DCIS is diagnosed in a biopsy. Identification of DCIS patients at low risk for developing invasive carcinoma will individualize future therapy and prevent over-treatment.

Keywords: ductal carcinoma in situ; recurrence; active surveillance; risk stratification; prognostic markers

Conflict of Interest

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10.1 Introduction

Ductal carcinoma in situ (DCIS) of the breast is a non-obligate precursor of invasive breast cancer, representing a heterogeneous group of lesions in terms of morphology and genetics[1]. DCIS is constituted by neoplastic epithelial cells which are confined within the ductal-lobular system by myo-epithelial cells and the basement membrane[1]. Before the advent of screening mammography, DCIS was only diagnosed when symptomatic (i.e. due to nipple discharge and/or the presence of a palpable mass) and constituted less than two percent of all diagnosed breast cancers[2]. Since the widespread introduction of mammographic screening, DCIS detection rates substantially increased to approximately twenty percent of all screen-detected breast cancers diagnosed at present[3–5]. Nowadays, around 9-19% of DCIS patients are symptomatic, whereas the majority has an occult screen-detected lesion[5–8].

Whether this increased detection rate mainly represents over-diagnosis remains subject for debate. Early breast cancer diagnosis is considered as beneficial for patients, since it is supposed to decrease the risk of both regional lymph node metastasis and distant metastasis. On the other hand, diagnosis of DCIS might also be considered as a negative side-effect of mammography screening, as it is often questioned whether every DCIS would have become symptomatic in the absence of screening[9]. It is currently unclear which DCIS lesions are able to progress to invasive cancer, and which DCIS lesions will remain indolent[10,11]. The identification of those indolent DCIS lesions remains a substantial challenge for future research. In the current narrative review, we discuss the available evidence on natural progression of DCIS, as well as the possible pitfalls of active surveillance trials. Additionally, we present a new research strategy which may serve as an efficient retrospective surrogate for active surveillance studies.

10.2 Current state-of-the-art treatment of DCIS

Per definition, DCIS itself does not yield a risk of (lymph)angio-invasion and metastasis. Therefore, the cornerstone of current DCIS treatment is to prevent the development of invasive carcinoma[12]. Patients with DCIS show excellent survival,[13] with a 20-year actuarial breast cancer-specific mortality rate of 3,8%[14]. However, women who develop ipsilateral invasive breast carcinoma after initial diagnosis of DCIS show reduced overall and breast cancer-specific survival[15–17], as they are 18 times more likely to die of breast cancer than women who do not develop an ipsilateral invasive in-breast recurrence[17]. At present, most DCIS patients undergo surgery. Depending on the size of the lesion and patient preferences, surgical treatment consists either of breast-conserving surgery (BCS; i.e. lumpectomy) or mastectomy[12]. Adjuvant radiotherapy halves the overall recurrence risk after BCS, regardless DCIS size, grade and margin status[15,18,19]. Despite its substantial influence on recurrence-free survival, adjuvant radiotherapy does not significantly alter overall survival for DCIS patients[19]. Adjuvant endocrine therapy with tamoxifen was shown to be associated with both a reduced ipsilateral recurrence risk and a reduced risk of contralateral invasive and in situ carcinoma, although the UK/ANZ DCIS trial and NSABP B-24 trial did not discern oestrogen receptor-positive (ER+) from ER-negative (ER-) DCIS patients[18,20,21]. Post hoc analysis of the NSABP B-24 cohort confirmed these results only for patients with ER+ DCIS treated with tamoxifen[22]. Despite these observations, adjuvant tamoxifen did not significantly influence the all-cause mortality risk for patients with ER+ DCIS[23]. A recently published

report on the UK Sloane Project, which prospectively studied a population-based cohort of patients with screen-detected DCIS, confirmed that both radiotherapy and endocrine therapy were associated with decreased ipsilateral recurrence risk[24]. A retrospective population-based analysis of endocrine treatment of DCIS patients in British Columbia showed similar results, thereby demonstrating the generalizability of these trial data at the population level[25].

10.3 The gaps in our knowledge on natural progression

Since most DCIS patients are treated upon diagnosis, little is known about the natural course of progression to invasive breast cancer. Evidence on the prevalence and spontaneous course of DCIS is restricted. A systematic review on thirteen autopsy studies reports a prevalence of undetected DCIS in 8,9% of adult women without a history of pre-existent breast disease[26]. Series of DCIS patients treated with biopsy only are scarce. Betsill and Rosen et al. observed that eight of fifteen DCIS patients, who were treated by biopsy only, developed an invasive carcinoma after a mean interval of ten years[27,28]. Collins et al. identified a series of thirteen DCIS patients who were initially diagnosed as having benign breast disease: four to eighteen years later, six of these thirteen untreated patients had developed invasive carcinoma, regardless DCIS grade[29]. Sanders et al. reported on a cohort of 45 patients with low grade DCIS, initially diagnosed as having benign breast disease and therefore 'treated' with biopsy only[30]. Sixteen of these low grade DCIS patients developed invasive carcinoma in the same breast quadrant within three to 42 years after initial biopsy[30]. More recently, Maxwell et al. reported a series of 89 DCIS patients who either declined or were unfit to undergo surgery[31]. One in three patients developed invasive carcinoma after a median interval of 45 months, and high grade DCIS patients showed a significantly higher risk for developing invasive carcinoma (48%) than intermediate or low grade DCIS patients (32% and 18%, respectively)[31].

These retrospective series probably underestimate the risk of spontaneous progression of DCIS, as most reports mainly concern low grade DCIS. Despite these limitations, useful information can be deduced: both low grade and high grade DCIS show the ability to progress to invasive carcinoma, but this occurs more frequently and after a shorter time interval in high grade DCIS[13,31]. Patients with high grade DCIS also have a higher risk of breast-cancer related death than patients with low or intermediate grade DCIS[13]. Notwithstanding the risk of progression, a significant number of unresected DCIS in the aforementioned retrospective series remained in situ, even among the patients with high grade DCIS. It is therefore generally accepted that a substantial number of DCIS patients is currently over-treated.

10.4 Is active surveillance a non-inferior alternative?

At present, three active surveillance trials are conducted to investigate whether watchful waiting is a non-inferior alternative strategy for low risk DCIS compared to conventional surgery with or without adjuvant irradiation and/or hormonal therapy, as per local protocol[32,33]. In the UK, the LoRis trial is open to women with a vacuum-assisted biopsy diagnosis of asymptomatic low or intermediate grade DCIS without necrosis and with low mitotic rate[34]. Upon central histopathological review of the biopsies, eligible patients are randomized between an active monitoring arm

and a surgery arm with conventional surgical and adjuvant treatment[35]. In mainland Europe, inclusion in the LORD trial is limited to women aged over 45 with asymptomatic screen-detected pure low grade DCIS[36]. The LORD trial does not require central histopathological review and randomizes patients between active surveillance and standard treatment according to local policy[36]. In the USA, the COMET trial is open to women aged over 40 with newly diagnosed hormone receptor-positive HER2-negative (HER2-) low or intermediate grade DCIS[37]. A fourth active surveillance study was announced in Australian and New Zealand with more stringent inclusion criteria than the aforementioned trials[38]. This LARRIKIN trial will include women aged over 55 with screen-detected or incidentally detected DCIS smaller than 25 mm on imaging. Additionally, only patients with hormone receptor-positive, HER2 non-amplified low or intermediate DCIS without comedonecrosis will be allowed to participate[38].

Accrual for these trials seems challenging: a report on the first 22 months of the LoRis trial mentions randomization of 38 of only 55 eligible patients[39], whereas the required sample size amounts 932 patients[35]. At that rate, accrual will take more than forty years. The stringent inclusion criteria limit the number of eligible patients. It is likely that patients feel anxious upon being allocated to the watchful waiting arm. Collaboration between these trials for combined data analysis in case of lack of power will be arduous, as all trials apply slightly different inclusion criteria. Even if these active surveillance trials prove that watchful waiting is not inferior compared to standard treatment, it will take many years before these data will be available for routine clinical use. Moreover, only a minority of DCIS patients will benefit from these findings, since only 9-12% of DCIS are low grade[13,24,40–42]. Additional inclusion criteria besides nuclear grade will further decrease the number of eligible patients. The overall impact of active surveillance trials on the population of DCIS patients might therefore be limited[43].

Risk stratification and thus treatment allocation based on nuclear grade remains an additional challenge, since nuclear grade is characterized by considerable inter-observer variability[40,44]. Pathologists disagree more often on the difference between low and intermediate grade, than on the difference between intermediate and high grade[45,46]. It would therefore be interesting to investigate the prognostic value of 2-tier grading as non-high grade versus high grade instead of a 3-tier grading system[47]. This 2-tier morphological grading is corroborated by several molecular and gene expression studies that indicate a low grade and high grade pathway in breast cancer development[48–50]. The identification of alternative robust prognostic markers besides nuclear grade is of utmost importance, because it is likely that overtreatment of DCIS patients will continue despite the potential usage of active surveillance strategies in this limited subpopulation of "low risk" DCIS. Notwithstanding these drawbacks, active surveillance trials are presumed to teach us a lot about natural progression in this particular subgroup. This new knowledge should enable us to approach DCIS biology from a completely different perspective.

10.5 Risk assessment for synchronous invasion at the biopsy level

Active surveillance might be hazardous, as up to 24% of patients with a biopsy diagnosis of "low risk" DCIS present a synchronous IBC component in the subsequent resection specimen[51–55]. Five studies applied the inclusion criteria of one or more active surveillance studies on pure DCIS diagnosed in biopsies to investigate the risk of under-treatment in case of a synchronous invasive carcinoma in the subsequent resection specimen (**Table 10.1**). Podoll et al. examined a series of

105 DCIS that were upstaged to invasive cancer in the subsequent resection specimen and applied the LORD and LoRis criteria on this cohort[56]. Only three (3%) upgraded DCIS met the LORD criteria, but twenty (19%) upgraded DCIS met the LoRis criteria[56]. A similar analysis by Alexander et al. reports 229 DCIS that were upstaged to invasive cancer, of which four (2%) met the LORD criteria, 37 (16%) met the LoRis criteria and 15 (7%) met the COMET criteria[57]. A combination of the LORD, LoRis and COMET trial eligibility criteria was retrospectively applied on a subset of 37.544 patients in the National Cancer Database of the American College of Surgeons and the American Cancer Society, which revealed an upstaging rate to invasive carcinoma of 21.8% in this eligible subgroup[58].

Table 10.1: Overview of retrospective analyses of active surveillance trials' eligibility criteria in relation to upstage rates to invasive cancer in the surgical resection specimen, after an initial diagnosis of pure DCIS at the biopsy level.

