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Ductal carcinoma in situ and invasive breast cancer: diagnostic accuracy and prognosis

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Ductal carcinoma in situ and invasive breast cancer: diagnostic accuracy and prognosis

Maartje van Seijen

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The work presented in this thesis was performed at the Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands, in cooperation with Leiden University Medical Center, the Netherlands Comprehensive Cancer Organisation (IKNL) and the nationwide network and registry of cytopathology and histopathology in the Netherlands (PALGA) foundation.

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Ductal carcinoma in situ and invasive breast cancer: diagnostic accuracy and prognosis

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Chapter 1

Scope of the thesis

General introduction

In general, diagnostic tests and medical assessments are fundamental for adequate health care management. This also holds true for ductal carcinoma in situ (DCIS), a non-obligate precursor of invasive breast cancer (IBC) and IBC itself. All tests might serve multiple purposes: first, to exclude or confirm whether a suspect breast lesion is present in both the setting of population-based breast cancer screening or at request. Second, if a lesion is confirmed, to classify the lesion and predict prognosis and benefit of treatment. Third, to monitor progress of the disease or to evaluate response to treatment. All these tests lead to detailed information about the subtype of DCIS or IBC, disease stage, risks of progression, and prediction of treatment effect. Obviously, all tests come with intrinsic limitations due to imperfect sensitivity, specificity and accuracy, which also depend on the context of the applied test[1]. For example, certain diagnostic tests can be highly accurate, e.g. detection of calcifications on mammography or recognizing a full blown (pre-)malignant abnormality in a breast biopsy, but the presence of such lesions does not imply lethal disease per se. Diagnosing such lesions that will never lead to symptoms or death is called overdiagnosis[2]. Strictly, the determined diagnosis is accurate, but treating these lesions is defined as overtreatment and will cause unnecessary harm to the patient. Therefore, knowledge about the follow-up of patients diagnosed with a DCIS or IBC is essential to understand the impact of the disease on individual patients as well as on society level in context of the 'benefit-to-harm' ratio. As such, epidemiological knowledge involving analyses about incidence, prevalence and outcome is interconnected with the interpretation of individual patients' test results.

Aim of this thesis

In this thesis, we evaluate the accuracy of diagnostic testing in context of the risk of progression of DCIS and IBC. This will ultimately help to optimize identification and classification of DCIS and IBC, to the disease course, including response to treatment, and to predict outcome.

Thesis Outline

Ductal carcinoma in situ: diagnostic accuracy and prognosis

Part one focuses on classification and associated risk of progression of ductal carcinoma in situ (DCIS). DCIS is proliferation of neoplastic epithelial cells confined to the ductal system of the breast. The incidence of DCIS has increased substantially since the introduction of population-based breast cancer screening while the breast cancer specific mortality is not decreased[3,4]. The majority of the DCIS diagnoses are identified by calcifications on mammograms acquired within the framework of population-based screening programs. Interestingly, only a minority of DCIS lesions

causes symptoms, for example a palpable lump in the breast or nipple discharge. We believe that most DCIS lesions will never progress to invasive breast cancer based on two major findings: i) since introduction of the screening the incidence of advanced stage breast cancer has not decreased[4,5] indicating that we mostly detect indolent breast lesions by screening instead of the lethal ones and ii) autopsy studies found a high incidence of DCIS indicating a DCIS reservoir in older women exists without clinical consequences[6]. At time of diagnosis it is unknown which DCIS lesions will progress to IBC, therefore all patients receive the same treatment as in the case of breast cancer leading to overdiagnosis and overtreatment for the patients with indolent DCIS, i.e. for those lesions that would never progress even if left untreated.

Chapter two *"Ductal Carcinoma in situ: to treat or not to treat that is the question"* provides an overview of the current knowledge of DCIS including how our initiative, PREvent ductal Carcinoma In Situ Invasive Overtreatment Now (PRECISION), manages to discriminate indolent from hazardous DCIS.

To guide treatment decisions, DCIS is classified diagnostically into well, intermediate or poorly differentiated DCIS. Since it is assumed that grade corresponds to prognosis[7–9] in terms of risk of a subsequent ipsilateral DCIS or IBC, this is used as a prognostic test. Multiple guidelines to classify DCIS exist and the interpretation of the same lesion shows variation between observers, resulting in substantial variability in DCIS grading. The study described in **chapter three** shows an evaluation of the differences in histological assessment of DCIS among pathologists around the world. In addition, we explored possibilities to decrease the interpretation differences.

Prediction of outcome in terms of risk of progression is dependent on the type of treatment of the primary DCIS. DCIS is nowadays often treated with breast conserving surgery supplemented with radiotherapy. The added benefit of radiotherapy has been studied in several clinical trials and has been estimated to be 15% absolute risk reduction for any ipsilateral breast event at ten years of follow-up[10]. In **chapter four**, we studied the association of initial DCIS treatment with long-term risk of subsequent ipsilateral in situ and invasive disease to evaluate the impact of treatment strategy in a non-randomized nationwide cohort. Ideally, we would develop a test, for example including age, tumor size and tumor grade, to select low risk patients to de-escalate (radio)therapy. This chapter provides insights in the long-term risks of treated DCIS on population level.

Invasive breast cancer: diagnostic accuracy and prognosis

Part two aims to explore how to optimize the accuracy of clinical tests in IBC patients treated with neoadjuvant systemic therapy. In addition to local surgery, systemic therapies are applied in breast cancer to eliminate metastasis undetected

at time of diagnosis. To determine who will benefit from systemic therapy, risk profiling is performed. Patient characteristics such as age, menopausal status and performance status, and tumor characteristics as hormonal status and HER2 status, tumor grade and size and lymph node status play a role in determining the risk profile [11,12]. These 'classic' characteristics capture only certain aspects of the tumor biology[11,13]. Molecular tests like the mammaprint[14] and Oncotype DX were developed to improve risk profiling. Based on genomic characteristics these assays try to classify patients in high and low risk breast cancer groups. In the MINDACT trial it was found that chemotherapy could be avoided in patients with clinical high, but a genomic low risk[11]. Hence, these assays are increasingly used in research and clinical setting. Before such a molecular test is performed, quality control (QC) measurements such as minimum tumor cell percentage and RNA quality are required. These inclusion criteria result in a selection of a specific group of breast cancer tissue samples. In **chapter five** we investigated if QC variables for gene expression analysis, could lead to a bias in sample selection.

Neoadjuvant systemic treatment (NST) – systemic treatment delivered prior to definitive breast surgery - could be applied whenever systemic therapy after surgery would be necessary according to the Dutch breast cancer guidelines[15]. NST is increasingly applied, because it intends to shrink the tumor permitting less extensive breast surgery and provides information regarding response during and quickly after treatment. NST compared to adjuvant systemic therapy has equivalent breast cancer recurrence and breast cancer mortality rates[16]. After surgery, the response of NST is evaluated in the resection specimen by the pathologist examining the vital tumor cell percentage. If all tumor cells disappear, a pathological complete response (pCR) is achieved corresponding to the best achievable prognosis at that timepoint[17]. Recently, the US Food and Drug administration mechanism for approval of newly systemic treatments is based on improved pCR figures[18,19]. Evaluation of residual tumor cells in these resection specimens is not standardized yet and various classification systems are used. In **chapter six** we evaluated different pathological classification systems: residual cancer burden (RCB), neoadjuvant response index (NRI) and Neo-Bioscore, and established the long-term prognosis based on various categories of residual disease.

Chapter seven summarizes how the results of the studies described above contribute to improve accuracy and clinical utility of diagnostic tests for breast cancer patients. Furthermore, future perspectives are discussed.

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Chapter 2

Ductal carcinoma in situ: to treat or not to treat, that is the question

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ABSTRACT

Ductal carcinoma in situ (DCIS) now represents 20–25% of all 'breast cancers' consequent upon detection by population-based breast cancer screening programs. Currently, all DCIS lesions are treated, and treatment comprises either mastectomy or breast-conserving surgery supplemented with radiotherapy. However, most DCIS lesions remain indolent; difficulty in discerning harmless lesions from potentially invasive ones can lead to overtreatment of this condition in many patients. To counter overtreatment and to transform clinical practice, a global, comprehensive, and multidisciplinary collaboration is required. Here, we review the incidence of DCIS, the perception of risk for developing invasive breast cancer, the current treatment options and the known molecular aspects of progression. Further research is needed to gain new insights for improved diagnosis and management of DCIS, and this is integrated in the PRECISION ('Prevent ductal Carcinoma In Situ Overtreatment Now') initiative. This international effort will seek to determine which DCIS requires treatment and prevent the consequences of overtreatment on the lives of many women affected by DCIS.

Background

Ductal carcinoma in situ (DCIS) was rarely diagnosed before the advent of breast screening, yet it now accounts for 25% of detected 'breast cancers'. Over 60,000 women are diagnosed with DCIS each year in the USA[1,2], more than 7,000 in the UK[3] and over 2,500 in the Netherlands.[4] DCIS is a proliferation of neoplastic luminal cells that are confined to the ductolobular system of the breast. If DCIS progresses to invasive breast cancer, DCIS cells penetrate the ductal basement membrane and invade the surrounding parenchyma. Individual lesions differ in aspects of the disease: presentation, histology, progression and genetic features.[5,6] Despite being pre- or non-invasive, DCIS is often regarded as an early form of (Stage 0) breast cancer. Therefore, conventional management includes mastectomy or breast-conserving surgery supplemented with radiotherapy; in some countries, adjuvant endocrine therapy is added. Regrettably, current therapeutic approaches result in overtreatment of some women with DCIS (Textbox 1). The Marmot Report in 2012 recognised the burden of overtreatment to women's wellbeing.[7] In effect, women with DCIS are labelled as 'cancer patients', with concomitant anxiety and negative impact on their lives, despite the fact that most DCIS lesions will probably never progress to invasive breast cancer. Due to the uncertainty regarding which lesions run the risk of progression to invasive cancer, current risk perceptions are misleading and consequently bias the dialogue between clinicians and women diagnosed with DCIS, resulting in overtreatment for some, and potentially many, women.

Improving the management and treatment of DCIS presents a central challenge: distinguishing indolent, harmless DCIS lesions from potentially hazardous ones. This poses a fundamental question to address: 'is cancer always cancer?'. To answer this question, we need to adopt an interdisciplinary and translational approach, merging fields of epidemiology, molecular biology, clinical research and psychosocial studies. How low does the risk need to be to refrain from treating DCIS? What are the prognostic markers and read-outs we can rely on? How do we frame and communicate the risks involved?

In this review, we describe the current approaches to diagnosing DCIS, the perception of the risk of developing invasive breast carcinoma, the treatment options available following a diagnosis, and a current knowledge of the progression of DCIS, before outlining future endeavours and the need for an integrated approach that blends clinical and patient insights with scientific advances.

Textbox 1: Consequences of overdiagnosis in DCIS: Impact of DCIS on a woman's life

The diagnosis of DCIS labels women as being at risk for invasive breast cancer. Despite the good prognosis and normal life-expectancy, women diagnosed with DCIS may experience substantial psychological distress[29] and overestimate the implications of a DCIS diagnosis.[34,35,92] Comorbidity of surgery, and prior depression have been reported as important factors related to worse quality of life in these women.[29] Critical questions yet to be answered include: (i) Can the way in which a diagnosis for DCIS is communicated be improved? (ii) Can the labelling effects of a diagnosis of DCIS be mitigated, whilst ensuring adequate follow-up of these high-risk women? And, finally, (iii) what is the impact on quality of life for active surveillance of women diagnosed with low grade DCIS? Addressing these questions requires central involvement of patient voices to improve clarity, not only for patients but also for healthcare providers, about the implications and risks of a diagnosis of DCIS.[93]

DCIS incidence

The number of women diagnosed with DCIS over the past few decades largely follows the introduction of population-based breast cancer screening.[8–12] The European standardised rate of in situ lesions has increased fourfold, from 4.90 per 100,000 women in 1989 (accounting for 4.5% of all diagnoses registered as breast cancer) to 20.68 in 2011 (accounting for 12.8% of all diagnoses registered as breast cancer; www.cijfersoverkanker.nl). Of all in situ breast lesions reported, 80% are DCIS.[12,13] Nevertheless, the incidence of mortality from early stage breast cancer has not decreased concurrently with DCIS detection and treatment, indicating that managing DCIS does not reduce breast cancer-specific mortality and therefore could be considered as overtreatment.[8,11] A review of autopsies in women of all ages revealed a median prevalence of 8.9% (range 0-14.7%). For woman over 40 years of age, this prevalence was 7-39%[14], whereas breast cancer is diagnosed in only 1% of women in the same age range.[13] These data suggest that a large number of women might have an undetected source of DCIS that will never become symptomatic.

Current diagnosis and imaging

DCIS is usually straightforward to detect by mammography because of its association with calcifications; the proliferation of cells itself is not visible on the mammogram. However, as only 75% of all DCIS lesions contain calcifications,[15] a

substantial percentage of DCIS lesions will not be detected by mammography, implying that some lesions might be mammographically occult or that the diameter of the area containing calcifications underestimates the extent of DCIS.[16,17] This suggests that DCIS might be left behind following breast-conserving treatment in a proportion of cases.

After detection, the lesion is classified by the pathologist by histological features as low-, medium- or high-grade, which is assumed to correspond to the level of aggressiveness. Surprisingly, many grading systems exist.[18] An agreement on classification was reached during a consensus meeting in the USA where consensus was reached to include nuclear grade, presence of necrosis, cell polarisation and architectural patterns in the pathology report.[19,20] Some studies showed a slight tendency for high-grade DCIS to progress to invasive breast cancer[21] but others demonstrated that grade is not significantly associated with the risk of local invasive recurrence.[22,23] Greater consistency in grading could result in more certainty about the association of morphology with progression and outcome. In addition, as grade is not a perfect discriminator for progression risk, other risk discriminators, such as molecular biomarkers, are examined (discussed alter in 'Molecular, cellular and microenvironmental aspects').

Perception of risk

Generally, patients diagnosed with DCIS have an excellent long-term breast-cancer-specific survival of around 98% after 10 years of follow-up,[24–27] and a normal life expectancy.[27] However, a consensus in the medical community is lacking on how to effectively communicate to patients about DCIS and the associated risk of development into invasive cancer.[28] It is essential to be aware of the fact that if the lower grade DCIS (considered as the lower risk lesions) progresses into invasive breast cancer, this will often be the lower grade, slow-growing and early-detectable invasive disease, with excellent prognosis.

Because both diagnosis and treatment of the condition can have a profound psychosocial impact on a woman's life, adequate perception of risk by both health professionals and patients is important in determining the appropriate modalities of treatment. Despite an excellent prognosis and normal life-expectancy, women diagnosed with DCIS experience stress and anxiety.[29] Studies report that most women with DCIS (and early stage breast cancer) have little knowledge and inaccurate perceptions of the risk of disease progression, and this misperception is associated with psychological distress.[30–36] Women with DCIS make substantial changes to their behaviour after diagnosis, including smoking cessation and decreasing the use of postmenopausal hormones.[37]

Similar to progression rates for DCIS, classic lobular carcinoma in situ (LCIS) confers a risk of 1–2% per year to develop into invasive disease.[38,39] First-line treatment for LCIS usually comprises active surveillance; unlike DCIS, doctors and patients accept the concept of active surveillance to monitor for progression of LCIS before administering any aggressive treatment. The need for effective doctor–patient communication is therefore essential for patients to understand the risk of recurrence.[40,41] According to Kim *et al.*[36], women in whom DCIS was detected experienced high decisional conflict in treatment options and were not satisfied with the information provided to them. The development of a prediction tool could help to classify patients into risk groups and provide accurate guidance to patients, as well as healthcare professionals, in their choice of an appropriate treatment option.[42] Nowadays, such a tool is even more important, as patients increasingly wish to engage in shared decision making about their disease.

Treatment of DCIS

Surgery and radiation therapy

Currently, breast-conserving treatment for DCIS is frequently recommended. A mastectomy is advised if the DCIS is too extensive to allow breast conservation.[43] According to Thompson *et al.*[21], the recurrence rates (for both invasive and in situ) with 5 years median follow-up are 0.8% after mastectomy, 4.1% after breast-conserving surgery followed by radiotherapy and 7.2% after breast-conserving surgery alone. According to Elshof *et al.*[22], invasive recurrence rates are 1.9%, 8.8%, and 15.4% respectively, after 10 years median follow-up. The 15-year cumulative incidence in the National Surgical Adjuvant Breast and Bowel Project (NSABP)17-trial of patients with clear margins is 19.4% after breast-conserving surgery alone and 8.9% after breast-conserving surgery followed by radiotherapy.[44] Four randomised clinical trials have been performed to investigate the role of radiotherapy in breast-conserving treatment for DCIS after complete local excision of the lesion. In a meta-analysis, these trials show a 50% reduction in the risk of local recurrences (for both in situ and invasive) after radiotherapy.[45] Radiotherapy was reported to be effective in reducing the risk of local recurrence in all analysed subgroups according to age, clinical presentation, grade and type of DCIS.