Ref.	Year of publication	Active surveillance eligibility criteria	Number of samples according to eligibility criteria n	Total upstage rate to invasive cancer n (%)	Upstage rate to invasive cancer according to nuclear grade n (%)
[51]	2013	LoRis	31	0 (0)	· Low grade : 0 (0) · Intermediate grade: 0 (0)
[52]	2016	LoRis	296	58 (20)	· Low grade : 4 (8) · Intermediate grade: 54 (22)
[53]	2017	LoRis	74	5 (7)	· Low grade : 1 (8) · Intermediate grade: 4 (7)
		LORD	10	1 (10)	· Low grade : 1 (10)
		COMET	81	5 (6)	· Low grade : 1 (8) · Intermediate grade : 4 (6)
[54]	2018	LoRis	25	6 (24)	Not mentioned in this report
		COMET	23	5 (22)	Not mentioned in this report
[55]	2017	LoRis	241	16 (7)	Not mentioned in this report

COMET: Comparison of Operative to Monitoring and Endocrine Therapy trial; DCIS: ductal carcinoma in situ; LORD: Low-Risk DCIS trial; LoRis: Low-Risk DCIS trial; Ref: reference

Overall, the prediction of synchronous invasive cancer when pure DCIS is diagnosed at the biopsy level remains challenging. Many studies have ascertained that upstaging is more frequent in high grade DCIS than in low and intermediate grade DCIS[52,59–62]. Nevertheless, a significant proportion of non-high grade pure DCIS shows synchronous invasive carcinoma in the subsequent resection specimen[57]. Attempts have been made to identify additional histological and immunohistochemical features for prediction of concurrent invasive carcinoma. Among them, increased stromal inflammation seems perpetually associated with increased risk for (micro-)invasive carcinoma,[63–65] but this promising histopathological feature requires further validation in larger independent patient cohorts. Likewise, HER2 positivity in pure DCIS at the biopsy level seems to be associated with increased risk of synchronous invasive cancer in the subsequent resection specimen[66]. This could be due to the fact that HER2-positive (HER2+) invasive carcinomas have an extensive HER2+ in situ component,[67] which is a risk factor for sampling error. However, further investigations are necessary as this upstage risk was not confirmed by others[63,68]. Of note, increased stromal inflammation is strongly correlated with HER2-positivity in pure DCIS,[69,70] and stromal inflammation is more frequently observed in DCIS admixed with invasive carcinoma than in pure DCIS[65]. It is currently unclear which feature is the most decisive factor in the progression of in situ to invasive carcinoma.

10.6 Innovation will discern indolence from agility in DCIS

Besides a lack of adequate markers to predict synchronous invasive carcinoma when pure DCIS is diagnosed at the biopsy level, there is also a need for reliable prognostic markers to assess recurrence risk after conventional treatment of DCIS patients. Despite decades of intensive research, adequate markers for the prediction of invasive recurrence after conventional treatment are lacking. This results in the current uniform treatment of DCIS patients: one size fits all. Why is this? Nearly all studies on prognostic markers in DCIS have applied the following strategy: all BCS-treated patients diagnosed with pure DCIS in the lumpectomy specimen are investigated and clinicopathological characteristics are noted. Subsequently, the initial DCIS lesions of the patients who have developed a recurrence (designated as "cases") are compared with the DCIS lesions of the patients who did not develop a recurrence (designated as "controls"). As a result, one or more clinicopathological features are significantly more or less often present in the DCIS lesions of the patients who developed a recurrence. Unfortunately, the prognostic power of these features can often not be confirmed by others in independent patient cohorts.

This lack of validation is probably due to the fact that most DCIS patients who undergo surgery, will be adequately treated: if the initial DCIS lesion is completely removed, the patient will never develop a recurrence. Contrariwise, patients who do develop a so-called "recurrence" should be considered as inadequately treated patients. These patients have residual, initially incompletely removed DCIS in their breasts. A so-called "recurrence" can then be regarded as an outgrowth from this residual disease. Evidently, these "outgrowths" should be discerned from new, independent breast lesions that are not clonally related to the initial DCIS lesion. Only few studies have investigated the relation between primary DCIS lesions and their recurrences. Although some studies were limited to histopathological and immunohistochemical features without molecular comparison, their results indicated that about 80-90% of recurrences are actually outgrowths from initially incompletely removed DCIS[71,72]. Other studies investigated copy number aberrations and loss of heterozygosity, which resulted in a similar high concordance between primary DCIS lesions and their recurrences[73–76]. To our opinion, retrospective studies should therefore only focus on these patients who recurred, and not on the entire initial patient cohort (**Figure 10.1**).

The key message is: pick the right cases and the right controls. The correct "controls" are the patients with incompletely removed DCIS that remained in situ, i.e. the patients who developed a so-called "in situ recurrence". Consequently, the right "cases" are not all patients who relapsed. Only those patients who had initially incompletely removed DCIS that has evolved into invasive carcinoma should be regarded as "cases", i.e. the patients who developed a so-called "invasive recurrence" (**Figure 10.1**). The patient group that has not developed a recurrence (yet) will probably contain a small but hitherto unidentifiable minority who does have incompletely removed DCIS. This small subgroup might either relapse somewhere in the future, or they might have initially incompletely removed DCIS that will remain clinically occult. However, this subgroup will not bias the study as it is not taken into account in the analysis. Such a comparison should enable us to answer the following question: why did some residual DCIS remain in situ, and why did other residual DCIS progress to invasive carcinoma? This novel approach can therefore be considered as a retrospective alternative for the current active surveillance trials: incomplete removal of DCIS allowed the residual DCIS lesion to "progress naturally", enabling us to retrospectively compare the DCIS lesions that remained in situ with the DCIS lesions that have progressed to in-

vasive cancer. The advantage of this strategy is that it will include all BCS-treated DCIS patients, and not just the patients with low grade DCIS. This novel approach should enable the identification of unequivocal robust markers that predict progression to invasive cancer. Eventually, these markers might allow adequate risk stratification when pure DCIS is diagnosed in core biopsies, by discerning indolent DCIS lesions from aggressive DCIS lesions that have an invasive carcinoma component, either on the short term (i.e. synchronous invasive component) or on the long term (i.e. metachronous invasive component). The results of such a study will aid to individualize therapy for DCIS patients and are therefore expected to significantly reduce over-treatment.

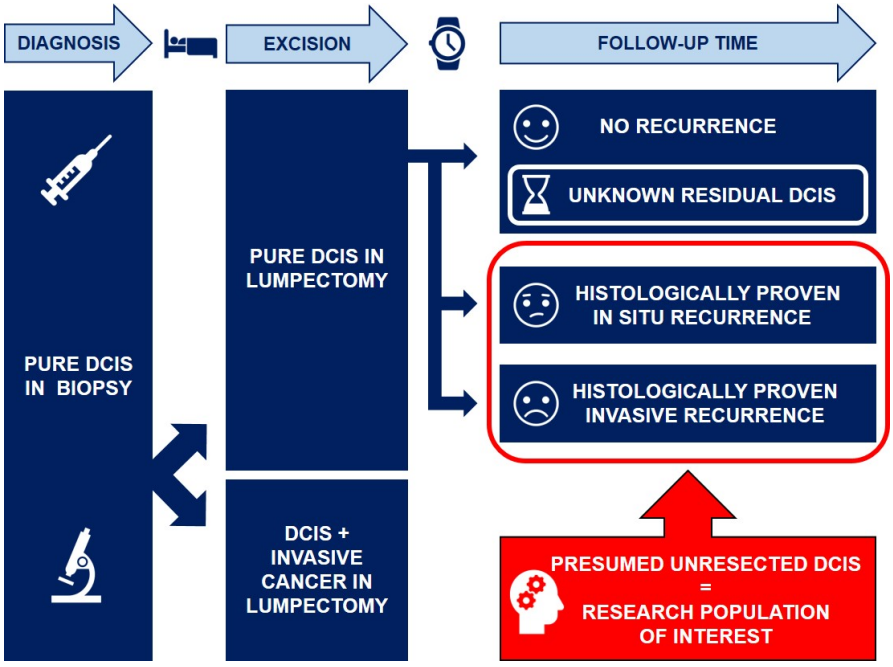

Figure 10.1: Schematic conceptualization of the novel research strategy. The characteristics of the primary DCIS lesions of patients with in situ and invasive recurrences after breast-conserving surgery are compared with each other. The majority of patients without recurrence are considered to be adequately treated, and are therefore not included in this study.

10.7 Practical implementation of the alternative research strategy

To our knowledge, this alternative strategy has been applied only once before. Zhou and colleagues investigated a series of 266 women with primary pure DCIS and a known ipsilateral breast event: 136 of these so-called recurrences were invasive carcinoma and 130 were DCIS[77]. Unfortunately, these authors did not investigate the clonal relationship between the primary DCIS lesion and its corresponding recurrence, since they regarded all ipsilateral new events as de facto recurrences[77]. Establishing whether a 'recurrence' is either a new second primary tumour

or an outgrowth of the initial DCIS lesion is essential for the success of this research strategy. To determine the clonal relationship between the primary DCIS lesion and the second breast event, morphological features such as nuclear grade and DCIS growth pattern can be studied, complemented by immunohistochemistry to assess the expression of ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). However, these histopathological characteristics enable only a rough comparison, and they should therefore be complemented by molecular studies (i.e. next-generation sequencing and/or copy number analyses). Such an approach seems feasible as it has been shown that there is a high degree of genomic concordance between both components in synchronous in situ and invasive carcinoma[78–82]. Although genomic profiling requires larger financial resources, it allows a more refined comparison than mere immunohistochemistry. This is especially important as protein expression profiles (e.g. ER, PR and HER2 status) can change during breast cancer progression[72]. An integrated approach of histopathological and molecular characteristics should enable to distinguish second primary breast lesions from true DCIS outgrowths with a higher degree of certainty than morphological and immunohistochemical features alone, and subsequent analyses can then be continued with the latter.

Despite the fact that Zhou et al. did not discern true DCIS outgrowths from new primary breast lesions,[77] their findings remain very interesting: patients with ER-HER2+ primary DCIS presented significantly more often with an in situ "recurrence", whereas ER+HER2- primary DCIS presented significantly more often with an invasive "recurrence"[77]. This might seem contradictory, as HER2 is associated with poor prognosis in invasive breast cancer. This apparent paradox is indirectly supported by other studies. For instance, HER2 protein overexpression in pure DCIS treated with BCS was associated with increased in situ recurrence risk, but not with invasive recurrence risk[83–85]. HER2+ invasive breast cancers are more often associated with adjacent DCIS than HER2- invasive breast cancers, and this adjacent DCIS shows more often a larger size and a higher rate of incomplete resection in HER2+ breast cancers[67]. HER2 amplification and its concurrent protein overexpression might act as a driver for intraductal clonal proliferation, instead of being a driver of cancer cell invasion. This hypothesis may explain the observations of Zhou et al.[77], and it may also explain the paradoxical observation of HER2 overexpression being much more common in DCIS than in invasive carcinoma[86,87]. Nevertheless, a nested case-control study identified HER2 as a marker for progression to invasive carcinoma,[88] and therefore additional investigations remain warranted to clarify the role of this intriguing receptor[87,89].

Practical implementation of the novel research strategy might be hampered by the low overall number of recurrences in a single centre. A multicentre approach seems therefore mandatory, to enable the inclusion of a sufficiently large number of DCIS patients with subsequent recurrence. Patient recruitment might be facilitated by including patients of previously conducted randomized clinical trials and retrospective studies, of which a non-exhaustive selection is summarized in **Table 10.2**[15,18,21,24,41,70,90–97]. Inclusion of these patients in a study conducted according to the new research strategy would allow for correction of treatment effects, since a substantial number of patients are treated with radiotherapy and/or tamoxifen after BCS. Additionally, it would be interesting to also include HER2+ DCIS patients with recurrences from the currently ongoing NSABP B-43 trial, wherein the value of adjuvant trastuzumab is investigated[98]. Inclusion of a sufficiently large number of patients does not only allow stratification according to type of adjuvant treatment, but it would also allow stratification according to DCIS grade and other clinicopathological parameters. It may therefore be possible to provide stronger evidence for

Table 10.2: A selection of prospective trials and retrospective studies with a substantial number of ipsilateral local recurrences (either in situ or invasive) after breast-conserving surgery for DCIS, with or without adjuvant radiotherapy and/or hormonal therapy. Patients with recurrent

Ref	Study / trial	FU time (months)° Mean Median	Total number of patients n	Overall recurrence n (%)	In situ recurrence n (%)	Invasive recurrence n (%)	No radiotherapy, no TAM		Radiotherapy without TAM		Radiotherapy with TAM	
							In situ recurrence n (%)	Invasive recurrence n (%)	In situ recurrence n (%)	**Invasive recurrence** n (%)	**In situ** recurrence n (%)	Invasive recurrence n (%)
[20]	NSABP B-17	207	813	222 (27)	99 (12)	123 (15)	62 (15)	79 (20)	37 (9)	44 (11)	NA	NA
[20]	NSABP B-24	163	1799	268 (15)	128 (7)	140 (8)	NA	NA	68 (8)	81 (9)	60 (7)	59 (7)
[90]	SweDCIS	204	1046	258 (25)	129 (12)	129 (12)	91 (17)	74 (14)	38 (7)	55 (11)	NA	NA
[19]	UK/ANZ DCIS *	152	1694	376 (22)	197 (12)	163 (10)	86 (16)	52 (10)	14 (5)	10 (4)	11 (3)	11 (3)
[17]	EORTC10853	190	1010	234 (23)	110 (11)	121 (12)	74 (15)	75 (15)	37 (7)	48 (10)	NA	NA
[24]	Sloane project	64	7007	368 (5)	#	#	#	#	#	#	#	#
[70]	Pruneri et al.	98	945	180 (19)	#	#	#	#	#	#	#	#
[41]	Punglia et al.	60	2762	79 (3)	#	#	#	#	#	#	#	#
[91]	E5194 cohort	138	327	53 (16)	26 (8)	27 (8)	#	#	#	#	#	#
[91]	Ontario cohort	118	446	65 (15)	27 (6)	38 (9)	#	#	#	#	#	#
[95]	Toss et al.	103	776	83 (11)	30 (4)	53 (7)	#	#	#	#	#	#
[97]	Wai et al.	113	460	60 (13)	32 (7)	28 (6)	32 (7)	28 (6)	NA	NA	NA	NA
[96]	Tunon-de-Lara et al.	118	812	71 (9)	24 (3)	47 (6)	#	#	#	#	#	#
[92]	Butler-Henderson et al.	113	1356	235 (17)	86 (6)	149 (11)	#	#	#	#	#	#
[93]	Collins et al.	58	2995	325 (11)	172 (6)	153 (5)	#	#	#	#	#	#
[94]	Rudloff et al.	67	1868	202 (11)	122 (7)	80 (4)	#	#	#	#	#	#

DCIS: ductal carcinoma in situ; EORTC: European Organization for Research and Treatment of Cancer; FU: follow-up; NA: not applicable; NSABP: National Surgical Adjuvant Breast and Bowel Project; Ref: reference number; TAM: tamoxifen; UK/ANZ: United Kingdom / Australia, New Zealand.

* The category "tamoxifen alone, without radiotherapy" from this report is not included in this table.

° Some study reports provided median follow-up time in years; for these reports the number of years was multiplied by 12 months to achieve median follow-up in months to enable comparison.

No details on different subgroups were provided in these study reports, and therefore data on different subgroups could not be provided in this table.

the so-called low grade and high grade pathway of breast cancer progression, wherein low grade DCIS gives rise to less aggressive low grade invasive carcinoma, and high grade DCIS gives rise to more aggressive high grade invasive carcinoma[50,99]. Implementation of this novel research strategy in a combined multicentre international effort could force a major breakthrough in the research on DCIS biology and its natural progression.

10.8 Conclusion

The breast is a rather unique organ regarding the clonality issue between DCIS and its recurrence, although a similar issue exists in the liver and the lungs, where intra-organ metastases have to be discerned from new, metachronous and synchronous carcinomas[100,101]. Based on the currently available evidence regarding prognostic markers in DCIS (or the lack thereof), we propose a new model as a retrospective surrogate for active surveillance trials, which may provide useful data on the short term. This novel strategy is based on the comparison of the initial DCIS lesion between the patients who developed an in situ recurrence and the patients who developed an invasive recurrence. We hope this model will be included in future scientific studies on risk stratification of DCIS, as we believe this strategy will enable identification of robust markers for prediction of the natural course of DCIS. If this new approach succeeds in the development of a reliable risk stratification tool, the direct impact on clinical management of DCIS will be enormous, as it is likely that many patients will be treated less aggressively than they are now.

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Chapter 11

Discussion and future perspectives

11.1 Discussion

The detection rate of ductal carcinoma in situ (DCIS) of the breast has exponentially increased in the past 20 years. A substantial number of patients with DCIS are being overtreated, while other patients might benefit from a more extensive treatment. We aimed to identify novel intrinsic and microenvironmental markers that could contribute to the diversity of DCIS behavior; thereby differentiating between low-risk DCIS and high-risk DCIS.

Our results showed that genetic changes such as somatic mutations and chromosomal aberrations are dynamic processes, which already occur in the early disease stage. Additionally, tumor heterogeneity remains during breast carcinogenesis, making it hard to discriminate driver mutations from 'passenger' ones. Some of these genetic changes were associated with the expression of the estrogen receptor (ER). PIK3CA mutations were more frequently observed in ER+ tumors in female while the loss of Y-chromosome in male breast cancer was mainly observed in ER- tumors. We did not find an association between loss of Y-chromosome and outcome, although ER+ tumors are generally associated with better prognosis. With regard to PIK3CA, previous studies showed an association between PIK3CA mutations and longer recurrence free survival[1–3]. More recently, the role of PIK3CA mutations in DCIS progression was demonstrated, suggesting that the presence of a mutation in the kinase domain (H1047R) could be a marker for low-risk DCIS[4]. Patients without a H1047R PIK3CA mutation were 4.54 time more likely to have an invasive component compared to patients with this PIK3CA mutation, irrespective of ER status. Next to PIK3CA mutations, other genetic changes have also been proposed for risk stratification of DCIS. Specifically, copy number gains in 1q32 and 8q24 were associated with progression and the mRNA expression of MMP11 and COL10A1 were reported as markers for high-risk DCIS[4–6]. These aberrations seem to persist during the invasive stage of the disease, suggesting their potential role in other stages of breast carcinogenesis.

Next, we demonstrated a potential role for tumor infiltrating lymphocytes (TILs) in DCIS biology, particularly HER2+ and triple negative DCIS. We identified attraction of high numbers of TILs in 27% of all DCIS cases, whereby the immune cell composition was site-specific. Periductal immune cells predominantly included CD4+ T cells and CD20+ B cells, while intraductal immune cells included CD68+ macrophages and CD8+ T cells. This finding is in line with the cellular properties of these immune cell subsets. CD4+ T cells and CD20+ B cells are predominantly helper cells that mediate the function of effector cells. On the other hand, CD8+ T cells and CD68+ macrophages are generally effector cells, which directly interact with DCIS cells in order to lyse and eliminate necrotic cells. The position of the immune cells might therefore be indicative for their function and activity. However, there is limited interaction between T cells and cancer cells at the DCIS stage[7]. We suggest that the DCIS-associated stroma might function as a physical barrier, since we observed less intraductal TILs in case of stromal changes. Within cancer immunology, roughly three immune phenotypes have been reported by Chen et al[8]. These phenotypes, listed as inflamed, excluded or ignored, can also be considered for the DCIS immune microenvironment. Based on our observations, we propose that the DCIS immune phenotype can be considered mainly as immune-excluded, since the vast majority of immune cells remain in the periductal stroma. However, the presence of intraductal immune cells suggests a dynamic process, towards a more inflamed phenotype[8]. This was specifically true in case of ER+HER2+ DCIS, which presented with the highest number of intraductal CD8+ T cells. These findings might explain the biological behavior of HER2+ DCIS, compared to TN DCIS. Additionally, in-

creased numbers of CD8+ T cells in HER2+ DCIS were associated with regressive changes of the periductal stroma, a dynamic process that suggests 'spontaneous healing'[9]. This further emphasizes the role of TILs in the behavior of DCIS, which is in line with the process of cancer immunoediting. This hypothesis, which was first described by Dunn et al., proposes that the immune system plays a role in preventing and promoting tumor growth through three sequential phases: elimination, equilibrium and escape[10,11]. During the elimination phase, cancer cells are eliminated and growth is suppressed. During the equilibrium phase, there is a balance between tumor cells and immune cells, keeping the tumor dormant. In the escape phase, tumor cells overrule the immune cells and are able to increase in growth and metastasize. Our data shows that the local immune response is associated with the DCIS immunohistochemical subtype, which is more prominent in HER2+ DCIS. We can therefore speculate that HER2+ DCIS are in an equilibrium phase of the process of cancer immunoediting. In addition, with regard to HER2+ DCIS, we suggest that alternative gene expression levels contribute to the attraction of TILs. This was also recently reported for invasive breast cancer, demonstrating subtype specific T cell clonality, distribution and antigenicity[12]. Immune interventions should therefore first consider the tumor subtype prior to treatment.