Adding radiotherapy to breast-conserving treatment reduces local recurrence rates, but does not influence overall survival or breast-cancer-specific survival.[27,45,46] The added value of conducting a sentinel node biopsy procedure is uncertain. In general, such a procedure is done with mastectomy for DCIS (since there is not the opportunity to perform a subsequent sentinel node biopsy) or where there is a high suspicion for invasive disease even where DCIS alone is present in the preoperative biopsy.[47,48]

A recent study based on an analysis of data from the American Cancer Registry of more than 100,000 women diagnosed with DCIS suggests that aggressive treatment might not be necessary to save lives.[24,49] A retrospective Surveillance, Epidemiology, and End Results (SEER) study demonstrated for the first time that patients with low-grade DCIS had the same overall survival and breast-cancer-specific survival rates with or without surgery.[49] These findings prompted the breast healthcare community to explore innovative studies that could circumvent the need for harsh therapeutic intervention for treating an indolent condition.[24,49]

Endocrine therapy

Due to the side effects of hormonal therapy and ambiguous results from clinical trials, postmenopausal women with DCIS are rarely treated with endocrine therapy in many countries. In addition, the notion of systemic treatment for a localised disease with an excellent outcome is perceived as being counterintuitive.[21,50] Two randomised clinical trials have investigated the role of tamoxifen – a drug that inhibits the oestrogen receptor (ER) – versus placebo in DCIS.[44,51] The risk of subsequent invasive ipsilateral breast cancer was found to be reduced by tamoxifen in the NSABP trial[44]; the UK, Australia, and New Zealand (UK/ANZ) DCIS trial demonstrated a reduction in recurrent DCIS but not in invasive breast cancer.[51] Tamoxifen administration did not influence overall survival in either trial[52] and appeared to be more effective at reducing the incidence of new breast events in patients who did not receive radiotherapy in the NSABP trial.[51] Yet, a non-significant reduction in the incidence of new breast events was seen in the prospective series from the UK, independent of whether the patients received radiotherapy or not.[53] Furthermore, to prevent one recurrence, 15 patients would need to be treated (the number needed to treat).[52] In terms of efficacy, tamoxifen and anastrozole (an aromatase inhibitor) are comparable, and the percentage of women who reported side effects were 91% and 93% for anastrozole and tamoxifen, respectively. Although anastrozole administration more often causes side effects such as musculoskeletal pain, hypercholesterolemia and strokes, tamoxifen is associated with muscle spasm, deep vein thrombosis and the development of gynaecological symptoms and gynaecological cancers.[54] In the USA, the uptake of endocrine treatment is higher than in other countries, nearly half of all ER positive patients are treated by additional adjuvant tamoxifen treatment, indicating a lack of consensus on the added value of this treatment.[55]

Active surveillance

To address the question whether some patients with DCIS are overtreated, a group of patients not treated with conventional therapies should be studied. A prospective study with long-term follow-up is the only way to gain confidence regarding the natural course of DCIS and therefore the potential need for interventions. Recently, three clinical trials (LORIS (United Kingdom, NCT02766881)[56], COMET (United States,

NCT02926911)[57,58] and LORD (The Netherlands, NCT02492607))[59] have opened to randomise patients with low risk DCIS between active surveillance and standard treatment. Lower grades of DCIS are enrolled (grade 1 and/or grade 2 with limitations depending on the trial). Patients receive annual mammography (in COMET biannual mammography) in the active surveillance arm to monitor the lesions. Patients in the control arm will get conventional treatment (surgery often supplemented with radiotherapy). The primary outcome assesses whether active surveillance is non-inferior to surgery in terms of ipsilateral invasive breast cancer free survival[56] (LORIS), ipsilateral invasive breast cancer free percentage at 2 years (COMET)[57] or at 10 years (LORD).[59] Because the primary outcomes of the trials are based on the occurrence of invasive disease during follow up, it is essential to exclude an invasive component at the time of enrolment. Missed invasive disease at DCIS diagnosis is reported up to 26%.[60] However, Grimm *et al.* found that among trial eligible patients, there was upstaging of 6%, 7% and 10% for COMET, LORIS and LORD trials respectively, compared to a general upstaging of 17% at the time of surgery for preoperatively diagnosed DCIS of all types.[61] All trials include only pure DCIS with the use of multiple biopsies, additional biopsies in extended lesions, and vacuum-assisted (large volume) biopsies.

From DCIS to invasive breast cancer

Proposed mechanisms for the development of invasive breast cancer

Although the natural course of the intraductal process is unknown, DCIS is considered to be a non-obligate precursor of invasive breast cancer. Four evolutionary models have been proposed to describe the progression of DCIS into invasive breast cancer (Figure 1).

The first model is the **independent lineage model**. On the basis of mathematical simulations of the observed frequencies of the histological grade of DCIS and the histological grade of invasive disease in the same biopsy sample, Sontag *et al.* proposed that *in situ* and invasive cell populations arise from different cell lineages and develop in parallel and independently of each other.[62–64] In support of this theory, Narod *et al.*[65] state that small clusters of cancer cells with metastatic ability spread concomitantly through various routes to different organs and can therefore give rise to DCIS, invasive breast cancer, and metastatic deposits simultaneously. Recent studies elucidating molecular differences between DCIS and invasive breast cancer further support the relevance of this model.[66]

The **convergent phenotype** model proposes that different genotypes of DCIS could lead to invasive breast cancer of the same phenotype. Furthermore, this model assumes that all the cells within the DCIS duct have the same genetic aberrations, but that the combination of aberrations could differ between ducts (within the same

DCIS lesion).[67,68] Hernandez *et al.* demonstrated similarity in the genomic profiles of DCIS and invasive breast cancer in the majority of the matched pairs. However, in some cases, DCIS and adjacent invasive breast cancer differ in copy number and gene mutations, supporting the notion that, at least in some cases, progression is driven by specific clones leading to the same phenotype.[69]

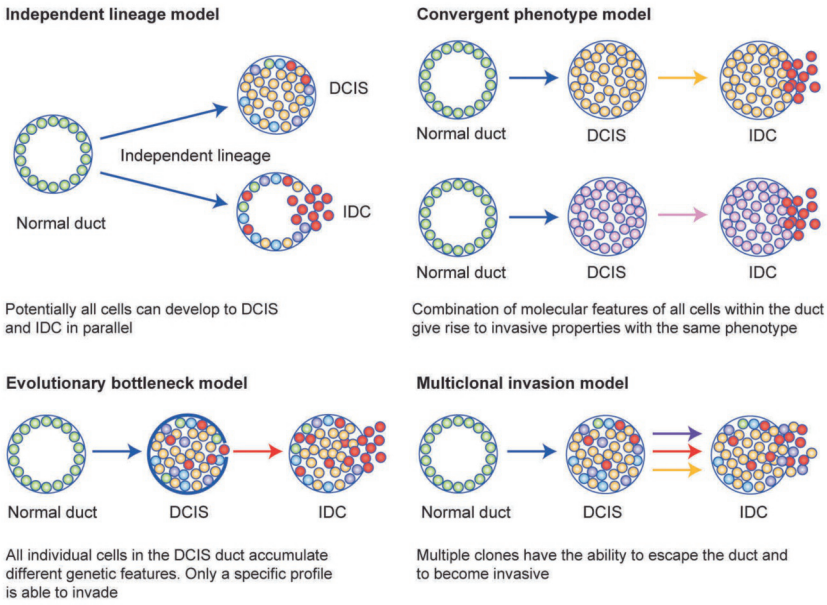


Figure 1. Overview of models showing four different theories of progression from DCIS to invasive breast cancer.

In the **evolutionary bottleneck model,** individual cells within a duct are considered to accumulate different genetic aberrations; however, only a subpopulation of cells with a specific genetic profile is able to overcome an evolutionary bottleneck and invade into the adjacent tissue.[63,64,68] This bottleneck model is supported by studies that report high genetic concordance between *in situ* and invasive lesions in addition to some differences between DCIS and invasive disease.[70]

In the **multiclonal invasion model**, multiple clones have the ability to escape from the ducts and co-migrate into the adjacent tissues to establish invasive carcinomas [63,64] Casasent *et al.* demonstrated, using single-cell sequencing, that most mutations and copy number aberrations evolved within the ducts prior to the process of invasion. Shifts in clonal frequencies were observed, suggesting that some genotypes are more invasive than others. The same subclones were present in both *in situ* and in invasive regions with no additional copy number aberrations acquired during invasion and few invasion specific mutations. These findings are,

however, limited by their small sample size and comparison of contemporaneous DCIS and invasive disease.[63]

These putative models illustrate the potential complexity of the invasion process in DCIS and indicate that indolent lesions might become invasive via a combination of more than one of the proposed mechanisms.[6]

Molecular, cellular and microenvironmental aspects

Many studies have focused on identifying molecular markers of the invasive process and recent studies[69–72] have linked mutations in *PIK3CA*, *TP53* and *GATA3* genes with aggressive DCIS; *TP53* mutations were reported to be exclusively associated with high-grade DCIS.[71,72] However, the requirement for fresh tissue and large amounts of DNA for whole exome- or genome sequencing has limited the extent of studies for determining the landscape of genetic mutations in DCIS.

Some molecular analyses have shown that pre-invasive lesions and invasive breast cancer display remarkably similar patterns,[73–76] indicating a common ancestor;[77] other groups have found that progression from DCIS to invasive breast cancer might be driven by a subset of cells with specific genetic aberrations, implying contribution to tumour initiation.[66,77–80] PAM50 is a gene signature that can classify invasive breast cancer into five intrinsic subtypes (luminal A, luminal B, HER2-enriched, basal- like and normal-like), which adds prognostic and predictive information.[81] Lesurf *et al.*[74]. applied the PAM50 signatures to DCIS, and showed substantial differences between the subtypes, indicating that each PAM50 subtype undergoes a distinct evolutionary course of disease progression. Strikingly, their results showed that these properties, specific for the PAM50 subtypes, reflect changes that involve the microenvironment rather than molecular changes specific for epithelial cells. This supports increasing evidence for the role of the microenvironment in tumour progression and disease outcome more generally.[74] Alcazar *et al.*[82] demonstrated a switch to a less active tumour immune environment during the *in situ* to invasive breast carcinoma transition and identified immune regulators and genomic alterations that shape tumour evolution. Their data suggest that the levels of activated CD8+ T cells might predict which DCIS is likely to progress to invasive disease.[82] In patients with invasive breast cancer — particularly those with triple-negative and HER2 positive subtypes — the presence of tumour-infiltrating lymphocytes (TILs), especially higher numbers of CD8+ cells, together with fewer FOXP3+ regulatory T cells, is associated with a better outcome.[83]

One of the key molecular differences between DCIS and invasive breast cancer is the prevalence of HER2 amplification: 34% for DCIS[84] versus 13% for invasive disease.[85] HER2 amplification might be a prognostic factor in predicting an *in situ* recurrence after DCIS, but it seems not to be predictive for an invasive

recurrence.[86] That said, one study with a long follow-up (mean follow-up > 15 years) counterintuitively demonstrated that HER2 positivity in primary DCIS was associated with a lower risk of late invasive breast cancer compared with HER2 negativity.[87] In HER2 positive DCIS, TILs are present at higher levels, but an association with an invasive recurrence risk after DCIS has not been reported.

A caveat of molecular studies on DCIS is the fact that most studies examine relatively small series of DCIS lesions with a contemporaneously adjacent invasive component, instead of a metachronous (subsequent) invasive lesion developing during follow up. Thus, these series are inherently biased, because the majority of the DCIS lesions will never develop an invasive component. In addition, most studies do not distinguish between in situ or invasive recurrences after DCIS. Two biomarker-based assays have been developed for DCIS,[88,89] which purport to predict the benefit of radiotherapy for DCIS. However, the assays only discriminate between the risk of an in situ versus an invasive recurrence after DCIS to a limited extent. This difference is important for the women involved, especially regarding treatment choices, prognosis and psychosocial impact. Furthermore, intratumoural heterogeneity complicates our understanding of the relationship between DCIS and its invasive counterpart, as most studies only analyse a small proportion of an often heterogeneous lesion, or analyse a bulk tissue sample, in which small cell populations are easily overlooked.[64] The low number of samples and lack of longitudinal follow-up data mean that our overall molecular knowledge of the landscape of changes in DCIS is limited.

Looking ahead

Uncertainty exists about how DCIS develops and global consensus is lacking as to how best to optimally manage this disease. A better understanding of the biology of DCIS and the natural course of the disease is required to support patients and healthcare professionals in making more informed treatment decisions, in turn reducing the current overtreatment of DCIS. In 2014, Gierisch *et al.*[90] described and prioritised knowledge gaps of patients and decision makers with regards to future research of DCIS for the Patient-Centered Outcomes Research Institute (PCORI), a private, nongovernmental, nonprofit, US-based institute created by The Patient Protection and Affordable Care Act of 2010 to 'help people make informed healthcare decisions, and improve healthcare delivery and outcomes'. By reviewing the existing literature and using a forced-ranking prioritisation method, a list of ten evidence gaps was created (Table 1). Issues that needed immediate attention include the effective communication of information about diagnosis and prognosis, and dedicated efforts to fill the knowledge gaps regarding long-term implications and risks of a diagnosis of DCIS.[90]

Table 1. How PRECISION addresses research needs for DCIS management (adapted from Gierisch et al., Ann Intern Med (2014) 160: 836-841)

Rank	Prioritization of research need according to Gierisch et al.	Recommended study design by Gierisch et al.	Addressed in Precision
1	Validate risk-stratification models	Meta-analysis or individual patient data analysis across RCTs or observational study using existing data sources	Combining retrospective case-control studies based on nation-wide, population-based cohorts
2	Compare safety and effectiveness of a management strategy involving no immediate treatment (i.e. monitoring/observation/active surveillance) vs. immediate treatment with surgery, RT, and/or medical therapy	Prospective observational study	Prospective RCT to test safety of active surveillance for low grade DCIS
3	Determine whether safety and effectiveness of DCIS management strategies differ depending on variations in clinical, pathologic, and genomic presentations of DCIS	Meta-analysis or individual patient data analysis across RCTs or observational study using existing data sources	Combining results from retrospective case-control studies and prospective RCTs
4	Comparative effectiveness of different approaches to communicating the diagnosis of DCIS to the patient	RCT	Evaluation level of being informed, QoL and HTA in prospective RCTs
5	Comparative effectiveness of decision-making tools compared with usual care.	RCT	Evaluation of prognostic factors, QoL and HTA in prospective RCTs
6	Comparative sensitivity and specificity of breast MRI, mammography, and other preoperative imaging evaluations for detecting occult invasive breast cancer	Observational study either collecting new data or using existing data sources	Analysis based on mammograms collected in prospective RCTs
7	Assess effect of DCIS management strategies on comorbid conditions	RCT	Prospective RCTs
8	Compare safety and effectiveness of partial-breast RT vs. whole-breast RT	RCT	Not addressed in this research proposed
9	Identify most important patient-centered outcomes for women diagnosed with DCIS	Observational study requiring new data collection	Prospective RCT for patient-centered outcomes
10	Assess effect of DCIS management strategies on rates of invasive cancer	Observational data using existing data	Retrospective case-control studies and prospective RCTs

RT denotes radiotherapy; RCT randomised controlled trial; QoL quality of life; HTA health technology assessment.

To address these priorities in DCIS, a multidisciplinary approach with scientific, clinical and patient expertise is needed. Data from large retrospective cohorts should be integrated with in vitro and in vivo studies and the results should be validated to transform clinical practise. To fund such a large multinational consortium, Cancer Research UK and the Dutch Cancer Society (KWF) partnered to support the Grand Challenge[91] award in 2017, the PREvent ductal Carcinoma In Situ Invasive Overtreatment Now (PRECISION) initiative (see Textbox 2 and Supplementary Material for more information about PRECISION).

Conclusion

Current perceptions of the risk-framing dialogue between clinicians and women diagnosed with DCIS are currently resulting in the overdiagnosis and overtreatment of DCIS. The need to reframe perceptions of risk and to avoid overtreatment is urgent, as overtreatment leads to physical and emotional harm for patients and to unnecessary costs for society. Specifically, knowing when a lesion could be or will not be life-threatening requires a thorough understanding of the progression and evolution of DCIS. To this end, initiatives, such as PRECISION, have been set out to reduce the burden of overtreatment of DCIS by gaining deep knowledge about the biology of DCIS. This knowledge will contribute to informed decision-making between patients and clinicians, without compromising the excellent outcomes for DCIS that are presently achieved. Dealing with this challenge demands an integrated approach that blends clinical and patient insights with scientific advances in order to improve the diagnosis, treatment and management of DCIS. To accomplish this, it is critical that patient advocates, scientists and clinicians work together, exemplified by a collaborative patient advocate and scientist in the PRECISION research team video**:** https://youtu.be/aoGSDDto1Gc

Textbox 2. The PRECISION initiative

The general aim of the CRUK/KWF Grand Challenge PRECISION Initiative (www.dcisprecision.org) is to prevent the burden of DCIS overtreatment. 'PRECISION' is the acronym for 'PREvent ductal Carinoma In Situ Invasive Overtreatment Now'. PRECISION ultimately aims to develop novel tests that promote informed and shared decision-making between patients and clinicians, without comprising the excellent outcomes for DCIS management that are presently achieved. The PRECISION initiative consists of seven interlinked work packages (WP). WP1 enables the collection of large tissue resources. These series will be used in WP 2-4 for genomic characterisation to find key drivers (WP2), characterising the function of the microenvironment in DCIS biology (WP3), and the role of imaging in DCIS prognosis and outcome (WP4). WP5 comprises functional validation of the key drivers in in vitro and in vivo models and WP6 will incorporate all the information obtained in a clinical risk prediction model. The three prospective studies will be used for overall validation through collection of blood and tissue samples (WP7). Importantly, patient advocates are actively involved in every part of the project. Ultimately, all these efforts may contribute to a more balanced perception of risk regarding non-life-threatening precancerous lesions in general, reducing anxiety and preserving quality of life.