In conclusion, we reported several associations between intrinsic changes of the DCIS cells, which occur in the early disease stage, and changes in the microenvironment. We suggest a protective immune response in case of HER2+ DCIS, which could play a role in its biological behavior. Due to lack of long-term clinical follow up and functional analysis, our findings remain merely associative and not causal. However, this thesis contributes to the increased knowledge of DCIS diversity, which facilitates the development of DCIS-specific treatment strategies such as preventive immune modulations.

11.2 Future Perspectives

We included a large cohort of patients with pure DCIS, which are associated with a low recurrence rate, which limited the possibility to evaluate the prognostic effect of our findings. Besides, the time span of our project did not allow functional analysis. Currently, there are several ongoing active surveillance trials to determine proper treatment for low-risk DCIS[13–15]. However, these studies remain time consuming and patient inclusion is challenging. As suggested in chapter 10 by van Bockstal et al, an alternative research strategy could bypass these challenges and provide robust markers for DCIS stratification[16]. Thanks to its retrospective setting, synoptic reporting and a national cancer registry in the Netherlands, this study design has a high feasibility. Historically, multiple cell lines have been used for functional analysis and evaluation of breast cancer. These cell lines are generally stable in culture and well characterized, and therefore great in identifying cellular mechanisms and new drug targets[17,18]. However, they do not represent the heterogeneity and spectrum of breast cancer. Additionally, new drug targets that are identified in vitro do not easily translate to the in vivo situation, since cell lines do not include the tumor microenvironment. Specific for DCIS, established DCIS cell lines represent the diversity of breast cancer subtypes[19]. However, the number of DCIS cell lines is very limited compared to the number of IBC cell lines. Additionally, the question remains how they differ from IBC cell lines, except from their origin. In culture, the myeoepithelial layer lacks, which is the major difference between in situ and invasive carcinoma. Animal models or xenograft models are a

potential alternative. When DCIS cell lines are grown in mice as xenografts, they capture DCIS structures and seem to represent the diversity of DCIS in terms of progression[19,20]. When grown in mice as xenografts, they develop into high grade DCIS of which some progress into IBC[21,22] . They are therefore, compared to cell lines, more suitable for developing prognostic biomarkers and study the role of the microenvironment during progression[17,23,24]. However, animal models or xenograft models are time consuming with variable success rates.

More recently, breast cancer organoids were developed as a potential alternative for these models. Breast cancer (including both DCIS and IBC) organoids are 3-D cell cultures derived from patient material with the same morphology, ER and HER2 status as the tumor they derived from[25]. Their ability to preserve all major epithelial markers and expression patterns of tissue of origin was also recently demonstrated[26]. These breast cancer organoids are currently used as models for therapy screening. Furthermore, 3-D DCIS cell line cultures can be co-cultured with cells form the microenvironment to analyze their interaction[22]. However, these models partly lack patient-specific tumor characteristics, which could hamper translation to the clinical setting. Another alternative is the study of fresh patient-derived breast cancer tissue[27]. These ex vivo tumor tissue slices can be obtained and generated within hours after surgery. This minimizes loss of morphology and cellular viability, thereby maintaining the original tumor characteristics, including its microenvironment. This allows for a fast throughput and real-time measurement of drug response, which can be used in a diagnostic setting. These organotypic tumor slices therefore allow personalized drug screens and other functional analysis[17,28,29]. Additionally, the isolation and expansion of TILs from ex vivo derived breast tumors was previously demonstrated[27].

Currently, our group is generating a breast cancer (including both DCIS and IBC) biobank, in collaboration with the Laboratory of Tumor Immunology of the Erasmus MC Cancer Institute. This will allow functional experiments in the future. After breast surgery, tumor sections are collected. First, freshly harvested TILs are analyzed by flow cytometry to determine the TILs composition. Secondly, cultured and frozen TILs will be used for functional assays. This will provide information on the functionality of TILs in DCIS behavior and possibly explain why some DCIS cases rapidly progress while others do not. The understanding of DCIS associated TILs might help in the development of preventive and targeted DCIS treatment.

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Summary

Since the introduction of population screening by mammography, over 20% of all breast cancers is diagnosed as ductal carcinoma in situ (DCIS), which accounted for only 5% three decades ago. DCIS is considered a non-obligate precursor of invasive breast cancer (IBC) and it is treated as such, with respect to local therapy. However, some DCIS lesions will remain indolent while others will progress into IBC. In order to understand DCIS behavior, several classifications have been proposed. Morphological grading, which is based on nuclear atypia, is the widely used method for DCIS classification. However, immunohistochemical surrogate subtypes, based on the expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), are increasingly used in DCIS research. Currently, none of these classifications is able to predict DCIS behavior. **The aim** of this thesis was therefore to identify novel internal (restricted to DCIS cells) and environmental markers that could contribute to DCIS behavior.

The first section of our manuscript focusses on internal changes during DCIS progression. In **chapter 2**, we aimed to gain insight into PIK3CA mutations and their variant allele frequency (VAF) in association with PTEN and p-AKT expression during the progression from DCIS to IBC. PIK3CA mutations are frequently observed in IBC. They lead to the hyper-activation of the AKT pathway, which results into increased cell growth and survival. This pathway is negatively regulated by PTEN, whereby loss or impaired function of PTEN also results in uncontrolled activation of the AKT pathway. Contra intuitively, PIK3CA mutations are generally associated with good prognosis, while PTEN loss is associated with worse prognosis. Previous studies correlating PIK3CA and PTEN mutations suggested mutual exclusivity although data regarding PTEN expression during breast cancer progression is limited. Using isolated DNA from DCIS (n=73), synchronous IBC (n=73) and metastasis (n=23), we demonstrated that the presence of PIK3CA mutations in these lesions was concordant. However, DCIS had a higher VAF compared to IBC. In case of a wild type PIK3CA, we observed higher PTEN expression in DCIS, while it remained similar in case of a mutated PIK3CA. The presence of PIK3CA mutations in DCIS and PTEN loss in IBC suggest an early role for PIK3CA mutation in breast carcinogenesis rather than DCIS progression, while PTEN might be more essential during DCIS progression into IBC in case of a wild type PIK3CA. These results may contribute to further unraveling the process of breast carcinogenesis and this could facilitate the development of patient-specific treatment.

In **chapter 3**, we focused on the role of the Y-chromosome in male breast cancer, whereby we aimed to assess the presence and prognostic effect of loss of Y-chromosome (LOY) during male breast cancer progression. Generally, LOY has been observed in several solid tumors and is associated with increased cancer mortality in male. We included a large national cohort of 796 patients and assessed LOY using fluorescent in situ hybridization (FISH). LOY was detected in 12.7% of all invasive cases and it was associated with ER and PR negative tumors. With regard to progression, the presence of LOY was assessed in patients with synchronous DCIS and IBC. A concordant LOY status between DCIS and the invasive component was detected in the majority of patients (17 out of 22) with LOY in the invasive component. No LOY was detected in the DCIS component when LOY was absent in the invasive component. There was no association between

LOY and outcome. Overall, we described the presence of LOY in the largest cohort of male breast cancer as an early event, which generally starts at the DCIS stage and is associated with ER and PR negative tumors.

In the final chapter of this section (**chapter 4**), we investigated the genetic differences in breast cancers with a heterogeneous HER2 status. HER2 expression is routinely used in the diagnostic setting, which allows for specific targeted therapy. It is amplified in 15% of all IBCs and these lesions are frequently associated with a DCIS component. Although the majority of the patients demonstrate a homogeneous HER2 amplification, heterogeneous HER2 expression is observed in 5-41% of all HER2-enriched IBCs. This suggests that not all cancers cells are dependent on HER2 for progression and other alternative drivers might be involved. Here, heterogeneous components were compared with next-generation sequencing using a customized targeted 53-gene panel in 10 patients with a heterogeneous HER2 status. Somatic mutations were detected in 26 different genes, which were (likely) pathogenic. Copy number gains were observed in five patients, whereby EGFR copy number gain resulted in EGFR protein overexpression in one patient. No common alternative drivers were identified in the HER2-negative tumor components. Conclusively, we demonstrated a dynamic process of breast carcinogenesis of which some genetic aberrations are likely to be crucial for cancer progression, and others will be mere 'passenger' molecular anomalies.

The second section of this thesis includes the role of the tumor microenvironment in DCIS behavior. We first reviewed the literature regarding the role of tumor infiltrating lymphocytes (TILs) in DCIS in **chapter 5**. In IBC, the presence of high numbers of TILs is frequently observed in high grade, ER- and/or HER2+ subtypes and was associated with improved outcome. This effect also seems to be dependent on molecular subtype and immune cell composition. Generally, CD8+ T cells are associated with favorable outcome while CD4+FOXP3+ T cells are associated with poor outcome. Research regarding the role of TILs in DCIS is relatively new and consensus therefore remains to be found. However, high numbers of TILs are also associated with high grade, ER-and/or HER2+ DCIS. High numbers of CD8+ T cells are associated with low risk for recurrence and low numbers of B cells are associated with longer recurrence free survival. Additionally, DCIS vaccine studies have demonstrated the effectiveness of treating HER2+ DCIS in an immune modulatory fashion. This supports the hypothesis of an active role for TILs during DCIS progression, although the exact role of the immune system during DCIS progression has to be elucidated.

In **chapter 6**, we aimed to study the DCIS-associated immune response by characterizing immune cell subsets according to DCIS immunohistochemical subtypes. DCIS subtypes were previously suggested to have a divergent biological behavior. HER2+ DCIS seems to either remain in situ for a longer period or has a rapid in situ growth rate. Triple negative (TN) DCIS on the other hand seems to have a relatively rapid progression to IBC. These behavioral differences could be explained by changes in the microenvironment and TIL composition. Using hematoxyn and eosin stained section from 473 patients, 27.7% cases were considered TIL-high. Of these, whole slide were stained for CD4, CD8, CD20, CD68, FOXP3 and PD-L1 (SP142 and SP263) to determine the immune cell subsets. There was no statistical difference in the immune cell composition according to immunohistochemical subtype. However, a higher proportion of CD8+ T cells was observed in ER+HER2+ DCIS compared to TN DCIS. Additionally, the HER2+ subgroup (independent of ER) was associated with the lowest PD-L1 expression according to SP142-based immunohistochemistry on tumor cells. Overall, our results suggest a more pronounced anti-

tumorigenic immune response in HER2+ DCIS, which might play a role in its distinct biological behavior.