Additional information

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of Data and Materials

Not applicable

Conflict of Interest

The authors declare no conflict of interests.

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Author contributions

EL, JW, JL and AT designed and wrote the manuscript. MS contributed to the revision and drafted figure 1. AT,SNZ,AF,ESH,EV,JJ,DR, revised the sections in their expertise. JW supervised and finalized the manuscript. All authors reviewed the manuscript and approved the final version.

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Supplementary files

PREvent ductal Carcinoma In Situ Invasive Overtreatment Now (PRECISION) initiative

In 2015, we commenced the PREvent ductal Carcinoma In Situ Invasive Overtreatment Now (PRECISION) initiative by applying to the Cancer Research UK Grand Challenge theme 'When is cancer not really cancer'. We brought together a complementary team of scientific, clinical and patient expertise needed to change clinical practice regarding the diagnosis and management of DCIS, to ignite new ideas and to hasten the translation of science to the clinical setting. Through this multidisciplinary approach we are aiming to address 9 out of the top 10 priorities in DCIS research as outlined by Gierisch *et al.*[90] (Table 1). In 2017, the PRECISION initiative was awarded £15 million to distinguish harmless from hazardous DCIS. The PRECISION initiative consists of seven interlinked work packages and four supporting working groups, as outlined in Supplementary Figure 1.

Curation of DCIS cohorts (Work Package (WP1): The PRECISION project enables the collection of large tissue resources from both retrospective and prospective clinical trials. Supplementary Table 1 gives an overview of the cohorts which will form the basis of the PRECISION effort. Clinical data, including long-time follow up, tissue blocks and imaging data obtained through pooling multiple retrospective clinical studies based in the USA, UK and the Netherlands, are available for an in-depth characterisation of indolent and aggressive DCIS. These series will be used in WP2, 3 and 4 to gain a deep and thorough understanding of DCIS. Most importantly, to address the heterogeneity issue in DCIS, the studies contain large datasets, collected in different settings (population/hospital based and screening setting) and capture different populations.

Comprehensive genomic characterisation of DCIS (WP2): To determine whether a lesion is life-threatening, a clear understanding of the biology of DCIS is required to identify the critical drivers of DCIS evolution and progression to invasive disease. To identify putative novel drivers, whole genome and whole exome sequencing are performed to identify the mutation spectrum, the sequence of each gene, the impact of coding substitutions (synonymous, missense, nonsense, splice site) and the variation of the mutation rate across genes. The landscape of base substitution mutational signatures in DCIS will be assessed and compared to what is already known for invasive breast cancer. A bank of genomic data is being created for future analysis. A key feature of our genomic studies is to capture both the interpatient and intratumoural heterogeneity. The first is addressed by profiling large sets of samples from various studies (see WP1). The latter is addressed by multiregim sequencing and single cell studies.

Supplementary Table 1. Sources for DCIS cohort for tissue collection and prospective DCIS trial for watchful waiting.

Retrospective series				
Series	**Description**	**Size (total n/ recurrences)**	**Median follow up (year)**	**Ref.**
Dutch DCIS cohort	population based cohort 1989-2004	10,096/ 1,200	11.6	Elshof et al, 2016[22]
Sloane project	UK NHS Breast Screening Prospective cohort	13,000/800	9.2	Thompson et al, 2018[21]
MDAnderson series	US MD Anderson retrospective cohort 2003-2015	2.500/200	5	NA
Duke series	US Duke retrospective cohort	658/NA	5	NA
Prospective clinical trials				
Trial acronym	**Description**	**Size (target number of participants)**	**Main outcome**	**Ref.**
LORIS	A trial comparing surgery with active monitoring for low risk DCIS (LORIS)	900	Ipsilateral invasive BC-free rate at 10 years	Francis et al, 2015[56] NCT02766881
COMET	Comparison of Operative to Monitoring and Endocrine Therapy (COMET) Trial For Low Risk DCIS (COMET)	1200	Proportion of new diagnoses of ipsilateral invasive cancer in GCC and AS arms at 2 years of follow up	NCT02926911[57,58]
LORD	Low risk DCIS study	1240	Ipsilateral invasive BC-free rate at 10 years	Elshof et al, 2015[59] NCT02492607

Characterising the role of the immune microenvironment as a determinant of DCIS clinical biology (WP3): The main hypothesis being explored is that features of the immune microenvironment are key in determining the risk of DCIS progression. The immune microenvironment of DCIS are profiled by multiplex immunohistochemistry and immunofluorescence assays. Furthermore, T-cell receptor clonality and neo-antigen prediction will be investigated.

Investigating the role of imaging in DCIS prognosis and outcome (WP4): The overarching goal is to identify a series of systematic differences in the radiographic and physicochemical characteristics of lethal versus non-lethal cancers captured on images to DCIS biology. The first objective is to develop novel molecular mapping approaches to quantitatively characterise DCIS tissue using mid-infrared absorption and Raman spectroscopic imaging of soft tissue and calcifications. The second objective is to create and test a computational learning algorithm to compare mammographic characteristics and diversity measures in pure DCIS compared to DCIS with IDC. The third objective is to validate the algorithm in PRECISION's retrospective cohorts (WP1) and in data obtained from the prospective clinical trials (see below; WP7).

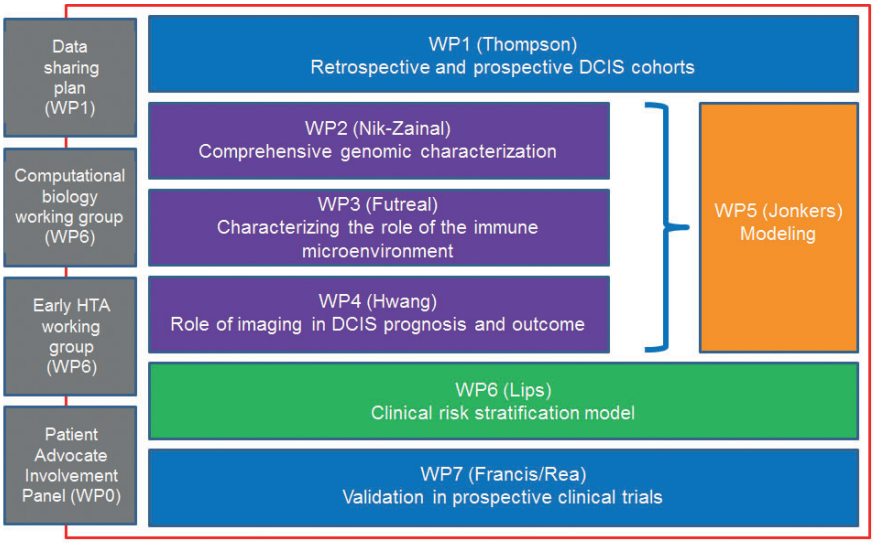
Functional validation of DCIS drivers (WP5): The aim of this WP entails the functional validation of candidate DCIS genes from the comprehensive genomic characterisation of DCIS samples. To critically assess the functional relevance, reliable animal models are essential. Recent advances in CRISPR/Cas9-based somatic gene editing, three-dimensional (3D) organoid culturing and patient-derived tumour xenografting have resulted in a number of novel approaches that can be applied to *in vitro/in vivo* validation of candidate DCIS genes and to *in vitro/in vivo* propagation of viable DCIS samples from patients.[94–99]

Building a clinical risk stratification model (WP6): All information obtained from the previous steps will be collated to develop a DCIS risk prediction model, integrating all clinical, morphological, molecular and imaging data. The most promising molecular markers will be combined in an easy-to-use clinical assay. The risk prediction model and clinical assay will be validated in the prospective clinical trials (LORIS, COMET and LORD, see below in next section).

Validation of molecular markers in active surveillance using the LORIS, LORD and COMET trials (WP7): The LORIS (United Kingdom, NCT02766881)[56], COMET (United States, NCT02926911)[57] and LORD (The Netherlands, NCT02492607)[59] randomised trials together present a unique opportunity. They have a common aim of assessing which low or intermediate grade DCIS requires primary surgical management and whether regular monitoring for disease progression by mammography can be safely performed, with intervention only in those women in whom there

is evidence of progression to high-risk DCIS or invasive cancer. The common research objectives of the three trials are to evaluate the safety, effectiveness, cost effectiveness and acceptability of non-surgical intervention in patients with newly diagnosed, mammogram detected asymptomatic, low or low-intermediate grade DCIS; and to define the natural history of low-risk DCIS and to identify those patients who require surgery because their DCIS is at risk of progression to invasive disease.

All the above trials are prospectively randomising patients with screen-detected or incidental low risk of recurrence DCIS to standard surgical treatment or active monitoring. The trials had started before the inception of the PRECISION initiative, but the initiative gave us a unique opportunity to collaborate, and to safeguard tissue and blood collections for translational biomarker research. LORD, LORIS and COMET are recruiting and expect to complete recruitment within 5 years. In addition to collaborating and exchanging valuable information regarding accrual and patient participation, we plan to assess the value of circulating tumour DNA and genomics approaches in blood and tissue samples of trial participants.



Supplementary Figure 1. Overview and links to the different work packages (WP) within the PRECISION project

Patient involvement

International expert patient advocates with previous experience of DCIS, cancer or another condition are centrally involved in PRECISION. Their contributions are at three levels: project governance; scientific work; and outreach to the general public,

patients and physicians, which helps to reshape clinical practice and the public perception of DCIS risk. The scope and depth of patient involvement is possible because all patient advocates are highly experienced and knowledgeable about the attitudes of their respective countries toward DCIS. Each patient advocate adds professional patient expertise combined with personal experience to the science that will be conducted in PRECISION. They are also directly involved with the prospective LORD, LORIS, and COMET trials in each country, and can relate to the long-term quality of life issues that women face from current DCIS treatments. Their goal is to replace fear of DCIS with confidence that each woman will receive effective, evidence-based treatment (or monitoring alone) that matches her specific type of DCIS, based on personalized invasive cancer risk that PRECISION will help to elucidate.



Chapter 3

Variability in grading of ductal carcinoma in situ among an international group of pathologists

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ABSTRACT

The prognostic value of cytonuclear grade in ductal carcinoma in situ (DCIS) is debated, partly due to high interobserver variability and by the use of multiple guidelines. The aim of this study was to evaluate the interobserver agreement in grading DCIS between Dutch, British and American pathologists. Hematoxylin and eosin-stained slides of 425 women with primary DCIS were independently reviewed by nine breast pathologists, based in the Netherlands, the UK and the USA. Chance corrected kappa (κ_{ma}) for association between pathologists was calculated based on a generalized linear mixed model using the ordinal package in R. Overall κ_{ma} for grade of DCIS (low, intermediate or high) was estimated as 0.50 (95% confidence interval (CI) 0.44-0.56), indicating a moderate association between pathologists. When the model was adjusted for national guidelines, the association for grade did not change (κ_{ma} = 0.53; 95% CI 0.48-0.57); subgroup analysis for pathologists using the UK pathology guidelines only had significantly higher association (κ_{ma} = 0.58; 95% CI 0.56-0.61). To assess if concordance of grading relates to expression of the estrogen receptor (ER) and HER2, archived immunohistochemistry (IHC) was analysed on a subgroup (n=106). This showed that non-high grade according to the majority opinion was associated with ER-positivity and HER2-negativity (100% and 89% of non-high grade cases, respectively). In conclusion, DCIS grade showed only moderate association using whole slide images scored by nine breast pathologists. Since therapeutic decisions and inclusion in ongoing clinical trials are guided by DCIS grade, there is a pressing need to reduce interobserver variability in grading. ER and HER2 might be supportive to prevent accidental and unwanted inclusion of high grade DCIS in such trials.

Introduction

Ductal carcinoma in situ (DCIS) is a non-obligate precursor of invasive breast cancer (IBC) in which the proliferating epithelial cells remain within the boundaries of the ducto-lobular system of the breast. DCIS is graded by pathologists using a three-tier system: well-differentiated (low nuclear grade, grade 1), intermediately differentiated (intermediate nuclear grade, grade 2), and poorly differentiated (high nuclear grade, grade 3). This histological assessment of grade is prognostic, in terms of subsequent ipsilateral in situ and invasive lesion risk, and is used to guide treatment decisions and to determine eligibility for inclusion in clinical trials. Although different guidelines are used to grade DCIS there seems to be a substantial difference in interpretation (interobserver variability) in grading, even using the same guidelines.[1] Consequently, the prognostic and clinical value of DCIS grade is still a subject of debate.[2–4] There are, however, no other histological features or widely tested biomarkers presently available that can be used to predict reliably the progression of DCIS lesions to IBC[5]. Because of this uncertainty, almost all women with DCIS receive similar treatment to that given for invasive breast cancer: i.e. mastectomy or breast conserving surgery often supplemented by radiotherapy and/or endocrine therapy.

To investigate how to distinguish indolent from potentially hazardous DCIS and to be able to stratify DCIS based on risk of progression to invasive disease, we established the international PREvent ductal Carcinoma In Situ Invasive Overtreatment Now (PRECISION) initiative[6]. PRECISION synergizes comprehensive prospective and retrospective DCIS studies[2,7], modelling and prospective clinical trials. Three ongoing prospective trials (COMET[8], LORIS[9], and LORD[10]) randomize patients between standard treatment and active surveillance for low risk DCIS. The identification of low risk DCIS based on morphological features is key for accrual into these trials, but also for international collaborations for conducting research studies of DCIS. We embarked on a DCIS interobserver variability study including whole slide digital images of hematoxylin and eosin (H&E) stained sections of DCIS including cohorts from three countries, namely the United States (USA), the United Kingdom (UK) and the Netherlands (NL) that were reviewed by breast pathologists practicing in these three countries. Our primary goal was to evaluate the extent of interobserver variability in DCIS grading between pathologists from the same and from different health care systems. Subsequently, we aimed to assess possible causes for the variability and then address strategies to establish greater uniformity of grading.

Methods

Slide collection

Four institutions, The Netherlands Cancer Institute (NKI; the Netherlands (NL)), Kings College London (KC; UK), MD Anderson Cancer Center (MDACC; USA) and Duke University Medical Center (DUMC; USA), participated in this study and contributed H&E stained whole slides images of tissue sections of DCIS. The cases were selected to represent the distribution of cytonuclear grade of DCIS (according to the pathology report or from previous review) from the participating countries or individual centers (supplementary table 1). The cases originated from the prospective, population-based Sloane DCIS cohort (KCL; UK)[2], the retrospective nation-wide Dutch DCIS cohort[5] and the retrospective, hospital-based DUMC and MDACC cohorts. Whole slides images of one representative H&E-stained section obtained from a formalin fixed paraffin embedded (FFPE) tissue block of a breast surgical resection were scanned at each centre, anonymized and uploaded to the NKI and evaluated using the web-based software platform Slidescore (supplementary table 1).[11] To assess the number of slides that had to be evaluated, power calculations were performed (see section power calculations, Supplementary file).

Histology & pathologists

To recapitulate pathology reporting in daily clinical practice, the breast pathologists interpreted the whole slide images of H&E tissue sections of DCIS without specific study-related guidelines for all evaluated variables (see supplementary table 2 for detailed information about the used diagnostic guidelines). The following histological variables were assessed (see scoring form, supplemental file): presence of DCIS/ atypical ductal hyperplasia (ADH)/ lobular carcinoma in situ (LCIS), DCIS grade (1, 2 or 3), DCIS grade (low or high), dominant histological architecture (comedo/solid, cribriform, (micro)papillary, flat/clinging, other), presence and semi-quantitative frequency of mitosis (sparse, many), lymphocytic infiltrate (absent, subtle, prominent), presence of calcifications (absent or present), presence of periductal fibrosis (absent, subtle, prominent) and presence and type of necrosis (absent, present - comedo, present – focal, present – comedo and focal).
Three breast pathologists from each country (NL, UK and USA) evaluated all the slides independently. The participating pathologists completed a short questionnaire to collect information about their experience and criteria for DCIS grading that they followed in their clinical practice (see supplementary table 3,).

Data analysis & statistics

The primary aim was the extent of variability between the nine pathologists for histological grade of DCIS based on review of the H&E scanned slides in Slidescore. Tissue slides of insufficient quality, as judged by more than 50% of

the participating pathologists for any histological variable were excluded from analysis (n=12).

As each slide was evaluated by each pathologist, generalized linear mixed models (GLMM) for cross-classified data structure were used to calculate kappa values as chance corrected association between pathologists (κ_{ma})[12,13]. κ_{ma} were obtained by taking into account levels of exact concordance, i.e. where pathologists assigned the exact same grade to a slide, and the level of disagreement among pathologists' classifications. κ_{ma} values were interpreted as measurement of agreement using the criteria suggested by Landis & Koch[14], which are based on the interpretation that 0.00 is pure coincidence and 1.00 is perfect agreement: <0.00 as no, 0.00-0.20 as poor to slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial and 0.81-1.00 as almost perfect agreement.