In **chapter** 7, we elute on gene-expression characteristics of HER2 positive DCIS cells that are associated with the presence of TILs. Although data are limited, TILs are likely to play a role in the biological behavior of HER2+ DCIS. To prevent invasiveness, the potential of targeted immune modulating treatment of HER2+ DCIS has been explored. HER2+ DCIS is often associated with a pronounced local immune response, marked by high numbers of TILs. Here, we included 23 patients of whom 11 were TIL-rich and 12 TIL-poor. We identified a 29 gene-expression profile that was associated with the density of TILs. These genes included *CCND3*, *DUSP10* and *RAP1GAP*, which were previously described in breast cancer and cancer immunity. Our data support a potential role of these genes in the local immune response and consequently, the biological behavior of DCIS. The identified gene-classifier may guide towards more rationalized choices with respect to immune mediated therapy in HER2+ DCIS, such as targeted vaccine therapy.

In addition to the attraction of TILs, previous studies reported that DCIS cells are also able to induce stromal changes and signs of regression, which can interact with TILs and mediate DCIS behavior. In **chapter 8**, we investigated how these stromal changes interact with TILs and if they could be mediators of DCIS behavior. Using a previously described cohort of 472 patients with pure DCIS, we identified stromal changes and DCIS regression in respectively 30% and 7% of the patients. The majority of the TILs remained periductal, however intraductal TILs were observed in 22.9% of the patients. We demonstrated a site-specific immune cell composition: periductal immune cells include a high proportion of CD4+ T cells, while intraductal predominantly consisted of CD68+ macrophages and CD8+ T cells. We observed less TILs influx in case of stromal changes and DCIS regression was associated with increased numbers of intraductal FOXP3+ T cells. Additionally, we observed a DCIS subtype specific immune response; the ER+HER2+ subtype had an increased number of intraductal CD8+ T cells. Our results therefore suggest a site- and subtype-specific immune response.

In the last section of this thesis, we studied DCIS treatment and suggested an alternative for surveillance trials. First, we compared the two widely used methods for pre-operative localization of DCIS in **chapter 9**. Using real-world data from the Dutch Pathology Registry, we compared the surgical resection margin status between patients treated with wire-guided localization and radioactive seed localization. We demonstrated comparable surgical margins between these localization methods in patients with DCIS. However, patients in the radioactive seed localization group were more often treated with breast-conserving therapy in case of a re-excision. This could be related to the volume of the first excision, although these data were not available.

In **chapter 10**, we proposed a retrospective alternative for the currently ongoing active surveillance clinical trials. Since a proportion of DCIS is likely to remain indolent, current DCIS treatment can be considered excessive. However, robust differentiation markers for DCIS progression are still lacking. Previous studies compared patients with a recurrence after breast conserving surgery to those without a recurrence. However, one can argue that the patient without a recurrence was probably treated adequately. Additionally, current active clinical surveillance studies apply different inclusion criteria and reaching the required number of patients remains challenging. In this chapter, we advocated a new research strategy, wherein only those patients with a recurrence are studied. Since approximately half of the recurrences are IBC and half are DCIS, the new approach allows us to ask: why did some residual DCIS evolve to invasive cancers and

others not? Identification of DCIS patients at low risk for developing invasive carcinoma will facilitate individualized future therapy and prevent over-treatment.

Finally, in **chapter 11** we conclude this thesis with a discussion of our current results including future perspectives. Our results show that genetic changes such as somatic mutations and chromosomal aberrations are early events that might be involved in breast carcinogenesis rather than DCIS progression. Additionally, we demonstrated a potential role for TILs in DCIS behavior, in particular HER2+ DCIS. We acknowledge that our major pitfalls are the lack of enough long-term clinical follow up details and functional analysis to confirm our findings. We therefore suggest in vitro or ex-vivo studies for future experiments. Currently, a breast cancer (including both DCIS and IBC) biobank is being generated which will allow functional experiments of TILs.

Samenvatting

Sinds de invoering van het borstkanker bevolkingsonderzoek door middel van mammografie wordt meer dan 20% van alle borstkankers gediagnosticeerd als ductaal carcinoma in situ (DCIS), terwijl dit drie decennia geleden slechts 5% was. DCIS wordt beschouwd als een voorloper van invasief borstkanker (IBC) en wordt als zodanig behandeld, met betrekking tot lokale therapie. Sommige DCIS laesies zullen echter indolent blijven, terwijl andere zich ontwikkelen tot IBC. Er zijn verschillende classificaties van DCIS voorgesteld. Een morfologische classificatie, die gebaseerd is op nucleaire atypie, is de meest gebruikte methode voor DCIS classificatie. Echter, immunohistochemische surrogaatsubtypes, gebaseerd op de expressie van de oestrogeenreceptor (ER), progesteronreceptor (PR) en humane epidermale groeifactorreceptor 2 (HER2), worden steeds vaker gebruikt in DCIS onderzoek. Op dit moment is geen van deze classificaties in staat om het gedrag van DCIS goed te voorspellen. **Het doel** van dit proefschrift was dan ook om nieuwe interne (beperkt tot DCIS cellen) en omgevingsmarkers te identificeren die een rol zouden kunnen spelen bij het gedrag van DCIS.

Het eerste deel van dit proefschrift richt zich op interne veranderingen tijdens de progressie van DCIS. In **hoofdstuk 2** hebben we getracht inzicht te krijgen in PIK3CA-mutaties en hun variant allel frequentie (VAF) in samenhang met PTEN en p-AKT expressie tijdens de progressie van DCIS naar IBC. PIK3CA-mutaties worden veelvuldig waargenomen in IBC. Ze leiden tot overactiviteit van de signaaltransductie route waar AKT onderdeel van is, wat resulteert in een verhoogde celgroei en overleving. Deze signaaltransductie route wordt negatief gereguleerd door PTEN, waarbij verlies of verminderde functie van PTEN ook leidt tot ongecontroleerde activatie van deze route. PIK3CA-mutaties worden over het algemeen geassocieerd met een goede prognose bij IBC, terwijl PTEN-verlies geassocieerd wordt met een slechtere prognose. Eerdere studies die PIK3CA- en PTEN-mutaties met elkaar in verband brachten suggereerden dat er sprake is van wederzijdse exclusiviteit, hoewel de gegevens over de expressie van PTEN tijdens de progressie van borstkanker beperkt zijn. Met behulp van geïsoleerd DNA uit DCIS (n=73), synchrone IBC (n=73) en metastasen (n=23) toonden we aan dat de aanwezigheid van PIK3CA-mutaties in deze laesies concordant was. DCIS had echter een hogere PIK3CA VAF in vergelijking met IBC. In het geval van een wild type PIK3CA, zagen we een hogere PTEN expressie in DCIS, terwijl het vergelijkbaar bleef bij een PIK3CA mutatie. De aanwezigheid van PIK3CA-mutaties in DCIS en PTEN-verlies in IBC suggereren een vroege rol voor PIK3CA-mutaties in de carcinogenese van de borst, in plaats van DCIS-progressie. PTEN zou daarentegen meer essentieel kunnen zijn tijdens de DCIS-progressie naar IBC in het geval van een wild type PIK3CA. Deze resultaten kunnen bijdragen aan het verder ontrafelen van het proces van carcinogenese in de borst. Dit zou de ontwikkeling van een patiënt specifieke behandeling kunnen stimuleren.

In **hoofdstuk 3** hebben we ons gericht op de rol van het Y-chromosoom bij mannen met borstkanker. We keken hierbij naar de aanwezigheid en het prognostische effect van het verlies van Y-chromosoom (LOY) tijdens de progressie van borstkanker. LOY is eerder waargenomen in verschillende soorten solide tumoren en is geassocieerd met een verhoogde kankermortaliteit bij mannen. We hebben een groot nationaal cohort van 796 patiënten bestudeerd en LOY beoordeeld met behulp van fluorescerende in situ hybridisatie. LOY werd ontdekt in 12.7% van alle

invasieve gevallen en was geassocieerd met ER en PR negatieve tumoren. Met betrekking tot de progressie hebben we de aanwezigheid van LOY beoordeeld bij patiënten met synchrone DCIS en IBC. Bij de meerderheid van de patiënten met LOY in de invasieve component (17 van de 22) werd een overeenkomende LOY-status tussen DCIS en de invasieve component gedetecteerd. Er werd geen LOY gedetecteerd in de DCIS-component wanneer LOY afwezig was in de invasieve component. We vonden geen verband tussen LOY en prognose. Samengevat hebben we de aanwezigheid van LOY in het grootste cohort van mannelijke borstkanker beschreven als een vroege gebeurtenis, die over het algemeen begint in het DCIS-stadium en geassocieerd is met ER- en PR-negatieve tumoren.

In het laatste hoofdstuk van dit deel (**hoofdstuk 4**) hebben we de genetische verschillen bij borstkanker met een heterogene HER2-status onderzocht. HER2 expressie wordt routinematig gebruikt in de diagnostische setting, wat gerichte therapie mogelijk maakt. Overexpressie van HER2 wordt beschreven in 15% van alle IBC's en deze laesies zijn vaak geassocieerd met een DCIS-component. Hoewel de meerderheid van de patiënten een homogene HER2 amplificatie vertoont, wordt heterogene HER2 expressie waargenomen in 5-41% van alle HER2+ IBC's. Dit suggereert dat niet alle kankercellen afhankelijk zijn van HER2 voor de progressie en dat er andere alternatieve routes bij betrokken kunnen zijn. Wij hebben de heterogene componenten bij 10 patiënten vergeleken met next-generation sequencing, met behulp van een op maat gemaakt 53-genen panel. Somatische mutaties werden gedetecteerd in 26 verschillende genen, die (waarschijnlijk) pathogeen waren. Bij vijf patiënten werd een toename van het aantal kopieën van een gen waargenomen. Bij een patiënt resulteerde de toename in het aantal EGFR-kopieën in overexpressie van het EGFR-eiwit. In de HER2-negatieve tumorcomponenten werden geen gemeenschappelijke alternatieve routes geïdentificeerd. Al met al hebben we een dynamisch proces van carcinogenese aangetoond, waarvan sommige genetische afwijkingen waarschijnlijk cruciaal zijn voor de progressie van borstkanker, en andere slechts 'passagiers' zullen zijn.

Het tweede deel van dit proefschrift gaat over de rol van de tumor micro-omgeving in het DCIS-gedrag. We hebben eerst de potentiele rol van tumor infiltrerende lymfocyten (TILs) in DCIS beschreven in **hoofdstuk 5**. In IBC is de aanwezigheid van hoge aantallen TILs geassocieerd met hooggradige, ER- en/of HER2+ subtypes en een betere prognose. Dit effect lijkt afhankelijk te zijn van het moleculaire subtype en de samenstelling van de immuun cellen. In het algemeen worden CD8+ T cellen geassocieerd met een gunstige prognose, terwijl CD4+FOXP3+ T cellen geassocieerd worden met een slechte prognose. Onderzoek naar de rol van TILs bij DCIS is relatief nieuw, er is daarom nog weinig bekend. Echter, hoge aantallen TILs worden ook geassocieerd met hooggradige, ER- en/of HER2+ DCIS. Hoge aantallen CD8+ T cellen worden geassocieerd met een laag risico op een recidief en lage aantallen B cellen zijn geassocieerd met een langere recidiefvrije overleving. Daarnaast hebben DCIS-vaccinstudies de effectiviteit van de behandeling van HER2+ DCIS aangetoond middels immuunmodulatie. Dit ondersteunt de hypothese van een actieve rol voor TILs tijdens DCIS progressie, hoewel de exacte rol van het immuunsysteem tijdens DCIS progressie nog moet worden opgehelderd.

In **hoofdstuk 6** hebben we geprobeerd de DCIS-geassocieerde immuunrespons te bestuderen door de samenstelling van de immuun cellen te karakteriseren in associatie met DCIS-immunohistochemische subtypes. Eerder werd beschreven dat DCIS-subtypes een verschillend biologisch gedrag vertonen. HER2+ DCIS lijkt voor een langere periode in situ te blijven ofwel een snelle in situ groei te hebben. Triple negatief (TN) DCIS daarentegen lijkt een relatief snelle progressie naar IBC te hebben. Deze gedragsverschillen zouden verklaard kunnen worden door

veranderingen in de micro-omgeving en de samenstelling van de immuun cellen. Met behulp van hematoxyn en eosine gekleurde diagnostische coupes van 473 patiënten, werden 27.7% gevallen beschouwd als TIL-hoog. Hiervan werden hele coupes gekleurd voor CD4, CD8, CD20, CD68, FOXP3 en PD-L1 (SP142 en SP263) om de immuuncel-subsets te bepalen. We vonden geen statistisch verschil in de samenstelling van de immuun cellen per immunohistochemisch subtype. Er werd echter een hoger aandeel CD8+ T cellen waargenomen in ER+HER2+ DCIS in vergelijking met TN DCIS. Bovendien werd de HER2+ subgroep (onafhankelijk van ER) geassocieerd met de laagste PD-L1-SP142 expressie op tumorcellen. Onze resultaten suggereren een meer uitgesproken immuunrespons tegen de tumorcellen in HER2 positieve DCIS, hetgeen een rol zou kunnen spelen bij het biologische gedrag.

In **hoofdstuk 7** wordt ingegaan op de genexpressiekarakteristieken van HER2+ DCIS-cellen die samenhangen met de aanwezigheid van TILs. Om ervoor te zorgen dat HER2+ DCIS niet invasief wordt, wordt gestreefd naar een HER2 specifieke behandeling. HER2+ DCIS is vaak geassocieerd met veel TILs. Deze zijn bij invasieve borstkanker geassocieerd met een betere prognose. Bij HER2+ DCIS is deze data beperkt, alhoewel het aannemelijk is dat TILs ook hier een rol spelen. In deze studie zijn 23 patiënten geïncludeerd, waarvan 11 met veel TiLs en 12 met weinig TiLs. We hebben een genexpressieprofiel van 29 genen gevonden die geassocieerd waren met de aanwezigheid van Tils, waaronder CCND3, DUSP10 en RAP1GAP. Deze zijn eerder beschreven bij borstkanker en tumorimmunologie. Onze data ondersteunen dat deze genen mogelijk een rol spelen in de lokale immuunrespons bij HER2+ DCIS, en daaraan gerelateerd het biologische gedrag. Dit genexpressieprofiel kan leiden tot optimalisatie van rationele keuzes omtrent het gebruik van immuun gemedieerde therapie bij HER2+ DCIS, zoals gerichte vaccinatie therapie.

Naast het aantrekken van TILs, rapporteerden eerdere studies dat DCIS cellen ook in staat zijn om stromale veranderingen en tekenen van regressie te induceren, wat op zijn beurt kan interageren met TILs en effect kan hebben op het gedrag van DCIS. In **hoofdstuk 8** hebben we onderzocht hoe deze stromale veranderingen interageren met TILs en of ze mediatoren van DCIS gedrag kunnen zijn. Met behulp van een eerder beschreven cohort van 472 patiënten met DCIS, identificeerden we stromale veranderingen en DCIS regressie in respectievelijk 30% en 7% van de patiënten. Het merendeel van de TILs bleef periductaal, maar intraductale TILs werden waargenomen bij 22.9% van de patiënten. We toonden een locatie-specifieke samenstelling van de immuun cellen aan: periductale immuun cellen bevatten een hoog aandeel CD4+ T cellen, terwijl de intraductale immuun cellen voornamelijk bestond uit CD68+ macrofagen en CD8+ T cellen. We zagen minder intraductale influx van TIL's bij aanwezigheid van stromale veranderingen. DCIS regressie was geassocieerd met een verhoogd aantal intraductaal gelegen FOXP3+ T cellen. Daarnaast hebben we een DCIS subtype specifieke immuunrespons waargenomen, waarbij een verhoogd aantal intraductaal gelegen CD8+ T cellen geassocieerd was met ER+HER2+ DCIS. Onze resultaten suggereren dus een locatie- en subtype-specifieke immuunrespons.

In het laatste deel van dit proefschrift hebben we de behandeling van DCIS bestudeerd en hebben we een alternatief voor surveillancestudies voorgesteld. In **hoofdstuk 9** vergeleken we de twee meest gebruikte methoden voor preoperatieve lokalisatie van DCIS. Met de gegevens uit het Nederlands Pathologisch Register, hebben we de status van de chirurgische resectiemarge vergeleken tussen patiënten behandeld met draadgeleide lokalisatie en radioactieve zaadlokalisatie. De chirurgische marges tussen deze lokalisatie-methoden bij patiënten met DCIS was vergelijkbaar. Echter, patiënten met radioactieve zaadlokalisatie werden vaker behandeld met een borstsparende therapie in het geval van een re-excisie. Dit zou gerelateerd kunnen zijn aan het volume

van de eerste resectie, maar deze data hadden we niet tot onze beschikking.

In **hoofdstuk 10** hebben we een alternatief voorgesteld voor de momenteel lopende klinische observatieonderzoeken. Aangezien DCIS bij een deel van de patiënten waarschijnlijk indolent zal blijven, is de huidige DCIS-behandeling waarschijnlijk deels overbodig. Het ontbreekt echter aan robuuste differentiatie markers voor de progressie van DCIS. In eerdere studies werden patiënten met een recidief na een borstsparende operatie vergeleken met patiënten zonder recidief. Een deel van de patiënten zonder recidief zal echter waarschijnlijk al adequaat behandeld zijn. Bovendien passen de huidige klinische observatiestudies verschillende inclusiecriteria toe en blijft de vereiste inclusie van het aantal patiënten een uitdaging. In dit hoofdstuk wordt gepleit voor een nieuwe onderzoeksstrategie, waarbij alleen de patiënten met een recidief worden onderzocht. Aangezien ongeveer de helft van de recidieven IBC zijn en de helft DCIS, zal deze nieuwe aanpak antwoord kunnen geven op de vraag: waarom evolueerden sommige residuele DCIS laesies naar invasieve kankers en andere niet? De identificatie van DCIS-patiënten met een laag risico op het ontwikkelen van een invasief carcinoom zal een geïndividualiseerde therapie in de toekomst vergemakkelijken en overbehandeling voorkomen.

We sluiten dit proefschrift af in **hoofdstuk 11**, met een bespreking van onze huidige resultaten, inclusief de toekomstperspectieven. Onze resultaten laten zien dat genetische veranderingen zoals somatische mutaties en chromosoomafwijkingen vroege gebeurtenissen zijn die betrokken kunnen zijn bij borstcarcinogenese. Daarnaast beschrijven we een potentiële rol voor TILs bij het gedrag van DCIS, in het bijzonder bij HER2+ DCIS. We erkennen hierbij onze belangrijkste valkuilen: het gebrek aan voldoende lange termijn klinische follow-up en functionele analyses om onze bevindingen te bevestigen. We stellen daarom in vitro en ex-vivo studies voor, waarbij we gebruik maken van zowel immuun cellen als tumorcellen voor toekomstige experimenten. Momenteel wordt er een biobank voor borstkanker (inclusief DCIS en IBC) gegenereerd die functionele experimenten met TILs in de toekomst mogelijk maakt.

Resumé

Depuis l'introduction de la mammographie , plus de 20% de tous les cancers du sein sont diagnostiqués comme des carcinomes canalaires in situ (CCIS), ce qui ne représentait que dans 5% il y a trois décennies. Le CCIS est considéré comme un précurseur non obligatoire du carcinome canalaire infiltrant (CCI) et il est traité comme tel, en ce qui concerne la thérapie locale. Or, certaines CCIS resteront indolentes tandis que d'autres évolueront en CCI. Afin de comprendre le comportement des CCIS, plusieurs classifications ont été proposées. La classification morphologique, qui est basée sur l'atypie nucléaire, est la méthode largement utilisée pour la classification CCIS. Récemment, on détermine aussi les caractéristiques des cellules immunohistochimiques, basés sur l'expression du récepteur d'œstrogène (ER), du récepteur de progestérone (PR) et du récepteur 2 du facteur de croissance épidermique humain (HER2). Ces déterminant sont de plus en plus utilisés dans la recherche CCIS. Actuellement, aucune de ces classifications n'est capable de prédire le comportement des DCIS. **L'objectif** de cette thèse était donc d'identifier de nouveaux marqueurs internes (limités aux cellules CCIS) et environnementaux qui pourraient contribuer au comportement des CCIS.