We modelled the histological variables separately and to analyze the influence of the tissue slides' and pathologists' characteristics on each of the histological variables, GLMM were adjusted for guidelines used, experience, country and using dominant or highest grade in case of heterogeneous DCIS as characteristics of the pathologists and origin of the slide (both country and centre) as characteristics of the slides. Since all the pathologists from the same country used the same guidelines (except in the USA; supplementary table 3), including both 'country of pathologists' and 'guidelines' in the same multivariable model resulted in collinearity. We therefore chose to use the 'guidelines' as covariate instead of country to evaluate variation. The different values of κ_{ma} from the different adjusted models were compared to the results of the intercept only models. The ordinal package within the open source software R was used for all the calculations.

Majority opinion and influence of ER, PR and HER2 expression

For each slide the majority opinion classification, defined as the grade given by most of the pathologists, was assigned. When there was no majority opinion (i.e. equal number of pathologists, for example, four pathologists graded 2, four pathologists graded 3 and one pathologists did not complete the form), the slide was assigned as not applicable (NA). The variable 'number of pathologists' was defined as the number of pathologists that make up the majority opinion and reflects the strength of agreement.

To investigate how to decrease interobserver variability, we retrospectively collected information about the status of estrogen receptor (ER), progesterone receptor (PR) and overexpression of HER2 through immunohistochemical stains (IHC) obtained from whole slides from the NKI, and the ER and PR status of the DUMC whole slides. MDACC had no IHC data available and KCL assessed biomarker IHC on tissue microarrays (TMAs) and was therefore excluded. For the IHC evaluated in NKI,

≥10% ER, ≥10% PR and ≥10% strong membrane expression of HER2 was considered positive, for 2+ HER2 expression (equivocal) silver in situ hybridization (SISH) was performed. The IHC from USA (DUMC) was examined by the Allred method[15] and a score of >2 was considered as positive. See supplementary table 4 for more details about the scoring details, antibodies used and IHC staining procedures.

Results

Cohort information & slide collection

In total, 425 slides were provided by the participating centers (110 by NKI, KCL, and DUMC and 95 by MDACC). All slides were independently evaluated by the international group of nine breast pathologists. Twelve of the 425 slides (2.8%) were excluded from all analyses based on quality issues, as noted by the majority of the participating pathologists. For the histological variables of grade and mitoses, two and five additional cases, respectively, were excluded based on quality issues. supplementary table 3 (Supplementary file) shows both the characteristics of the included cases, and of the participating pathologists.

Differences between pathologists

Figure 1A demonstrates both the individual evaluation and the majority opinion of grading as low (grade 1), intermediate (grade 2) and high (grade 3) per pathologist. It demonstrates substantial variability in grading the same lesion (see supplementary figure 1 for histological examples of concordant and discordant slides). In addition, some pathologists had a tendency for lower grading, while others had a tendency for higher grading; variability diminished only slightly when grade 1 and 2 were grouped together (Figure 1B).

Associations measure between pathologists, κ_{ma}

According to the GLMM model, the probability that an individual H&E section of DCIS was classified into grade 1, 2 or 3 was 8%, 44% and 48%, respectively. The model-based chance corrected measure of association, κ_{ma}, was estimated as 0.50 (95% confidence Interval (CI) 0.44-0.56, table 1), indicating moderate association between the nine pathologists. For dichotomized grade 1 and 2 vs 3, the κ_{ma} also indicated moderate association (0.51; 95% CI 0.43-0.59). When the pathologists had to select between low or high grade as a binary grading system for all cases, the κ_{ma} was 0.52 (95% CI 0.45-0.59). The highest association was achieved for the category of dominant architectural pattern with κ_{ma} of 0.61 (95% CI 0.57-0.64, table 1), indicating substantial association.

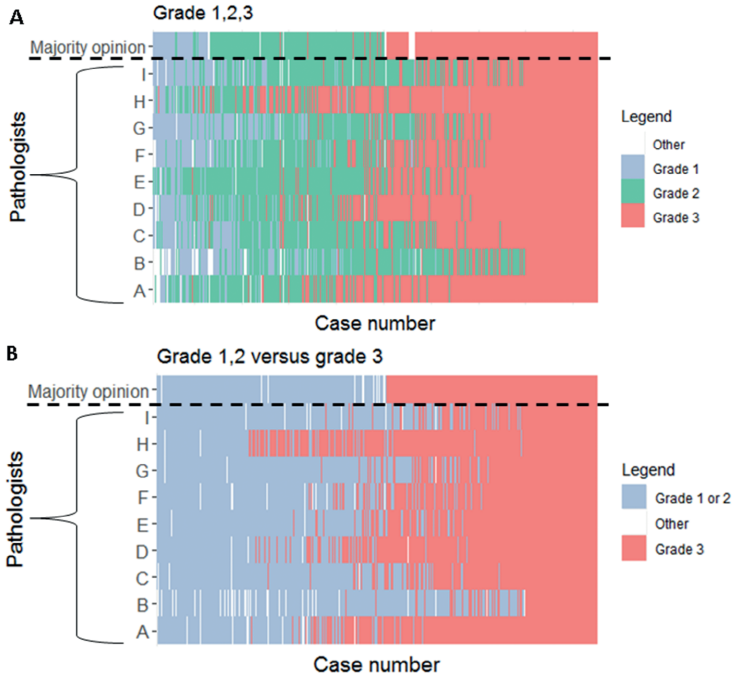


Figure 1. DCIS grades by pathologist (y-axis) and by case (x-axis). The upper row reflects the majority opinion. A) for grade 1 or 2 or 3. B) for grade 1 or 2 vs 3.

Table 1. Model-based measure of association (κ_{ma}) for histological variables.

Histological variable	Model based weighted kappa (κ_{ma})	95% CI
DCIS grade 1, 2, 3 (n=411; intercept only model)	0.50	0.44 - 0.56
DCIS grade 1 and 2 vs 3 (n=411)	0.51	0.43 - 0.59
DCIS grade 1 vs 2 and 3 (n=411)	0.45	0.41 - 0.50
DCIS grade as binary, low vs high (n=411)	0.52	0.45 - 0.59
Necrosis; absent vs present (n=413, manually dichotomized)	0.55	0.51 - 0.59
Calcifications; absent vs present (n=413)	0.51	0.48 - 0.55
Lymphocytic infiltrate; absent vs subtle vs prominent (n=413)	0.47	0.38 - 0.55
Periductal fibrosis; absent vs subtle vs prominent (n=413)	0.35	0.03 - 0.31
Mitoses; sparse vs many (n=408)	0.33	0.24 - 0.42
Architectural pattern; solid and comedo vs cribriform, flat and (micro)papillary (n=413)	0.61	0.58 - 0.64

DCIS grade 1 denotes low grade, 2 intermediate grade, 3 high grade

When incorporating guidelines used as covariate on the pathologist level, the κ_{ma} in the univariable GLMM model for DCIS grade did not change in comparison to the intercept only model (κ_{ma} =0.53; 95% CI 0.48-0.57; p=0.52, table 2). We aimed to investigate whether the κ_{ma} improved when we only included pathologists using the same guideline into the GLMM model. A minimum of three observers was necessary enabling us to analyze the UK and WHO guidelines. Pathologists utilizing the UK pathology guideline had better association between each other (κ_{ma} 0.58, 95% CI 0.56- 0.61) compared to pathologists using the WHO guidance, which showed a κ_{ma} of 0.48 (95% CI 0.36-0.61; p= 0.80), and a model including use of UK pathology guideline shows better association between pathologists compared to the standard model (p=0.02).

For DCIS cytonuclear grading, the associations between pathologists did not change when the following covariates were separately added to the model on pathologist and case level: pathologist's experience (κ_{ma}=0.50; 95% CI 0.44-0.57), country of the pathologist (κ_{ma}=0.51; 95% CI 0.44-0.57) and country of origin of the case (κ_{ma}= 0.49; 95% CI 0.42-0.55). When the model was adjusted for additional histological variables separately, the κ_{ma} for DCIS nuclear grade did not improve (table 2). Multivariable modelling including the variables characterizing the pathologists (i.e. use of guidelines, experience and manner of reporting cases of heterogeneous DCIS) showed an increased but not statistically improved κ_{ma} of 0.57 (95% CI 0.55-0.60; p=0.06). When the model was adjusted for all other histological variables together, the reproducibility for DCIS grading decreased (κ_{ma} = 0.31; 95% CI 0.26-0.36, table 2).

Majority opinion and influence of ER and HER2 expression

Grade 3 DCIS showed less variability than grade 1 or grade 2 disease: 62% of lesions were scored by eight or nine pathologists as grade 3 (see figure 2). We then explored whether ER and/or HER2 expression could help in the identification of grade 3 (high grade) lesions (see figure 3 and supplementary table 5). Figure 3, representing only NKI cases (n=106), shows that lesions categorized as grade 1 DCIS by the majority opinion were all ER positive and HER2 negative, those categorized as grade 2 were predominantly ER positive (100%) and HER2 negative (88%). Grade 3 DCIS cases, determined by the majority opinion, were heterogeneous for ER and HER2 expression, with both positive and negative cases represented. We were able to validate the results of ER expression in the IHC data from DUMC (USA) (see Supplementary table 5); none of the low grade cases of DCIS according to majority opinion were ER negative.

Table 2. Univariable and multivariable analysis with pathological and histological features as covariates to determine the influence on DCIS grade (1 or 2 or 3).

Variable	Model based weighted kappa (κ_{ma})	95% CI	P-value for kappa comparison with the outcome only
DCIS grade 1, 2 or 3	0.50	0.44 - 0.56	
Univariable analysis – adjusted for features of the pathologists			
Experience	0.50	0.44 - 0.57	0.95
Country of pathologist	0.51	0.44 - 0.57	0.91
Heterogeneous DCIS; highest vs most prominent vs other	0.53	0.48 - 0.57	0.54
Guideline used	0.53	0.48 - 0.57	0.52
Split according to guideline used			
1 Consensus Conference	*Only used by one pathologist, not possible*		
2 UK Royal College of Pathologists	0.58	0.56 - 0.61	0.02*
3 College of American Pathologists	*Only used by two pathologists, not possible*		
4 WHO	0.48	0.36 - 0.61	0.80
Univariable analysis - histological features			
Necrosis; absent vs present	0.45	0.39 - 0.52	0.31
Calcification; absent vs present	0.50	0.44 - 0.57	0.97
Lymphocytic infiltrate; absent vs subtle vs prominent	0.46	0.41 - 0.52	0.37
Periductal fibrosis; absent vs subtle vs prominent	0.48	0.43 - 0.54	0.72
Mitoses; sparse vs many	0.46	0.40 - 0.52	0.40
Architectural pattern; solid and comedo vs. cribriform, flat and (micro)papillary	0.45	0.39 - 0.52	0.33
Multivariable analysis – adjusted for features of the pathologists			
Guidelines + experience + solution to heterogeneity of DCIS	0.57	0.54 - 0.59	0.06
Country + experience + solution to heterogeneity DCIS	0.53	0.49 - 0.58	0.41
Multivariable analysis –adjusted for histological features			
Necrosis + calcification + lymphoid infiltrate + periductal fibrosis + mitosis + architectural pattern	0.31	0.26 - 0.36	<0.01*

* p-value showing a significant effect, i.e. p-value<0.05

DCIS grade 1 denotes low grade, 2 intermediate grade, 3 high grade, WHO world health organization

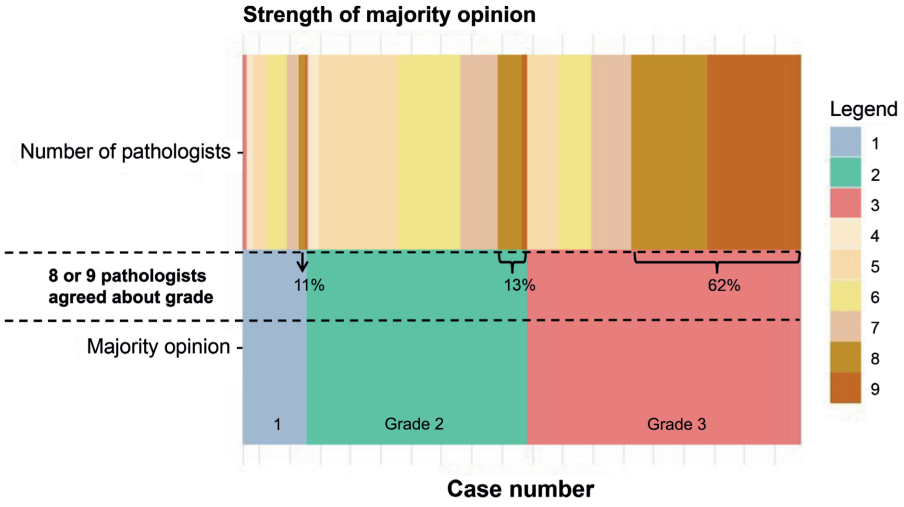


Figure 2. The strength of the majority opinion for low, intermediate and high grade. The bottom row shows the distribution of DCIS grade according to the majority opinion and the upper row the number of pathologists that represent the majority opinion.

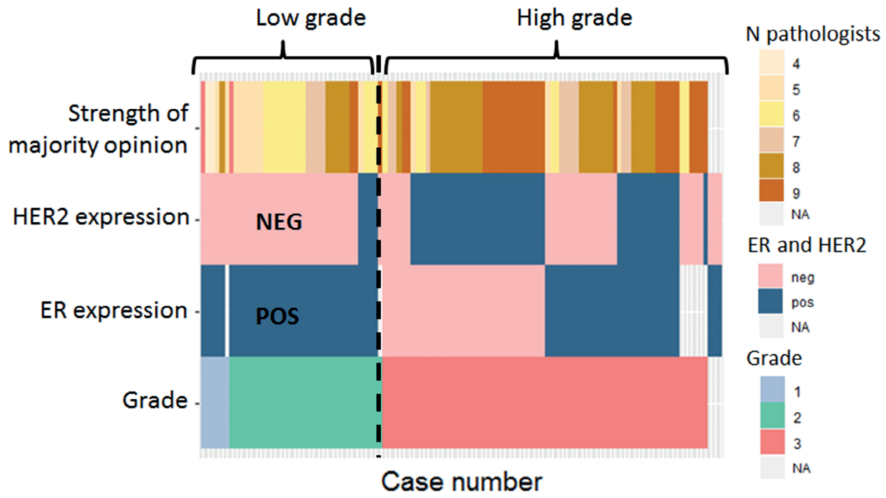


Figure 3. ER and HER2 expression in relation to low (grade 1), intermediate (grade 2) and high (grade 3) grade according to the majority opinion and to the strength of the majority opinion including the NL (NKI)cases (n=110) only.

Discussion

Although reproducibility of the *diagnosis* of DCIS has been demonstrated to have substantial agreement[16], this international study among nine pathologists showed kappa values of 0.5-0.6 for assessment of DCIS *grade*, based on a generalized linear mixed model, indicating only a moderate association between pathologists. Including guidelines as a covariate in to the GLMM model did not improve the association; analyzing the data specifically for the UK pathology guidelines[17] showed a statistically significant improvement in association between pathologists compared to the standard model. Linking the interobserver variability data to immunohistochemical stains demonstrated that almost all non-high grade DCIS lesions according to the majority opinion were ER-positive (100%) and HER2-negative (89%), whereas 55% high grade DCIS were ER-negative and/or HER2-positive (62%). Applying these biomarker stains might be helpful to prevent accidental selection of high grade DCIS, for example in active surveillance protocols.

The significance of cytonuclear grade of DCIS, whilst generally regarded as a predictor of risk of recurrence as subsequent in situ or invasive disease [2,18], is not universally accepted [3,7]. We show here variability in grading DCIS; twenty percent of cases were highly discordant as different pathologists categorized the exact same lesion, on a single identical H&E scanned slide, as grade 1, 2 or 3. This discrepancy might result in a low correlation between prognosis and grade. Multiple studies have shown high interrater variability of DCIS grade and have suggested methods for improvements in consistency, such as dichotomous scoring[19–21], assessing the proportions of DCIS heterogeneity[22], adding uniform e-learning[23] and using second opinions[24]. Our results are based on a GLMM model taking into account the same pathologists examined the same slides[25]. Such variability in grading of DCIS has profound consequences for inclusion of cases of DCIS in active surveillance trials (COMET[8], LORIS[9], LORD[10]), where low or intermediate grade (or low and lower portion of intermediate grade in LORIS) are inclusion criteria. Regarding the COMET and LORD, where no central review is performed, patients are deemed eligible or ineligible based on examination by an individual local pathologist. For all these reasons, it is essential to achieve a globally reproducible scoring system.