La première section de notre manuscrit se concentre sur les changements internes au cours de la progression du CCIS. Dans le **chapitre 2**, nous avons cherché à mieux comprendre les mutations PIK3CA et leur fréquence allèle variante (VAF) en association avec l'expression de PTEN et de p-AKT pendant la progression de CCIS à CCI. Les mutations PIK3CA sont généralement associées à un bon pronostic, tandis que la perte de PTEN est associée à un pronostic plus défavorable. Des études précèdent on mit en corrélation les mutations PIK3CA et PTEN ont suggéré une exclusivité mutuelle, bien que les données concernant l'expression de PTEN pendant la progression du cancer du sein soient limitées. À l'aide d'ADN isolé provenant de CCIS (n=73), de CCI synchrone (n=73) et de métastase (n=23), nous avons montré que la présence de mutations PIK3CA dans ces lésions était concordante. Le CCIS avait un VAF plus élevé que le CCI. Dans le cas d'un PIK3CA sans mutation, nous avons observé une expression de PTEN plus élevée dans le CCIS, alors qu'elle restait la même dans le cas d'un PIK3CA muté. La présence de mutations de PIK3CA dans le CCIS et la perte de PTEN dans le CCI suggèrent un rôle précoce de la mutation de PIK3CA dans la carcinogenèse du sein plutôt que dans la progression du CCIS, alors que PTEN pourrait être plus essentiel pendant la progression du CCIS dans le CCI dans le cas d'un PIK3CA de type sauvage. Ces résultats pourraient contribuer à éclaircir davantage le processus de la carcinogenèse mammaire, ce qui pourrait faciliter la mise au point d'un traitement spécifique pour chaque patient.

Dans le **chapitre 3**, nous nous sommes concentrés sur le rôle du chromosome Y dans le cancer du sein chez l'homme. On à évaluer la présence et l'effet pronostique de la perte du chromosome Y (LOY) pendant la progression du cancer du sein chez l'homme. Le LOY a été observé dans plusieurs tumeurs solides et est associé à une augmentation de la mortalité par cancer chez l'homme. Nous avons inclus une large cohorte nationale de 796 patients et évalué LOY en utilisant l'hybridation fluorescente in situ (FISH). LOY a été détecté dans 12.7% de tous les cas invasifs et a été associé à des tumeurs ER et PR négatives. En ce qui concerne la progression, la présence de LOY a été évaluée chez les patients présentant un CCIS et un CCI synchrones. On a détecté

un statut LOY concordant entre le CCIS et la composante invasive chez la majorité des patients (17 sur 22), avec LOY présent dans la composante invasive. Aucun LOY n'a été détecté dans la composante CCIS lorsque LOY était absent dans la composante invasive. On n'a pas trouvé un effet pronostique de LOY. Dans l'ensemble, nous avons décrit la présence de LOY dans la plus grande cohorte de cancer du sein chez l'homme comme un événement précoce, qui commence généralement au stade CCIS et est associé à des tumeurs ER et PR négatives.

Dans le dernier chapitre de cette section (**chapitre 4**), nous avons étudié les différences génétiques dans les cancers du sein ayant un statut HER2 hétérogène. L'expression de HER2 est couramment utilisée dans le cadre du diagnostic, ce qui permet une thérapie spécifique. Elle est amplifiée dans 15 à 20% de tous les CCI et ces lésions sont fréquemment associées à un composant CCIS. Bien que la majorité des patients présentent une amplification homogène de HER2, une expression hétérogène de HER2 est observée dans 5 à 41% de tous les CCI enrichis en HER2. Cela suggère que toutes les cellules cancéreuses ne dépendent pas de HER2 pour leur progression et que d'autres facteurs alternatifs pourraient être impliqués. Ici, les composants hétérogènes ont été comparés avec le séquençage de la prochaine génération (NGS) en utilisant un panel de 53 gènes ciblés, sur mesure chez 10 patients ayant un statut HER2 hétérogène. On a détecté des mutations somatiques dans 26 gènes différents, qui étaient (probablement) pathogènes. Des gains de nombre de copies (CNV) ont été observés chez cinq patients. Le gain de nombre de copies génétique de l'EGFR chez un patient ayant entraîné une surexpression de la protéine EGFR. Aucun facteur alternatif commun n'a été identifié dans les composants tumoraux HER2-négatifs. En conclusion, nous avons démontré un processus dynamique de cancérogenèse du sein dont certaines aberrations génétiques sont susceptibles d'être cruciales pour la progression du cancer, et d'autres ne seront que des anomalies moléculaires "passagers".

La deuxième partie de cette thèse porte sur le rôle du microenvironnement dans le comportement du CCIS. Nous avons d'abord examiné le rôle des lymphocytes infiltrant les tumeurs (TILs) dans le CCIS au **chapitre 5**. Dans les CCI, la présence d'un nombre élevé de TIL est fréquemment observée dans les sous-types de haut grade, ER- et/ou HER2+ et a été associée à une amélioration des résultats. Cet effet semble également dépendre du sous-type moléculaire et de la composition des cellules immunitaires. En général, les cellules T CD8+ sont associées à un résultat favorable tandis que les cellules T CD4+FOXP3+ sont associées à un résultat médiocre. Les recherches concernant le rôle des TIL dans le CCIS sont relativement récentes et un consensus reste donc à trouver. Cependant, un nombre élevé de TIL est également associé à des CCIS de haut grade, ER et/ou HER2+. Un nombre élevé de cellules T CD8+ est associé à un risque réduit de récidive et un nombre réduit de cellules B est associé à une survie sans récidive plus longue. De plus, les études sur le vaccin CCIS ont démontré l'efficacité du traitement du CCIS HER2+ de manière immunomodulatoire. Ceci soutient l'hypothèse d'un rôle actif des TILs pendant la progression du CCIS, bien que le rôle exact du système immunitaire pendant la progression du CCIS doive être élucidé.

Dans le **chapitre 6**, nous avons étudier la réponse immunitaire associée au CCIS en caractérisant la composition de cellules immunitaires selon les sous-types immunohistochimiques du CCIS. Les sous-types CCIS ont été précédemment suggérés comme ayant un comportement biologique divergent. Le sous-type HER2+ CCIS semble soit rester in situ pendant une période plus longue, soit avoir un taux de croissance in situ rapide. Les CCIS triple négatif (TN), en revanche, semblent avoir une progression relativement rapide vers les CCI. Ces différences de comportement pourraient s'expliquer par des changements dans le microenvironnement et la composition

des TILs. En utilisant la section colorée à l'hématoxine et à l'éosine de 473 patients, 27.7% des cas ont été considérés comme ayant un TIL élevé. Parmi ceux-ci, la lame entière a été colorée pour les CD4, CD8, CD20, CD68, FOXP3 et PD-L1 (SP142 et SP263) afin de déterminer la composition de cellules immunitaires. Il n'y a pas eu de différence statistique dans la composition des cellules immunitaires selon le sous-type immunohistochimique. On a observé une proportion plus élevée de cellules T CD8+ dans les CCIS ER+HER2+ par rapport aux CCIS TN. En outre, le sous-groupe HER2+ (indépendant de ER) était associé à la plus faible expression de PD-L1-SP142 sur les cellules tumorales. Globalement, nos résultats suggèrent une réponse immunitaire anti-tumorigène plus prononcée dans les CCIS HER2+, ce qui pourrait jouer un rôle dans son comportement biologique distinct.

Dans le **chapitre** 7, nous éluons sur les caractéristiques d'expression génique des cellules de CCIS HER2+ qui sont associées à la présence des TILs. Bien que les données soient limitées, les TILs sont susceptibles de jouer un rôle dans le comportement biologique de CCIS HER2+ . Pour prévenir la progression invasif, le potentiel d'un traitement immuno-modulant de CCIS HER2+ a été exploré. Le CCIS HER2+ est associé à une réponse immunitaire locale prononcée, marquée par un nombre élevé de TILs. Ici, nous avons inclus 23 patients dont 11 étaient riches en TILs et 12 pauvres en TILs. Nous avons identifié un profil d'expression génique de 29 gènes associé à la densité des TILs.

Nous avons identifié un profil d'expression génique 29. Ces gènes comprenaient CCND3, DUSP10 et RAP1GAP, qui ont été précédemment décrits dans le cancer du sein et l'immunité contre le cancer. Nos données soutiennent un rôle potentiel de ces gènes dans la réponse immunitaire locale et par conséquent, le comportement biologique du CCIS. Le classificateur génique identifié peut guider vers des choix plus rationalisés en ce qui concerne la thérapie à médiation immunitaire dans les CCIS HER2+, telle qu'une thérapie vaccinale ciblée.

En plus de l'attrait des TIL, des études ont rapporté que les cellules CCIS sont également capables d'induire des changements stromal et des signes de régression, qui peuvent interagir avec les TILs et servir de médiateur dans le comportement des CCIS. Au **chapitre 8**, nous avons étudié comment ces changements stromal interagissent avec les TIL et s'ils peuvent être des médiateurs du comportement des CCIS. En utilisant une cohorte précédemment décrite de 472 patients atteints de CCIS pur, nous avons identifié des changements stromal et une régression CCIS chez respectivement 30% et 7% des patients. La majorité des TILs sont restés périductal, mais des TILs intraductal ont été observés chez 22.9% des patients. Nous avons démontré une composition cellulaire immunitaire spécifique a l'endroit: les cellules immunitaires périductales comprennent une grande proportion de cellules T CD4+, tandis que les cellules intraductales sont principalement constituées de macrophages CD68+ et de cellules T CD8+. Nous avons observé un afflux moindre de TILs en cas de changements stromal et la régression CCIS a été associée à une augmentation des cellules T FOXP3+ intraductales. En outre, nous avons observé une réponse immunitaire spécifique au sous-type, montrant un nombre augmenté de cellules T CD8+ intraductales uniquement dans le sous-type ER+HER2+ CCIS. Nos résultats suggèrent donc une réponse immunitaire spécifique à l'endroit et au sous-type.

Dans la dernière partie de cette thèse, nous avons étudié le traitement CCIS et proposé une alternative pour les essais de surveillance. Tout d'abord, nous avons comparé les deux méthodes largement utilisées pour la localisation préopératoire du CCIS dans le **chapitre 9**. En utilisant des données réelles du registre de pathologie néerlandais, nous avons comparé l'état de la marge de résection chirurgicale entre les patients traités par localisation filoguidée et par localisation de

grains radioactifs. Nous démontrons des marges chirurgicales comparables entre ces méthodes de localisation chez les patients atteints de CCIS. Pourtant, les patients du groupe localisation de grains radioactifs ont été plus souvent traités avec une thérapie de conservation du sein en cas de ré-excision. Cela pourrait être lié au volume de la première excision, mais ces données n'était pas disponibles.

Au **chapitre 10**, nous avons proposé une alternative rétrospective pour les essais cliniques de surveillance active actuellement en cours. Étant donné qu'une partie des CCIS est susceptible de rester indolente, le traitement actuel des CCIS peut être considéré comme excessif. Cependant, il manque encore de solides marqueurs de différenciation pour la progression du CCIS. Des études antérieures ont comparé des patientes ayant subi une récidive après une chirurgie conservatrice du sein à celles qui n'ont pas eu de récidive. Pourtant, on peut avancer que la patiente sans récidive a probablement été traitée de manière adéquate. En outre, les études actuelles de surveillance clinique active appliquent des critères d'inclusion différents et il reste difficile d'atteindre le nombre de patients requis. Dans ce chapitre, nous avons préconisé une nouvelle stratégie de recherche, dans laquelle seules les patientes présentant une récidive sont étudiées. Étant donné qu'environ la moitié des récidives sont des CCI et l'autre moitié des CCIS, la nouvelle approche nous permet de nous demander: pourquoi certains CCIS résiduels ont-ils évolué vers des cancers invasifs et d'autres pas? L'identification des patients CCIS aux risque réduit de développer un carcinome invasif facilitera la thérapie future individualisée et empêchera un sur traitement.

Enfin, au **chapitre 11**, nous concluons cette thèse par une discussion de nos résultats actuels, y compris les perspectives futures. Nos résultats montrent que les changements génétiques tels que les mutations somatiques et les aberrations chromosomiques sont des événements précoces qui pourraient être impliqués dans la cancérogenèse du sein plutôt que dans la progression de la CCIS. En outre, nous avons démontré un rôle potentiel des TILs dans le comportement des CCIS, en particulier des HER2+ CCIS. Nous reconnaissons que nos principaux écueils sont le manque de détails sur le suivi clinique à long terme et d'analyses fonctionnelles suffisantes pour confirmer nos conclusions. Nous suggérons donc des études in vitro ou ex vivo pour de futures expérimentés. Actuellement, une biobanque sur le cancer du sein (comprenant à la fois des CCIS et des CCI) est en cours de création, ce qui permettra des expériences fonctionnelles de TIL.

Appendices

Curriculum Vitae

Marie Colombe Agahozo was born on the 5th of august 1990 in Kigali, Rwanda. When she was about 9 years old, she came to Amsterdam, the Netherlands with her family where she finished primary school and started secondary school. At the age of 17, her family moved to the Hague where she finished secondary school. In 2011, she attended the VU university in Amsterdam, where she obtained her Bachelor's degree in Health and Life Sciences and Master's degree in Biomedical Sciences, with a specialization in Immunology and Neurobiology. Additionally, her master thesis got published, turning into her first scientific publication. During her time at the VU, she was a member of the Faculty Students Council, president of the Social activities and Photography committee and an active member of the study association Anguilla. She also got exposed to teaching, which sparked up her interest. With a scientific/academic carrier in mind, she started her PhD at the Erasmus Medical Center in Rotterdam with Dr. C.H.M. van Deurzen, Prof.dr. F. van Kemenade and Prof.dr. J.W.M. Martens, a few months after graduation in November 2016. During a period of 4 years, she not only focused on her research, but also continued teaching and obtained her University Teaching Qualification. She also intensively collaborated with the Laboratory for Pathology Dordrecht. She is now aiming for an academic position which combines both research and teaching.

PhD portfolio

Name PhD student: Marie Colombe Agahozo

Department: Pathology

Promotor: prof. dr. F. van Kemenade and prof. dr. J.W.M. Martens

Copromotor: dr. C.H.M. van Deurzen

1. PhD Training

General Courses

Name	Domain/Subject	Duration (days)	ECTs
Advanced Immunology	Immunology	5	1.8
Basic and Translational oncology	Oncology	5	1.8
Basic Course on R	Statistics and programming	5	1.8
Basisdidactiek voor docenten (TtT I)	Teaching	2	12
Biomedical Scientific English Writing (short)	English writing	5	2
Flipping the classroom	Teaching	0.5	0.3
Grant Proposal Writing (GPW)	Proposal writing	0.5	0.5
NGS in DNA diagnosis	Sequencing	3	1
Photoshop and Illustrator CS6	Illustration	1	0.3
Schriftelijke tentamenvragen ontwerpen	Teaching	0.5	0.3
Scientific integraty	Medical ethics	1	0.3
SCORE	Regenerative Medicine	4	1
Techniques in Biomedical sciences	Research methods	5	1.5
Total ECTs			24.6

Oral Presentations

Name meeting	Place	Domain/Subject	Duration (min)	ECTs
AACR	Atlanta, USA	TILs in DCIS subtypes	20	1
BCR 2018	Rotterdam, NL	Overview project	30	1
BCR 2019	Rotterdam, NL	PIK3CA during BC progression	30	1
Cancer research day	Rotterdam, NL	PIK3CA during BC progression	10	0.5
MolMed day 2018	Rotterdam, NL	PIK3CA during BC progression	15	0.5
PALM 2018	Rotterdam, NL	Overview project	30	1
PALM 2019	Rotterdam, NL	TILs in DCIS subtypes	20	0.5
Total ECTs				5.5

(Inter)National Congress

Name Congress	Place	Domain/Subject	Duration (days)	ECTs
AACR 2019	Atlanta, USA	Cancer research	4	1.2
Erasmus MC PhD Day 2018	Rotterdam, NL	EMC Carrier	0.5	0.2
Erasmus MC PhD Day 2019	Rotterdam, NL	EMC Carrier	0.5	0.2
ESMO Breast 2019	Berlin, DE	Breast cancer Research	3	0.9
JNI cancer research day 2017	Rotterdam, NL	Cancer research	1	0.3
JNI cancer research day 2018	Rotterdam, NL	Cancer research	1	0.3
MolMed day 2017	Rotterdam, NL	EMC research	1	0.3
MolMed day 2018	Rotterdam, NL	EMC research	1	0.3
MolMed day 2019	Rotterdam, NL	EMC research	1	0.3
Total ECTs				4

2. Teaching

Assistant Teaching Integrated Skills

Name	Subject/Domain	Date	Duration (days)	ECTs
VO: Bouw vrouwelijke genitaliën	Gyn	dec.2018	4	1.2
VO: Bouw vrouwelijke genitaliën	Gyn	nov.2019	5	1.5
VO: Epitheliale tumoren	Carcinoma	sept.2017	4	1.2
VO: Epitheliale tumoren	Carcinoma	sept.2018	4	1.2
VO: Epitheliale tumoren	Carcinoma	sept.2020	4	1.2
VO: Mamma tumoren	Breast	nov.2017	4	1.2
VO: Mamma tumoren	Breast	nov.2018	4	1.2
VO: Mamma tumoren	Breast	nov.2018	4	1.2
VO: Mamma tumoren	Carcinoma	nov.2019	4	1.2
VO: Diagnose & stadium Kanker	Carcinoma	Sept. 2020	3	0.9
Total ECTs				12.0

Supervision and Tutoring

Name	Subject/Domain	Date	Duration (days)	ECTs
Erasmus MC PhD Day 2019	Organizing	aprl-juli2019	6	1.8
Supervising medical students	Writing systematic review	dec.2018-jan.2019	10	3
Supervising medical students	Writing systematic review	dec.2019-jan.2020	12	3.6
Supervising medical students	Conducting Ethical debates	dec.2019	2	0.6
Supervising medical students	Writing systematic review	may-juli2020	5	1.5
Total ECTs				10.5

3. Personal Achievements

Travel Grant, from ESMO Breast 2019, Berlin Germany Best Poster Award, at Molecular Medicine Day 2019 (3 out of 60 posters)

List of Publications

1. **M.C. Agahozo**, M. Smid, R. van Marion, T.P.P. van den Bosch, A.M. Timmermans, C.J. Heijerman, P.J. Westenend, J.W.M. Martens, C.H.M. van Deurzen, Transcriptomic properties of HER2+ ductal carcinoma in situ of the breast associate with absence of immune cells. Biology. in press.

2. **M.C. Agahozo**, A.M.S. Berghuis, E. van den Broek, L.B. Koppert, I.M. Obdeijn and C.H.M. van Deurzen, Radioactive seed versus wire-guided localization for ductal carcinoma in situ of the breast: comparable resection margins. Ann Surg Oncol. 2020 Jun 23.

3. **M.C. Agahozo**, P.J. Westenend, T. Hasum, J. Giang, S.E. Maltung and C.H.M. van Deurzen, Immune response and stromal changes in ductal carcinoma in situ of the breast are subtype-dependent. Mod Pathol. Feb 2020.

4. M.R. van Bockstal, **M.C. Agahozo**, R. van Marion, P.N. Atmodimedjo, F.B.M. Sleddens, W.N.M. Dinjens, L.L. Visser, E.H. Lips, J. Wesseling and C.H.M. van Deurzen, Somatic mutations and copy number variations in breast cancers with heterogeneous HER2 implication. Mol Oncol. 2020 Apr;14(4): 671-685.

5. **M.C. Agahozo**, A.M. Timmermans, H.F.B.M. Sledens, J.W.M. Martens, W.N.M. Dinjens, C.H.M. van Deurzen, A role for loss of Y-chromosome in the progression from male ductal carcinoma in situ to invasive breast cancer. Cancers (Basel). 2020 Mar 9;12(3):631.

6. **M.C. Agahozo**, M.R. van Bockstal, F.H. Groenendijk, T.P.P. van den Bosch, P.J. Westenend and C.H.M. van Deurzen, Ductal carcinoma in situ of the breast: immune cell composition according to subtype. Mod Pathol. 2020 Feb;33(2):196-205.

7. M.R. van Bockstal, **M.C. Agahozo**, L.B. Koppert and C.H.M. van Deurzen, A retrospective alternative for active surveillance trials for ductal carcinoma in situ of the breast. Int J Cancer. 2020 Mar 1;146(5):1189-1197.

8. A.M. Sieuwerts, S.C. Doebar, V. de Weerd, E.I. Verhoef, C.M. Beaufort, **M.C. Agahozo**, J.W.M. Martens, C.H.M. van Deurzen, APOBEC3B Gene Expression in Ductal Carcinoma In Situ and Synchronous Invasive Breast Cancer. Cancers (Basel). 2019 Jul 27;11(8):1062.

9. **M. C. Agahozo**, A. M. Sieuwerts, S. C. Doebar, E.I. Verhoef, C.M. Beaufort, K. Ruigrok-Ritstier, V. de Weerd, H.F.B.M. Sleddens, W.N.M. Dinjens, J.W.M. Martens and C.H.M. van Deurzen, PIK3CA mutations in ductal carcinoma in situ and adjacent invasive breast cancer. Endocr Relat Cancer. 2019 May;26(5):471-482.

10. **M.C. Agahozo**, D Hammerl, R. Debets, M. Kok and CHM. Van Deurzen, Tumor-infiltrating lymphocytes and ductal carcinoma in situ: friends or foes? Mod Pathol. 2018 Jul;31(7):1012-1025.

11. **M.C. Agahozo**, L. Peferoen, D. Baker and S. Amor, CD20 therapies in multiple sclerosis and experimental autoimmune encephalomyelitis – Targeting T or B cells? Mult Scler Relat Disord. 2016 Sep;9:110-7.

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