As noted, some pathologists tended to score substantially more DCIS lesions as low grade than others while the opposite also occurred. In the case of heterogeneous DCIS, one pathologist categorized the lesion according to the most prominent grade while the majority (7/9) classified the DCIS by the highest cytonuclear grade present, which could explain some of the differences presented. One guideline (UK) clarifies that the highest grade should be recorded when, uncommonly, more than one form is present[17]. Other, previous, guidelines like the 2012 WHO[26] or

1997 Consensus conference[27] have advised that all grades present should be noted. Specifically, in this study we sought to simulate daily clinical practice and therefore did not provide specific guidelines beforehand for grading or for any of the other histological features recorded. Compared to the standard model, pathologists who followed the UK pathology guidelines[17] showed significantly more mutual concordance (κ_{ma} =0.58; p=0.02; table 2) than those who used the 2012 WHO guidance[26] (κ_{ma} =0.48; p=0.80). However, when exploring the details of the various guidelines no major differences were apparent that could explain the better concordance for the UK guideline compared to the others[26–28] (supplementary table 2). It is the case that in the UK, adherence to the breast reporting guidelines is mandated for breast screening pathologists, as is participation in a twice yearly national breast external quality assurance slide review scheme (that includes cases of DCIS) as well as attendance at regional meetings to discuss these. However, two of the three UK breast pathologists are central reviewers in the LORIS trial (through which they have also provided advice and educational webinars for other UK pathologists) and two work in the same department (albeit where cases are reported by the individual). It is therefore difficult to know if the greater concordance of the 3 UK pathologists represents the recent focus on consistency of grading of DCIS in the UK, the overall educational and quality assurance mechanisms in place, or simply that they have had the opportunity to work together, discuss problematic cases and align their approach to DCIS grading. Nevertheless, this supports the use of one international DCIS grading system along with a uniform training program, as also suggested by other studies[1,19–21,29].

To improve guidance for clinical decision making, we explored the use of IHC. In our data on the NKI-series, majority opinion low and intermediate grade DCIS was characterized by ER positivity and HER2 negativity. We were able to validate this in DUMC (USA) slides for ER expression, scored by an alternative (Allred[15]) method (supplementary table 5). This is in line with other studies which also showed that ER was frequently expressed in low and intermediate grade DCIS, whereas HER2 positivity was much more frequent in high grade disease[30,31]. The proportion of pure DCIS that is ER positive is 68%-83%[5,30–33] whilst HER2 positivity ranges from 25%-35%[5,31,32,34]. IHC scoring for ER and HER2 is reported to have high interobserver agreement between pathologists (intra class coefficient >0.8)[5], which is better than the interobserver agreement for grade (presented here and other studies[19–22,35–37]). Globally, the use of IHC within DCIS is variable; no marker is at present included in international DCIS pathology minimum datasets, although in some national datasets (e.g. USA) ER assessment is mandated. In the USA, half of the patients with ER positive DCIS are treated with endocrine therapy[38], but this is still a subject of debate, and is much lower in other countries [2–4]. Positive ER/PR and negative HER2 status is used in the COMET trial as inclusion criteria for the active surveillance regimen[8] in keeping with the data presented

here; when DCIS shows ER negativity and/or HER2 positivity, classification as high grade DCIS should be considered.

The present study has several limitations. Firstly, only limited outcome data was available for many of the cases and therefore the primary outcome was histological interobserver variability, instead of recurrence or progression of disease. Unfortunately we were not able to validate the results of the N=106 NKI cases in another cohort. To our knowledge, only one single centre study has correlated interobserver variability with progression to invasive breast cancer and found that using majority opinion based scores of grade (grade 1+2 versus 3), mitotic activity and growth pattern were associated with outcome in patients treated with breast conserving surgery (BCS) only and not in patients treated with BCS plus radiotherapy. Furthermore, we sought to simulate daily clinical practice and therefore did not require adherence to guidelines assigned specifically for the study. The concordance may have been better if we had provided guidance for assessment of the slides. It should also be noted that most of the study pathologists are not using digital slides to diagnose cases in their daily practice, although digital pathology will become daily practice in the near future. In this study, a DCIS case was represented by one slide, while in daily practice multiple slides are typically examined in evaluating DCIS. Moreover, increasing the number of (international) pathologists would have provided more information about the differences between countries and the guidelines used. Lastly, independent validation of the data on ER and HER2 expression presented is necessary in order to prove the association between low and intermediate grade DCIS with immunohistochemical ER positivity and HER2 negativity.
The strength of this study is the international character of both the cases of DCIS and the participating pathologists. Moreover, the data has been analysed using a method that takes into account the cross-classified data structure.

In conclusion, in this international study we show a moderate concordance for a range of histological features of DCIS between nine specialist breast pathologists. As cytonuclear grade of DCIS plays a role as a prognostic parameter in treatment decisions there is an urgent need for adherence of pathologists to a more objective scoring system. As a first step in improving reproducibility, we suggest that ER negativity and/or HER2 positivity of an individual DCIS lesion is indicative of a high grade lesion, which may be of value in distinguishing this from low and intermediate grade DCIS, although validation is required.

Additional Information

Conflicts of interest

All the authors declare no conflict of interests.

Data availability

The data generated and analysed during this study will be available from the corresponding author upon reasonable request.

Ethics Approval and consent to participate

Local IRB's approved the use of the tissue blocks of NKI, MD Anderson Cancer Center and Duke University with the waiver of informed consent because of the retrospective character of the study.
For the UK slides held at Guy's and St Thomas' Hospitals in the King's Health Partner's Cancer Biobank facility, this is licensed by the Human Tissue Authority (license 12121). Ethics Committee approval was not required for this prospective cohort study originally conducted under the NHS Cancer Screening Program's application to the Patient Information Advisory Group.

Consent for publication

Not applicable

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Supplementary files

Power calculation method

Based on a statistical power calculation, we aimed to have at least 379 tissue slides all evaluated by nine pathologists, i.e. three pathologists from each country. This number was obtained taking into consideration the proportion of high-grade DCIS anticipated between three countries and taking correlations within pathologists from the same country into account. We expected overall proportions of high-grade DCIS from the NL, US and UK to be 42%, 52% and 62% respectively, and we assumed that correlations between randomly chosen grades of DCIS within pathologists from the same country was 0.60. Such a design would give us at least 80% power to detect all pairwise comparisons of proportions between different countries using a corrected for multiple testing significance level of 0.016.

Scoring form for evaluating the DCIS slides

☐ **Disease present: DCIS**
☐ **Disease present: ADH**
☐ **Disease present: LCIS**

Dominant architectoral pattern:

- Not assessable
- Comedo
- Solid
- Cribiform
- Flat/Clinging
- (Micro)papillary

Calcification present:

- Not assessable
- Absent
- Present

Necrosis present:

- Not assessable
- Absent
- Present: Comedo
- Present: Focal
- Present: Comedo and focal

Periductal fibrosis present:

- Not assessable
- Absent
- Subtle
- Prominent

Lymphocytic infiltrate (in relation with DCIS) present:

- Not assessable
- Absent
- Subtle
- Prominent

Histological grade DCIS (1/2/3):

- Not assessable
- Low grade
- Intermediate grade
- High grade

Histological grade DCIS (low/high):

- Not assessable
- Low grade
- High grade

Frequency of mitoses:

- Not assessable
- Sparse
- Many

Comments (other diagnosis or otherwise):
open text field

Supplementary table 1. Information regarding included slides

Before inclusion in the study, all slides were evaluated to ensure that they were in focus. Pilot study was performed to check if the quality between the slides from the different centers was similar.

	UK (KCL)	**NL (NKI)**	**USA (Duke)**	**USA (MDACC)**
Type of scanner	NanoZoomer 2.0 HT Slide Scanner (Hamatsu Photonics)	Aperio AT2 Slide Scanner (Leica Biosystems)	Leica Aperio scanner	Aperio AT2 Slide Scanner (Leica Bio systems)
Magnification	40x	20x	20x	20x
Type of slides	Whole breast images	Whole breast images	Whole breast images	Whole breast images
Format	.ndpi	.svs	.svs	.svs
Grade to original pathology reports (N, %)				
Grade 1	12 (11%)	19 (17%)	5 (5%)	14 (16%)
Grade 2	34 (31%)	36 (33%)	41 (45%)	38 (45%)
Grade 3	64 (58%)	55 (50%)	46 (50%)	33 (39%)
Excluded*	0	0	18	10

* Slides originally evaluated as grade 1-2 or 2-3 were excluded

Supplementary table 2. Criteria of the guidelines used.

	WHO 2012	RCPath Guidelines UK	College of American pathologists (Lester et al)	Consensus conference on classification of DCIS
Grade 1 (low)				
Cell appearance	Small monomorphic cells with regular chromatin inconspicuous nucleoli	Monomorphic, evenly spaced cells with rounded, centrally placed nuclei and inconspicuous nucleoli	Monomorphic cells, usually diffuse finely dispersed chromatin, only occasional nucleoli	Monomorphic, usually exhibit diffuse, finely dispersed chromatin, only occasional nucleoli and mitotic figures
Pattern	Arcades, micropapillae, cribiform, or solid	Generally arranged in micropapillary and cribriform patterns		
Orientation	Polarized cells around rosettes	Usually polarisation of cells covering the micropapillae	Polarized toward luminal spaces	Usually associated with polarization of constituent cells
Nuclear Size	Nuclear: Uniform size,	Nuclear: 2x-3x erythrocyte	Nuclear: 1x-2x size of normal RBC or normal duct epithelial cell nucleus	Nuclear: 1.5-2.0 RBC or duct epithelial cell nuclear dimensions
Mitosis	Rare	Few		
Necrosis	uncommon	rarely individual cell necrosis		
Calcifications	Often psammomatous type			

Supplementary table 2. Continued.

	WHO 2012	RCPath Guidelines UK	College of American pathologists (Lester et al)	Consensus conference on classification of DCIS
Grade 2 (intermediate)				
Cell appearance	Mild to moderate variability in shape, variably coarse chromatin, variably prominent nucleoli	Moderate pleomorphism, nuclear to cytoplasmic ratio is often high, and one or two nucleoli may be identified. one or two nucleoli. Clear cell or apocrine types often fall into this category	Intermediate pleomorphism, intermediate chromatin, nucleoli	Nuclei that are neither NG1 or NG2
Pattern		Solid, cribriform or micropapillary.		
Orientation	Cell polarization is not well developed as in low-nuclear grade	some degree of polarization	Intermediate polarization	
Size	Nuclear: variability in size	Nuclear: 2-3x size of an erythrocyte	Nuclear: intermediate	
Mitosis	Maybe present		Intermediate	
Calcifications	Distribution of amorphous of or laminated microcalcifications is generally similar to low-nuclear-grade			
Necrosis	Puncate or comedo necrosis maybe present			

Supplementary table 2. Continued.

	WHO 2012	RCPath Guidelines UK	College of American pathologists (Lester et al)	Consensus conference on classification of DCIS
Grade 3 (high)				
Cell appearance	Highly atypical cells with pleiomorphic nuclei	pleomorphic, irregularly spaced and, nuclei exhibiting marked variation in size with irregular nuclear contours, coarse chromatin and prominent nucleoli	Markedly pleomorphic, usually vesicular with irregular chromatin distribution, prominent nucleoli	Markedly pleiomorphic, usually vesicular and exhibit irregular chromatin distribution and prominent often multiple nucleoli
Pattern	Solid, cribiform or micropapillary patterns	It is often solid with comedo-type central necrosis. Also micropapillary and cribriform patterns frequently associated with central comedo type necrosis		
Orientation	Poorly polarized	rarely any polarization of cells	Usually not polarized toward the luminal space	
Size	Lesion: usually >5mm	Nuclear: >3x the size of erythrocytes	Nuclear : >2.5x size of RBC or normal duct epithelial cell nucleus	Nuclear: usually>2.5 x RBC or duct epithelial cell nuclear dimensions
Mitosis	Usually common (not required)	usually frequent and abnormal forms may be seen		Might be conspicuous
Calcifications	Amorphous microcalcifications are common and usually associated with intraluminal debris			
Necrosis	Frequently presence of comedo necrosis (not obligatory)			

Supplementary table 3. Characteristics of participating pathologists and examined tissue slides

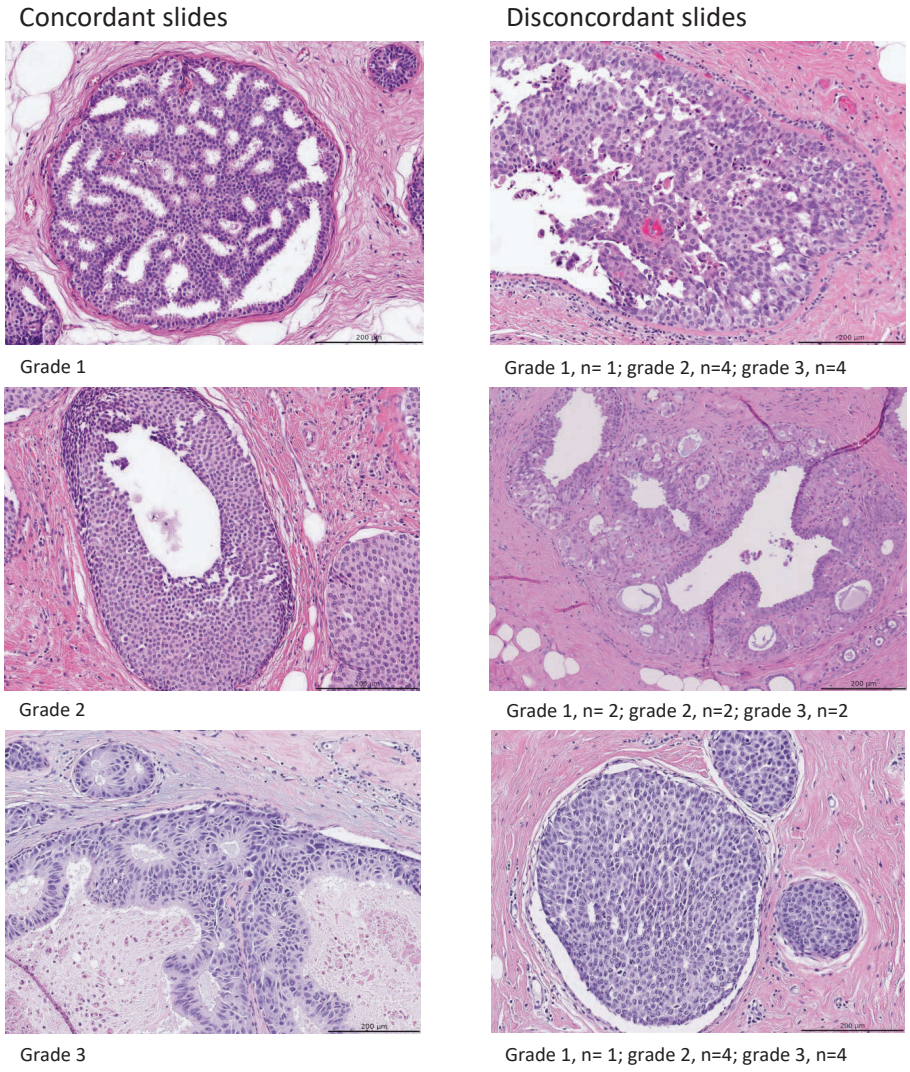
Pathologists (N, %)	**9 (100%)**	**Slides (N, %)**	**425 (100%)**
Country		*Center*	
The Netherlands (NL)	3 (33%)	NKI	110 (26%)
United Kingdom (UK)	3 (33%)	KCL	110 (26%)
United States (US)	3 (33%)	Duke	110 (26%)
		MDACC	95 (22%)
Experience		*Grade according to majority opinion*	399 (100%)
Median	12.0 years	1	45 (11%)
<10 yrs	5 (56%)	2	158 (40%)
>=10 yrs	4 (44%)	3	196 (49%)
Guidelines			
WHO	3 (33%)		
UK RCPath Guidelines	3 (33%)		
College of American pathologists	2 (22%)		
Consensus conference on classification of DCIS	1 (11%)		
In case of heterogeneous DCIS			
Highest grade	7 (78%)		
Most prominent grade	1 (11%)		
Other	1 (11%)		

Supplementary table 4. Details about the scoring of the immunohistochemical stains and characteristics of the used antibodies

	Antigen	NL (NKI)	USA (Duke)
Clone	ER	SP1	1D5 and ER-2-123
	PR	1E2	PgR1294
	HER2	4B5	*Not used in this study*
Dilution	ER	ready-to-use	ready-to use
	PR	ready-to-use	ready-to-use
	HER2	ready-to-use	*Not used in this study*
Manufacturer	ER	Ventana medical systems	Dako / Agilent
	PR	Ventana medical systems	Dako / Agilent
	HER2	Ventana medical systems	*Not used in this study*
Type of slides		Whole slides	Whole slides
Scorings method	ER	% of positive cells; ≥10% is positive	Allred method; >2 is considered as positive
	PR	% of positive cells; ≥10% is positive	Allred method; >2 is considered as positive
	HER2	% membrane staining; ≥10% is positive(3+), if incomplete or weak (2+) SISH was performed	*Not used in this study*
Number of observers		7 (5 pathologists)	1 out of 5 breast pathologists
More details		Supplementary table Visser et al. Clin Can Res 2018	

Supplementary table 5. ER, PR and HER2 expression in relation to interobserver variability in a subset. Grade 1, grade 2, or grade 3 are established according to the majority opinion. For 'certain' cases eight or nine pathologists agreed and 'uncertain' cases <8 pathologists agreed.

NL	**ERneg**	**ERpos**		**PRneg**	**PRpos**		**HER2neg**	**HER2pos**	
Grade 1	**0**	**5**	**5**	**0**	**5**	**5**	**6**	**0**	**6**
Certain (8,9)	0	1		0	1		1	0	
Uncertain (<8)	0	4		11	4		5	0	
Grade 2	**0**	**31**	**31**	**13**	**18**	**31**	**28**	**4**	**32**
Certain (8,9)	0	7		2	5		8	0	
Uncertain (<8)	0	24		11	13		20	4	
Grade 3	**34**	**28**	**62**	**47**	**15**	**62**	**26**	**42**	**68**
Certain (8,9)	27	18		34	11		14	35	
Uncertain (<8)	7	10		13	4		12	7	
Total	**34**	**64**	**98**	**60**	**38**	**98**	**60**	**46**	**106**
USA (Duke)	**ERneg**	**ERpos**		**PRneg**	**PRpos**				
Grade 1	**0**	**8**	**8**	**0**	**8**	**8**			
Certain (8,9)	0	0		0	0				
Uncertain (<8)	0	8		0	8				
Grade 2	**0**	**46**	**46**	**2**	**42**	**44**			
Certain (8,9)	0	5		0	5				
Uncertain (<8)	0	41		2	37				
Grade 3	**16**	**33**	**49**	**21**	**26**	**47**			
Certain (8,9)	14	15		17	12				
Uncertain (<8)	2	18		4	14				
Total	**16**	**87**	**103**	**23**	**76**	**99**			



Supplementary figure 1. Histological examples of concordant and discordant slides.



Chapter 4

Long-term risk of subsequent ipsilateral lesions after a diagnosis of ductal carcinoma in situ

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ABSTRACT

Background
Radiotherapy (RT) following breast conserving surgery (BCS) for ductal carcinoma in situ (DCIS) reduces ipsilateral breast event rates in clinical trials. This study assessed the impact of DCIS treatment on 20-year risk of ipsilateral in situ (iDCIS) and invasive breast cancer (iIBC) in a population based cohort.

Methods
The cohort comprised all women diagnosed with DCIS in the Netherlands during 1989-2004 with follow-up until 2017. Cumulative incidence of iDCIS and iIBC following BCS and BCS+RT were assessed. Associations of DCIS treatment with iDCIS and iIBC risk were estimated in multivariable Cox models.

Results
The 20-year cumulative incidence of any ipsilateral breast event was 30.6% (95% confidence interval (CI);28.9-32.6%) after BCS compared to 18.2% (95%CI;16.3-20.3%) following BCS+RT.
Women treated with BCS compared to BCS+RT had higher risk to develop iDCIS and iIBC within five years after DCIS diagnosis (for iDCIS: $HR_{age<50}$ 3.2(95%CI;1.6-6.6); $HR_{age\geq50}$ 3.6(95%CI;2.6-4.8) and for iIBC: $HR_{age<50}$ 2.1(95%CI;1.4-3.2); $HR_{age\geq50}$ 4.3(95%CI;3.0-6.0)). After ten years, risk of iDCIS and iIBC after initial therapy no longer differed (for iDCIS: $HR_{age<50}$ 0.7(95%CI;0.3-1.5); $HR_{age\geq50}$ 0.7(95%CI;0.4-1.3) and for iIBC: $HR_{age<50}$ 0.6(95%CI;0.4-0.9); $HR_{age\geq50}$ 1.2(95%CI;0.9-1.6)).

Conclusion
Radiotherapy strongly reduces iDCIS and iIBC risk in the first decade after BCS for DCIS, but this benefit wanes thereafter.

Introduction

Since the introduction of population-based mammography breast cancer screening in the 1990s ductal carcinoma in situ (DCIS) comprises approximately 15% of all newly diagnosed neoplastic breast lesions[1,2]. DCIS is considered a non-obligate precursor of invasive breast cancer (IBC) and consists of neoplastic epithelial cells confined to the ductal system of the mammary gland. Because of its potential to become invasive, patients diagnosed with DCIS are usually treated as for invasive breast cancer with a mastectomy or with breast conserving surgery (BCS) often followed by radiotherapy to the whole breast (RT). DCIS itself, however, is not life-threatening and these treatment strategies by definition lead to overtreatment for those lesions that will never progress to IBC.

Radiotherapy as an adjunct to BCS as treatment for DCIS was evaluated in several clinical trials (NSABP B17, EORTC 10853, SweDCIS, UK/ANZ) and a meta-analysis demonstrated a 15% absolute ten-years risk reduction of both subsequent ipsilateral in situ (iDCIS) and invasive (iIBC) lesions for BCS+RT versus BCS only, without effect on breast cancer specific and overall survival[3–7]. However, how these trial data translate into reduction of ipsilateral breast events in large, population-based patient cohorts on the longer-term is unclear. We previously showed an absolute risk for iIBC of 15.4% for patients treated with BCS only compared to 8.8% for patients treated with BCS+RT at 15 years after diagnosis in a cohort with nationwide coverage[8]. Importantly, we also observed a trend towards a diminishing effect of radiotherapy after longer follow-up. In the same cohort, now with up to 28 years follow-up, we assess the very long-term risk of both iDCIS and iIBC after a diagnosis of primary DCIS and asses associations with initial DCIS treatment overall and in subgroups based on age and elapsed time since diagnosis.

Methods

Data collection

Our cohort comprises all women diagnosed with primary pure DCIS in the Netherlands between January 1st,1989 and December 31st, 2004[8]. Diagnoses of subsequent ipsilateral invasive breast (iIBC) lesions were derived from the Netherlands Cancer Registry (NCR) as well as through linkage of the NCR database with the nationwide registry of histology and cytopathology in the Netherlands (PALGA). Subsequent ipsilateral ductal carcinoma in situ (iDCIS) lesions are not registered within the NCR and therefore identification is solely based on pathology reports provided by the PALGA registry. iDCIS was defined as any ipsilateral ductal carcinoma in situ lesion including micro-invasive growth <1 mm at least 3 months after diagnosis of the index DCIS; iIBC was defined as any ipsilateral invasive breast lesion diagnosed at

least 3 months after diagnosis of the index DCIS. Follow-up for both NCR and PALGA has been completed until January 1st, 2017. Initial treatment was categorized into three groups: breast conserving surgery alone (BCS only), BCS with additional whole breast radiotherapy (BCS+RT) or mastectomy (independent of subsequent RT). Chemotherapy and endocrine therapy was almost never administered to women with DCIS in the Netherlands during the time of the cohort accrual and patients who received chemotherapy or endocrine therapy for DCIS were excluded (n=123). For patients treated with a mastectomy the risk of iDCIS recurrences was not assessed. Intercurrent mastectomies were defined as mastectomies of the ipsilateral breast ≥3 months after primary DCIS diagnosis and applied for other reasons than our events of interest (iDCIS or iIBC) as identified from pathology reports provided by the PALGA registry. In this paper, subsequent ipsilateral lesions are referred to as 'recurrence' although we do not know whether these lesions are biologically related to the primary DCIS or represent independent secondary primaries.

Statistical analyses

Time at risk started at date of primary DCIS diagnosis and ended at date of the first event of interest (iDCIS or iIBC), date of death, emigration or January 1st, 2017, whichever came first. The cumulative incidence of iDCIS, iIBC and the combination of iDCIS and iIBC was estimated using the Aalen-Johanson estimator with death as the only competing risk and emigration as censoring event. If laterality of a subsequent iDCIS was unknown, this resulted in censoring at date of iDCIS (n=10). For the iIBC cumulative incidence analysis treatment was considered a time-varying variable. As a consequence if a patient initially treated with BCS or BCS+RT underwent an intercurrent mastectomy (i.e. for benign disease or for iDCIS), she contributed all person time from the date of mastectomy to the mastectomy group. In all other analyses an intercurrent mastectomy resulted in censoring.

Multivariable Cox proportional hazard analysis was used to examine the effects of treatment strategies on iDCIS and iIBC risk. Attained age was used as time-scale. The proportional hazard assumption was assessed using residual-based and graphical methods. Because the hazard ratios (HRs) for treatment were non-proportional with time since treatment, the models for iDCIS and iIBC risk were stratified by time since treatment, using intervals of 0-4, 5-9 and ≥10 years after diagnosis and an interaction term for treatment and time since treatment, using the above intervals, was added to the models[9]. Additionally, the HRs for treatment differed with age at diagnosis ($p_{interaction}$<0.001). Using the Aikake Information Criterion the iIBC model demonstrated the best fit when age at DCIS diagnosis was fitted as a dichotomous categorical variable (<50 years versus ≥50 years old) and an age-treatment interaction term was added to the model. For iDCIS, the best model fit was achieved by adjusting for age at DCIS diagnosis as a continuous variable. To keep the models for iDCIS and iIBC comparable, we, however, included

age as a dichotomous categorical variable (<50 years versus ≥50 years old), while also including an age-treatment interaction term, although for iDCIS this age-treatment interaction was non-significant ($p_{interaction}$=0.06).

The association of histological grade of the primary DCIS and iDCIS and iIBC risks was evaluated only among patients diagnosed in the period 1999-2004, as information on DCIS grade was incomplete before 1999. In the analysis of iDCIS risk among patients diagnosed in 1999-2004 the proportional hazards assumption was not violated and no interaction term for treatment and time since treatment was included and age neither modified the effect of treatment.
All analyses were performed in open source software R version 3.5.1 using the 'survival' and 'etm' packages[10].

Results

The study cohort comprised 10,045 women of whom 2,647 (26%) received BCS only, 2,604 (26%) received BCS+RT, and 4,794 (48%) underwent mastectomy as primary treatment. Additional patient characteristics are summarized in table 1. The median follow-up was 15.7 years (interquartile range: 9.2-22.3 years). During follow-up in total 774 (7.7%) iIBC and 497 (4.9%) iDCIS lesions were identified. The 10- and 20-year cumulative incidence of subsequent ipsilateral breast disease (iDCIS or iIBC) for women treated with BCS only was 24.6% (95% confidence interval (CI) 23.0-26.3) and 30.6% (95%CI 28.9-32.6), respectively, whereas for women treated with BCS+RT the cumulative incidence was 9.6% (95%CI 8.6-10.8) and 18.2% (95%CI 16.3-20.3) at 10 and 20 years, respectively (figure 1). The competing risk, death, varied for the different treatment strategies between 8.7% and 14.7% after 10 years and between 26.8% and 35.2% after 20 years since DCIS diagnosis (supplementary figure 1).

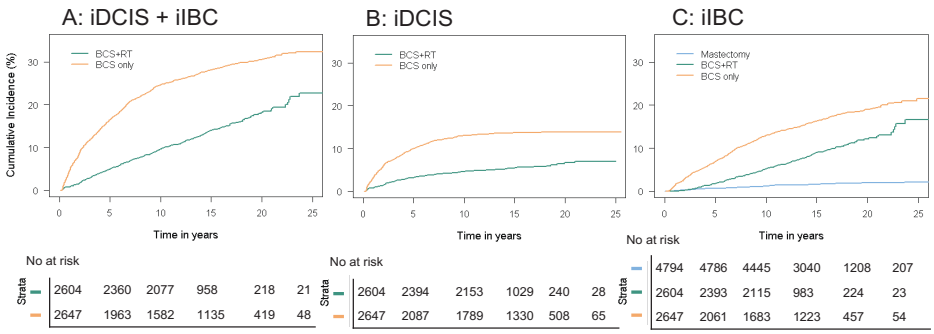


Figure 1. Cumulative incidence with death as competing risk by treatment strategy. A) in situ and invasive recurrences, B) iDCIS only, C) invasive recurrences only.

Table 1. Patient characteristics.

Initial DCIS treatment	BCS only N=2647	BCS+RT N=2604	Mastectomy N=4794	Total N=10045
Follow-up in years, median (IQR)	17.0 (9.7-24.4)	14.5 (9.9-19.1)	16.0 (9.0-22.9)	15.7 (9.2-22.3)
Age at DCIS diagnosis, years, median (IQR)	58.9 (43.0-74.8)	57.2 (43.2-71.2)	57.2 (40.6-73.8)	57.6 (41.9-73.3)
Age <50	474 (17.9%)	457 (17.5%)	1212 (25.3%)	2143 (21.3%)
Age ≥50	2173 (82.1%)	2147 (82.5%)	3582 (74.7%)	7902 (78.7%)
DCIS grade (1999-2004[a])				
Low (1)	302 (40.9%)	215 (13.7%)	190 (10.2%)	707 (16.9%)
Intermediate (2)	234 (31.7%)	578 (36.7%)	553 (29.7%)	1365 (32.7%)
High (3)	202 (27.4%)	780 (49.6%)	1121 (60.1%)	2103 (50.4%)
Unknown	240	285	342	867
Subsequent iIBC	445	240	89	774
Subsequent iDCIS	352	145	NA	497

[a]Data on grade is presented for patients diagnosed with primary DCIS from 1999-2004 (n=5042). iIBC denotes ipsilateral invasive breast cancer; iDCIS: ipsilateral ductal carcinoma in situ; BCS: breast conserving surgery; RT: radiotherapy; N: number; IQR: interquartile range; DCIS: ductal carcinoma in situ.

Subsequent iDCIS risk

Among patients treated with BCS only 352 iDCIS occurred compared to 145 iDCIS among patients treated with BCS+RT. Most iDCIS occurred within the first 10 years of follow-up with only 19 patients developing a late iDCIS (10 years or more after their initial DCIS diagnosis) after BCS only and 27 after BCS+RT (supplementary table 1). For women treated with BCS only, the 10- and 20-year cumulative incidence of iDCIS was 13.0% (95%CI 11.8-14.4) and 13.9% (95%CI 11.6-15.3), respectively, versus 4.6% (95%CI 3.9-5.5) and 6.7% (95%CI 5.5-8.1), respectively, for women treated with BCS+RT (figure 1, supplementary table 1).

Women <50 years treated with BCS only had a 3.2-times higher HR (95%CI 1.6-6.6) for iDCIS in the first five years after diagnosis compared to women treated with BCS+RT, while women ≥50 years treated with BCS only had a 3.6-times higher HR for iDCIS (95%CI 2.6-4.8) then women treated with BCS+RT (table 2). The relative risk to develop iDCIS among patients treated with BCS only compared to BCS+RT decreased in the interval 5-9 years after DCIS and risks no longer differed from 10 years after initial DCIS in both age groups (table 2). Women diagnosed between 1999 and 2004 had a slightly lower risk to develop iDCIS than women diagnosed between 1989 and 1998 (HR 0.9; 95%CI 0.7-1.0).
Among all women diagnosed with primary DCIS between 1999 and 2004, women with grade 1 DCIS had half the risk (HR 0.5; 95%CI 0.3-0.8) of iDCIS of women with grade 2 lesions (supplementary table 2). iDCIS risk did not differ for women with grade 3 lesions compared to those with grade 2 lesions.

Table 2. Multivariate Cox analysis to estimate the association of treatment with the risk of subsequent ipsilateral ductal carcinoma in situ (iDCIS) and ipsilateral invasive breast cancer (iIBC).

Age at DCIS	Time since DCIS	Treatment	iDCIS	iIBC
years	years		HR (95%CI)	HR (95%CI)
<50	0-5	BCS+RT	Ref	Ref
		BCS only	3.2 (1.6-6.6)	2.1 (1.4-3.2)
		Mastectomy[a]	-	0.4 (0.2-0.6)
	5-10	BCS+RT	Ref	Ref
		BCS only	2.5 (1.1-5.3)	1.0 (0.7-1.5)
		Mastectomy[a]	-	0.1(0.1-0.3)
	≥10	BCS+RT	Ref	Ref
		BCS only	0.7 (0.3-1.5)	0.6 (0.4-0.9)
		Mastectomy[a]	-	0.1 (0.1-0.2)
≥50	0-5	BCS+RT	Ref	Ref
		BCS only	3.6 (2.6-4.8)	4.3 (3.0-6.0)
		Mastectomy[a]	-	0.3 (0.2-0.4)
	5-10	BCS+RT	Ref	Ref
		BCS only	2.7 (1.8-4.1)	2.1 (1.6-2.8)
		Mastectomy[a]	-	0.1 (0.1-0.2)
	≥10	BCS+RT	Ref	Ref
		BCS only	0.7 (0.4-1.3)	1.2 (0.9-1.6)
		Mastectomy[a]	-	0.1 (0.1-0.1)

[a]Information regarding mastectomy treatment was not available for iDCIS.
(Attained) age as primary time-scale, adjusted for period of initial DCIS diagnosis (1989-1998 vs 1999-2004) and age at DCIS diagnosis (<50 vs ≥50) including an age-treatment interaction term.
HR denotes hazard ratio; 95%CI: 95% confidence interval; Ref: Reference category; BCS: Breast conserving surgery; RT: radiotherapy; iDCIS: ipsilateral ductal carcinoma in situ; iIBC ipsilateral invasive breast cancer; DCIS: ductal carcinoma in situ.

Subsequent iIBC risk

Among patients treated with BCS only the 10- and 20-year cumulative incidence of iIBC was 13.9% (95%CI 11.7-14.3) and 19.1% (95%CI 17.5-20.8), respectively. The 10- and 20-year cumulative incidence was 5.2% (95%CI 4.4-6.2) and 12.1% (95%CI 10.5-14.0), respectively, in patients treated with BCS+RT and 1.1% (95%CI 0.9-1.5) and 1.9% (95%CI 1.6-2.4), respectively, in patients treated with mastectomy (figure 1, supplementary table 1). Women <50 years diagnosed with DCIS between 1999-2004 and treated with BCS+RT showed continuously lower absolute iIBC risks compared to those treated with BCS only (figure 2). In contrast, women <50 years diagnosed in the period 1989-1998 had approximately similar cumulative incidences after either BCS only or BCS+RT treatment from 10 years or more after DCIS diagnosis.

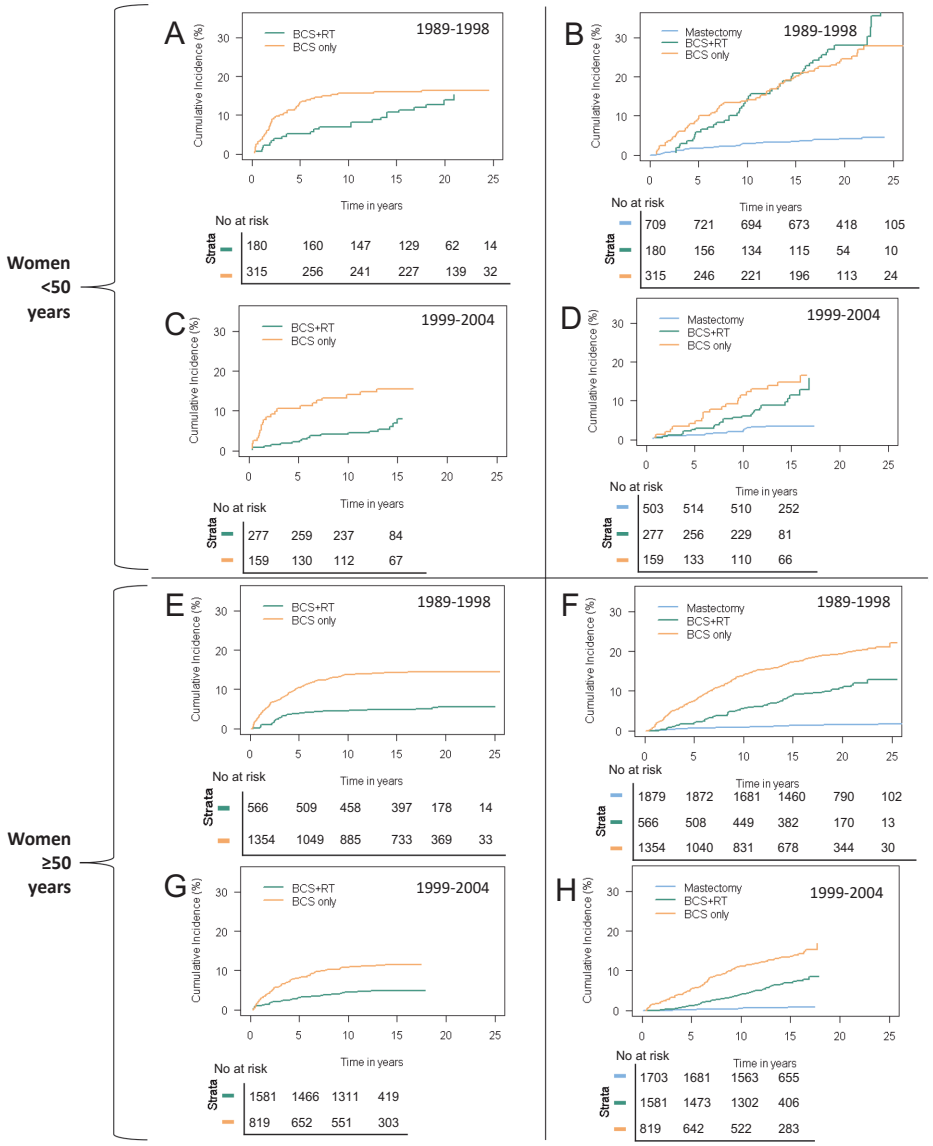


Figure 2. Cumulative incidence with death as competing risk in A) iDCIS risk of women < 50 years diagnosed between 1989-1998 for primary DCIS, B) iIBC risk of women < 50 years diagnosed between 1989-1998 for primary DCIS, C) iDCIS risk of diagnosed in women < 50 years diagnosed in 1999-2004 for primary DCIS and D) iIBC risk women < 50 years diagnosed between 1999-2004 for primary DCIS, E) iDCIS risk of women ≥50 years diagnosed between 1989-1998 F) iIBC risk of women ≥50 years diagnosed between 1989 -1998, G) iDCIS risk of women ≥50 years diagnosed between 1999-2004 iIBC risk of women ≥50 years diagnosed between 1999-2004.

In women <50 years at DCIS diagnosis, the HR for iIBC was 2.1-times (95%CI 1.4-3.2) higher in the first five years after diagnosis among those treated with BCS only compared to women treated with BCS+RT; the HR for iIBC was even 4.3-times (95%CI 3.0-6.0) higher for women ≥50 years treated with BCS only within the first five years after treatment compared to BCS+RT (table 2). The risk for developing an iIBC no longer differed from 5 years after DCIS diagnosis for women <50 years between those treated with BCS only or with BCS+RT (HR1.0; 95%CI 0.7-1.5). While for women ≥50 years this risk did not longer differed from 10 years after DCIS diagnosis (HR 1.2; 95%CI 0.9-1.6). Women treated with mastectomy had much lower risk to develop iIBC compared with women treated with BCS, irrespective of age at diagnosis or time since DCIS treatment (table 2). Women diagnosed with primary DCIS between 1999 and 2004 had a slightly lower risk to develop iIBC than women diagnosed between 1989 and 1999 (HR 0.8; 95%CI 0.6-0.9).
Inclusion of histological grade in the analysis did not affect the association of DCIS treatment with iIBC risk ($HR_{age\geq50}$ for BCS only versus BCS+RT in year 1-5: 4.8; 95%CI 2.7-8.5) for a model including grade and 4.8 (95%CI 2.7-8.6) for a model without grade, see supplementary table 3 for all estimates) and grade did not modify the association of initial treatment with iIBC risk ($p_{interaction}$=0.3).

Discussion

In this population-based study among 10,045 women treated for DCIS we showed, that patients treated with BCS only had an absolute risk of 14% to develop iDCIS and of 19% to develop iIBC at 20 years after treatment, while for BCS+RT treatment these risks were 7% and 12%, respectively. iDCIS predominantly occurred in the first 10 years after primary DCIS. Furthermore, from 5 years for younger and from 10 years for older women following the diagnosis of primary DCIS, the rate of iIBC recurrences did no longer differ between women treated with BCS only versus BCS+RT, indicating that the beneficial effect of RT is most prominent within the first years after DCIS diagnosis.

Although our study is based on a population-based cohort with complete follow-up provided by two registries, it has some limitations. Firstly, margin status and tumor size were not available for our patients while DCIS grade was only available for approximately half of the cohort. We had no information regarding the rationales underlying administering BCS only, BCS+RT or mastectomy. Additionally, patients in our cohort were diagnosed and treated sometimes decades ago and diagnosis and treatment strategies have evolved overtime.
Nonetheless, our data clearly show that late in situ recurrences, ≥10 years after DCIS diagnosis, rarely developed while incidence of iIBC continued to rise over time irrespective of initial treatment. This is concordant with the SweDCIS trial[11]

and with the Vermont cohort[12], which both reported few iDCIS occurrences after five years of follow-up.

An explanation for this plateau in risk of subsequent iDCIS lesions after 10 years might be that recurrent DCIS lesions were less detected after 10 years either due to the fact that patients were discharged from routine surveillance or were no longer within the age range invited for the population breast cancer screening program. Alternatively, the lack of in situ recurrences after 10 years may reflect the biology of these DCIS lesions, which would suggest that almost all subsequent iDCIS lesions originate from residual primary DCIS. This is supported by the high frequency of clonal relatedness of iDCIS to primary DCIS, reported to be 82% by Waldman et al.[13] while Shaw et al[14] even reported complete clonal relatedness of iDCIS to primary DCIS. Within our consortium, PREvent ductal Carcinoma In Situ Invasive Overtreatment Now (PRECISION)-initiative[15], we are conducting genomic studies to determine the clonal relatedness of in situ recurrences to the primary DCIS in order to better understand the relationship between the initial DCIS diagnosis and subsequent breast events.

Radiotherapy importantly reduces the risk of iDCIS and iIBC, particularly in the first 10 years after initial DCIS diagnosis. This is in line with prior meta-analysis which showed that radiotherapy reduced the absolute 10-years risk by 15% (28.1% any recurrence in BCS only group versus 12.9% in BCS+RT group[4)] and with several cohort studies which all showed that radiotherapy reduced breast events after radiotherapy in addition to BCS[12,16–18]. However, our analysis also showed that 10 years or more after DCIS diagnosis, the incidence of new iIBC is approximately similar in the BCS only and BCS+RT group (figure 1 and supplementary table 1). This is consistent with results of Rakovitch et al.[19] who showed lower risks of second breast events with increasing follow-up time after DCIS diagnosis. Since extensive clonal diversity is generated by mutations gradually evolving overtime[20], it becomes more likely that newly developed tumors represent an independent second primary tumor more than 10 years after initial DCIS. However, to our knowledge the association of follow-up time with clonal relatedness between primary DCIS and subsequent lesions has not yet been assessed. In addition, we cannot exclude the possibility that RT may induce (secondary) invasive breast tumors which may become apparent long after exposure to RT. Actually a meta-analysis by Akdeniz et al. did demonstrate a slightly increased risk of contralateral breast cancer after RT mainly in breast cancer patients treated<45 years of age[21].

Women <50 years diagnosed with primary DCIS between 1989 and 1998 had similar absolute late iIBC risk irrespective of treatment with BCS only or BCS+RT (figure 2). The SweDCIS trial neither showed a long-term beneficial effect of RT following BCS on iIBC risk in young women (<52 years)[11]. In our models we split age

at 50 years, because the Dutch nationwide breast cancer screening starts at the age of 50 and thus a diagnosis of primary DCIS in women <50 is rarely based on breast screening. These women may present with a different type of DCIS including more frequent symptomatic presentation (i.e. a lump) and/or be diagnosed in the light of familial genetic susceptibility syndromes, which may be accompanied by an increased risk of iIBC. In addition, some studies[19,22] showed that younger patients in general have higher risk of invasive recurrences compared to older patients. However, Ryser et al[23] did not found that iIBC risks were different between women aged <50 and ≥50 years, although this study was not powered to examine age differences. Therefore, we would caution against the interpretation that younger women benefit less from radiotherapy.

This large population-based DCIS cohort provides insight in the long-term risks of ipsilateral breast recurrences in women treated for DCIS. As DCIS is a not life-threatening disease, our ultimate goal should be to de-escalate treatment. There are efforts ongoing to determine whether molecular profiles of DCIS, such as Oncotype DX DCIS score[24] or DCISionRT signature[25] could support selection of women in whom radiotherapy could be safely omitted. Furthermore, three ongoing clinical trials (LORIS[26], LORD[27] and COMET[28] trials) currently randomize between active surveillance and conventional treatment to omit therapy for women with low risk DCIS. Understanding the dynamics of long-term residual breast cancer risk following treatment of DCIS contributes to the understanding of this disease and finally to reducing overtreatment.

Additional information

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Ethics approval and consent to participate

The Central Committee on Research involving Human Subjects determined that this study did not require approval from an ethics committee. The privacy review board of the NCR approved the study.

Data availability

The data generated and analysed during this study will be available from the corresponding author upon reasonable request.

Conflicts of interest

All the authors declare no conflict of interests.

Authors' contribution

Conception and design: MvS, EL, LE, JW, MS
Statistical support: DG, MR, MS
Data collection: MvS, LF, LM, LE, MS
Data analysis and interpretation: MvS, EL, DG, FvD, LM, AT, LE, MR, SH, ES, MS, PE, JW, MS
Manuscript writing: all authors
Final approval of manuscript: all authors

Consent for publication

Not applicable

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Supplementary files

Supplementary table 1. Absolute numbers of events and cumulative incidence, both split in periods of five years and split for initial DCIS treatment.

Type of Event	**iDCIS**				**iIBC**					
Treatment	**BCS only**		**BCS+RT**		**BCS only**		**BCS+RT**		**Mastectomy**	
	Events (n)	Cum inc % (95%CI)	Events (n)	Cum inc % (95%CI)	Events (n)	Cum inc % (95%CI)	Events (n)	Cum inc % (95%CI)	Events (n)	Cum inc % (95%CI)
0-4 year	258	10.0 (8.9-11.3)	84	3.3 (2.6-4.0)	173	6.9 (5.9-7.9)	46	1.8 (1.4-2.4)	33	0.7 (0.5-1.0)
0-9 year	333	13.0 (11.8-14.4)	118	4.6 (3.9-5.5)	315	13.9 (11.7-14.3)	131	5.2 (4.4-6.2)	55	1.1 (0.9-1.5)
0-14 year	349	13.7 (12.4-15.1)	137	5.5 (4.7-6.5)	390	16.3 (14.9-17.9)	207	9.0 (7.9-10.3)	79	1.6 (1.3-2.0)
0-19 year	352	13.9 (12.6-15.3)	144	6.7 (5.5-8.1)	432	19.1 (17.5-20.8)	231	12.1 (10.5-14.0)	87	1.9 (1.6-2.4)
0-24 year	352	13.9 (12.6-15.3)	145	7.0 (5.7-8.6)	444	21.6 (19.3-24.0)	239	16.6 (13.4-20.6)	89	2.2 (1.7-2.8)
0 - 4 year	258	10.0 (8.9-11.3)	84	3.3 (2.6-4.0)	173	6.9 (5.9-7.9)	46	1.8 (1.4-2.4)	33	0.7 (0.5-0.9)
5 - 9 year	75	3.6 (2.9-4.5)	34	1.4 (1.0-2.0)	142	7.0 (6.0-8.2)	85	3.6 (2.9-4.4)	22	0.5 (0.3-0.7)
10 - 14 year	16	0.9 (0.6-1.5)	19	1.1 (0.7-1.7)	75	4.7 (3.7-5.8)	76	4.4 (3.5-5.5)	24	0.6 (0.4-0.9)
15 - 19 year	3	0.3 (0.1-1.0)	7	1.5 (0.7-3.2)	42	4.4 (3.3-6.0)	24	4.2 (2.8-6.4)	8	0.4 (0.2-0.7)
20 - 24 year	0	NA	1	NA	12	5.1 (2.6-9.9)	8	7.4 (3.6-15.0)	2	0.4 (0.09-1.6)

iIBC denotes ipsilateral invasive breast cancer; iDCIS: ipsilateral ductal carcinoma in situ; BCS: breast conserving surgery; RT: radiotherapy; n: number; Cum inc: cumulative incidence; 95%CI: 95% confidence interval.

Supplementary table 2. Multivariable Cox analysis for iDCIS for patient diagnosed from 1999 – 2004.

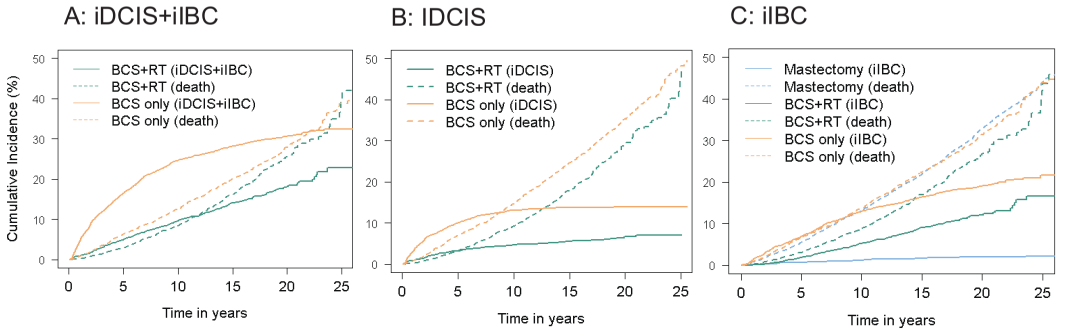
		iDCIS
		HR (95%CI)
Treatment	BCS+RT	Ref
	BCS only	3.2 (2.3-4.4)
Age	<50	0.6 (0.3-1.0)
	≥50	Ref
Grade*	Low (1)	0.5 (0.3-0.8)
	Intermediate (2)	Ref
	High (3)	1.2 (0.9-1.8)

*Patients with unknown grade were excluded (n=525).
**Age as primary time-scale and adjusted for age at DCIS diagnosis (<50 vs ≥50).
HR denotes hazard ratio; iDCIS: ipsilateral ductal carcinoma in situ; 95%CI 95% confidence interval; Ref: reference; BCS: breast conserving surgery; RT: radiotherapy.

Supplementary table 3. Multivariable Cox analysis for iIBC for patient diagnosed from 1999 – 2004 with and without including grade.

Age at DCIS	**Time since DCIS**	**Treatment**	**Model without grade**	**Model including grade**
years	years		HR (95%CI)	HR (95%CI)
<50	0-5	BCS+RT	Ref	Ref
		BCS only	2.7 (1.2-6.1)	2.8 (1.2-6.4)
		Mastectomy	0.5 (0.2-1.4)	0.5 (0.2-1.4)
	5-10	BCS+RT	Ref	Ref
		BCS only	1.8 (0.9-3.5)	1.8 (0.9-3.7)
		Mastectomy	0.2 (0.1-0.6)	0.2 (0.1-0.6)
	≥10	BCS+RT	Ref	Ref
		BCS only	0.8 (0.4-1.7)	0.8 (0.4-1.8)
		Mastectomy	0.2 (0.1-0.5)	0.2 (0.1-0.6)
≥50	0-5	BCS+RT	Ref	Ref
		BCS only	4.2 (2.4-7.4)	4.2 (2.4-7.6)
		Mastectomy	0.2 (0.1-0.4)	0.2 (0.1-0.4)
	5-10	BCS+RT	Ref	Ref
		BCS only	2.7 (1.8-4.2)	2.7 (1.8-4.3)
		Mastectomy	0.1 (0.0-0.2)	0.1 (0.0-0.2)
	≥10	BCS+RT	Ref	Ref
		BCS only	1.3 (0.7-2.1)	1.3 (0.8-2.2)
		Mastectomy	0.1 (0.0-0.2)	0.1 (0.0-0.2)
Grade*		Low (1)	-	0.9 (0.6-1.2)
		Intermediate (2)	-	Ref
		High (3)	-	0.9 (0.7-1.2)

*Patients with unknown grade were excluded (n=867)
** Age as primary time-scale, including a time-treatment interaction term and an age-treatment interaction term ($p_{interaction}$=0.002), adjusted for age at DCIS diagnosis (<50 vs ≥50)
HR denotes hazard ratio; iDCIS: ipsilateral ductal carcinoma in situ; 95%CI: 95% confidence interval; Ref: reference; BCS: breast conserving surgery; RT: radiotherapy; DCIS ductal carcinoma in situ



	iDCIS+iIBC		iDCIS		iIBC		
Treatment	**BCS only**	**BCS+RT**	**BCS only**	**BCS+RT**	**BCS only**	**BCS+RT**	**Mastectomy**
	% (95%CI)	% (95%CI)	% (95%CI)	% (95%CI)	% (95%CI)	% (95%CI)	% (95%CI)
Cumulative incidence of event of interest							
0-9 year	24.6 (23.0-26.3)	9.6 (8.6-10.8)	13.0 (11.8-14.4)	4.6 (3.9-5.5)	13.9 (11.7-14.3)	5.2 (4.4-6.2)	1.1 (0.9-1.5)
0-19 year	30.6 (28.9-32.6)	18.2 (16.3-20.3)	13.9 (12.6-15.3)	6.7 (5.5-8.1)	19.1 (17.5-20.8)	12.1 (10.5-14.0)	1.9 (1.6-2.4)
*Cumulative incidence of competing risk (death)**							
0-9 year	12.6 (11.4-13.9)	8.4 (7.4-9.6)	14.7 (13.4-16.1)	9.2 (8.1-10.4)	13.7 (13.4-16.1)	8.7 (8.2-10.4)	13.2 (12.3-14.2)
0-19 year	27.9 (26.0-29.9)	25.7 (23.3-28.4)	35.2 (33.2-37.4)	29.2 (26.6-32.1)	31.4 (29.3-33.6)	26.8 (26.7-32.0)	32.7 (31.3-34.3)

Supplementary figure 1. Cumulative incidence of A) in situ and invasive recurrences, B) in situ recurrences only, C) invasive recurrences only including the competing risk (death) at 10 and 20 years following DCIS diagnosis.
*Death in absence of iDCIS and/or iIBC.
iIBC denotes ipsilateral invasive breast cancer; iDCIS: ipsilateral ductal carcinoma in situ; BCS: breast conserving surgery; RT: radiotherapy; 95%CI: 95% confidence interval.



Chapter 5

Enrichment of high grade tumors in breast cancer gene expression studies

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ABSTRACT

Purpose

Gene expression (GE) profiling for breast cancer classification and prognostication has become increasingly used in clinical diagnostics. GE profiling requires a reasonable tumor cell percentage and high quality RNA. As a consequence a certain amount of samples drop out. If tumor characteristics are different between samples included and excluded from GE profiling, this can lead to bias. Therefore, we assessed whether patient and tumor characteristics differ between tumors suitable or unsuitable for generating GE profiles in breast cancer.

Methods

In a consecutive cohort of 738 breast cancer patients who received neoadjuvant chemotherapy at the Netherlands Cancer Institute, GE profiling was performed. We compared tumor characteristics and treatment outcome between patients included and excluded from GE profiling. Results were validated in an independent cohort of 812 patients treated with primary surgery.

Results

GE analysis could be performed in 53% of the samples. Patients with tumor GE profiles more often had high-grade tumors (odds ratio 2.57 (95%CI 1.77-3.72), $p<0.001$) and were more often lymph node positive (odds ratio 1.50 (95%CI 1.03-2.19), $p=0.035$) compared to the group for which GE profiling was not possible. In the validation cohort, tumors suitable for gene expression analysis were more often high grade.

Conclusions

In our gene expression studies, tumors suitable for GE profiling had more often an unfavourable prognostic profile. Due to selection of samples with a high tumor percentage we automatically select for tumors with specific features, i.e. tumors with a higher grade and lymph node involvement. It is important to be aware of this phenomenon when performing gene expression analysis in a research or a clinical context.

Introduction

DNA microarray analyses (e.g. tiling arrays, mRNA arrays, and direct sequencing of complementary DNA) have significantly advanced our understanding of breast cancer. They showed that breast cancer is not a single disease with variable morphologic features, but a group of molecular distinct neoplasms[1]. Furthermore, in certain clinical settings it can help determine whether or not adjuvant chemotherapy is justified[2].

Several assays, resulting in risk scores, have been developed and are partially commercially and partially clinically available. The 21-gene Recurrence Score (OncotypeDx assay, Genome Health inc, Redwood city, CA)[3], the Amsterdam 70-gene profile, commercially known as the Mammaprint (Agendia, Huntington Beach, CA) assay[4] and the Risk of Recurrence (ROR) score, derived from Predictor Analysis of Microarray 50 (PAM50)[5] are mostly used. Reliable results of the assays require a good quality tumor sample with high cellularity. To illustrate this, Elloumi et al.[6] revealed a systematic bias when too much normal tissue was present in a tumor sample. However, tumor percentage is also dependent on tumor morphology. For example, a tumor with solid growth more easily reaches a high tumor percentage than a tumor with glandular or lobular growth (a feature important for grade). Furthermore, presence of sclerosis as well as stromal and inflammatory cells can reduce the tumor cell percentage substantially. Another feature that influences the ability to perform gene expression analysis is the RNA quality. Pre-analytical factors such as time to fixation, fixation duration and storage temperature have an impact on the RNA quality [7].

Summarizing the above, a high tumor percentage and good quality RNA are prerequisites for successful gene expression analysis. These requirements lead to the dropout of samples not fulfilling these criteria. As a higher histological grade is associated with a higher tumor cellularity, gene expression analysis might be more successful in high grade tumors. To assess if indeed clinico-pathological variables are associated with successful gene expression analysis, we compared clinical and tumor characteristics of tumors suitable and unsuitable for gene expression analysis in two large (neo) adjuvant treated patient cohorts.

Materials and Methods

Patient selection

Tissue samples of patients treated in the neoadjuvant setting for breast cancer were collected at the Netherlands Cancer Institute (NKI) between 2004 and 2012. For participation in the neoadjuvant program, the tumor diameter should exceed 3

cm or axillary lymph node metastasis should be proven by fine-needle aspiration. Part of these patients participated in two ongoing clinical trials of which details have been described previously[8]. Both studies were approved by the ethical committee and informed consent for gene expression analysis was obtained for all included patients. At least two tumor biopsies were taken under ultrasound guidance, using a 14G core needle to assure sufficient tissue for both adequate diagnostics as well as for research purposes. To facilitate such analyses, at least one biopsy was snap frozen in liquid nitrogen and stored at -80 degrees.

Pathology

Paraffin-embedded sections were all stained by a hematoxylin and eosin (H&E) stain and reviewed by a consultant breast pathologist (JW) for immunohistochemically assessment and histological classification (including subtype and grade) on biopsy material (of which the details are described previously)[9]. In brief, samples were scored as positive for oestrogen receptor (ER) and progesterone receptor (PR) if at least 10% of the tumor cells showed nuclear staining. *HER2* (or *ERBB2*) was scored as positive when there was strong membranous staining in more than 30% of the tumor cells (3+) by immunohistochemistry or if chromosome in situ hybridization (CISH) revealed amplification. Percentage nuclear staining of tumor cells in Ki67 (MIB1) was scored as a marker for proliferation. Chemotherapy response was determined by pathological examination of resection specimens. Pathological complete remission (pCR) was defined as the absence of invasive tumor in both the breast and axillary lymph nodes after neoadjuvant chemotherapy.

Imaging data

For a subset of the patients, detailed imaging data were available. A dedicated breast radiologist (CL) assessed according to BIRADS lexicon [10] whether pre-treatment MRIs showed the tumor to be either mass-like, or non-mass like. For analysis purposes, these two categories were used. Metabolic activity was assessed using baseline 18F-fluorodeoxyglucose (FDG) positron emission tomography combined with computed tomography (PET/CT) scans. FDG uptake was quantified using maximum standardized uptake values (SUVmax) measured with Osirix DICOM viewer (Pixmeo SARL, Geneva, Swiss).

Gene expression assay and tumor percentage

mRNA was isolated from the frozen material as described previously[9]. Briefly, a 5-micrometer section of the biopsy was H&E stained. A pathologist evaluated if the overall tissue quality of the frozen biopsy was sufficient for further analysis (i.e., samples dropped out if the biopsy was too small, too fatty or in the absence of invasive tumor). The pathologist also estimated the tumor percentage and only the samples with a tumor percentage ≥ 50% were selected for microarray analysis. Gene expression analysis was only performed if the RNA integrity number

≥ 6.5 and the quantity ≥3 µg. Samples obtained between 2004 and 2010 were analyzed using Illumina microarray analysis (WG6 v3 microarray chips); RNAseq was performed on the samples from 2011 to 2012.

Validation cohort

As a validation, an independent cohort obtained from the microarRAy PrognoSTics in Breast cancER (RASTER) study was used. Study design is described before[11]. In short, 812 women were enrolled in 16 hospitals in The Netherlands after having given informed consent. Patients received surgery (mastectomy or breast conserving surgery) as primary treatment. Within 1h after surgery, a tumor sample was procured at the pathology department of the participating hospitals and sent to Netherlands Cancer institute by mail. After samples were received at the Netherlands Cancer Institute they were snap frozen at -70 degrees. Pathologists analyzed paraffin-embedded tumor samples of the validation dataset at the pathology department of the participating hospitals. Histological tumor grade according Elston and Ellis, ER status, PR status and ERBB2 status were established by each hospital according to locally used methods[11]. Frozen sections of the tumor samples of the validation set were obtained and stained with H&E stain, and subsequently analyzed by an experienced breast pathologist. Eligible samples had to contain ≥ 50% tumor cells. Agendia Laboratories performed the microarray analysis using the Mammaprint (Agilent microarray, Santa Clara USA)[11].

Data analyses and statistics

The variables age, histologic subtype of tumor, grade, T-stage, N-stage, and response (pathological complete remission (pCR) of breast and axilla) were compared between samples suitable or unsuitable for gene expression analysis. The $\chi 2$ (Spearman) was used to compare dichotomized variables. We also assessed differences in clinical characteristics for each exclusion criteria as described above, i.e. tissue quality of the frozen biopsy, tumor cell percentage, and RNA quantity as well as quality. Multivariate logistic regression analysis was performed to assess the independent association of various clinical variables with the ability to perform GE analysis. Recurrence-free survival was assessed with Kaplan-Meier plots and the log rank test. A cox proportional hazard model was built to assess if the ability to perform GE analysis was independently associated with recurrence-free survival. The SPSS Package 23.0 was used for statistical analyses and p-values (two-sided) <0.05 were considered statistically significant. This study was designed according to the Reporting recommendations for tumor MARKER prognostic studies (REMARK) guidelines [12].

For the validation dataset the variables age, histologic type of tumor, subtype, histological grade, and T-stage were compared between tumor samples suitable or ineligible for gene expression analysis. Statistical analysis was performed as described above.

Results

Patient selection in cohort

A total of 738 breast cancer patients were treated in the NKI with neoadjuvant chemotherapy between 2004 and 2012. From 665 patients a frozen biopsy was available. Seventy-seven tissue samples were not processed because the biopsies were too small or too fatty, or did not contain invasive tumor. Of the remaining 587 tissue specimens, 461 had a tumor percentage of more than 50%; 391 of these samples met the criteria for sufficient RNA quality and quantity, allowing gene expression analysis (53% of the total cohort). Figure 1 shows the sample selection flow diagram.

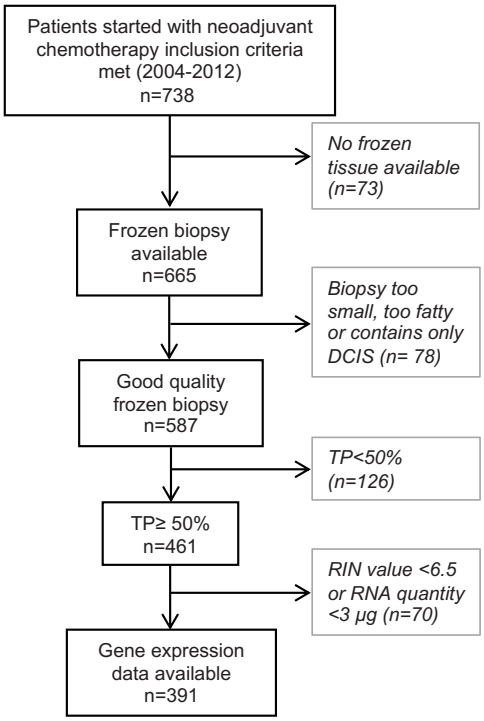


Figure 1. Flow diagram of included patients and in- and exclusion steps of the fresh frozen biopsies for gene expression studies. *TP denote tumor percentage, DCIS ductal carcinoma in situ, RIN RNA integrity number*

Association with clinical characteristics

Comparisons of baseline characteristics between the tumors for which a gene expression (GE) profile could be generated (GE+; n=391) and the tumors for which this was not possible (GE-; n=347) are shown in Table 1. GE+ tumors were more often high grade and had a higher SUV max value than GE- tumors. When we

stratify for subtype, the effect of grade is still significant in the ER+HER2- and in the triple negative subgroup (see Supplementary, Table 1). In the ER+HER2- subgroup these samples also have a higher SUV max value. Multivariate analysis shows that a high tumor grade and positive lymph node status are independently associated with GE+ tumors (Table 2).

Table 1. Characteristics of patients with gene expression profiles versus patients without gene expression profiles. *Due to rounding, some percentages do not count up to 100%. GE denoted gene expression, IDC invasive ductal carcinoma, ILC invasive lobular carcinoma, ER oestrogen receptor, HER2 ERBB2, SUV standardized uptake value.*

		no GE data available (n=347)	**GE data available (n=391)**	**p-value**
Age	<50	189 (55%)	237 (61%)	0.13
	>=50	153 (45%)	153 (39%)	
	Unknown	5	1	
Histology	IDC	244 (87%)	291 (90%)	0.25
	ILC	38 (13%)	34 (10%)	
	Unknown	65	66	
Subtype	ER+,Her2 -	182 (53%)	182 (47%)	0.25
	Her2+	73 (21%)	96 (25%)	
	Tripleneg	91 (26%)	113 (29%)	
	Unknown	1	0	
ER	ER Neg	122 (35%)	149 (38%)	0.44
	ER Pos	223 (65%)	242 (62%)	
	Unknown	2	0	
HER2	Her2 Neg	272 (79%)	293 (75%)	0.26
	Her2 Pos	73 (21%)	96 (25%)	
	Unknown	2	2	
Grade	Grade 1 & 2	184 (70%)	158 (48%)	**<0.01**
	Grade 3	80 (30%)	170 (52%)	
	Unknown	83	63	
T-stage	T1/T2	232 (68%)	270(70%)	0.65
	T3/T4	109 (32%)	118 (30%)	
	Unknown	6	3	
N-stage	neg	104 (30%)	94 (24%)	0.06
	pos	239 (70%)	294 (76%)	
	Unknown	4	3	
Response	No pCR	270 (79%)	293 (75%)	0.22
(breast and lymph node)	pCR	72 (21%)	97 (25%)	
	Unknown	5	1	
Ki-67	>15%	103 (49%)	98 (49%)	0.95
	≤15%	107 (51%)	103 (51%)	
	Unknown	137	190	
Mass (MRI)	Non-mass	47 (55%)	53 (55%)	0.94
	Mass	39 (45%)	43 (45%)	
	Unknown	261	295	
Maximal SUV-uptake mean (sd)		7.19 (4.55)	10.03 (6.79)	**0.03**
Unknown		259	294	

Table 2. Multivariate analyses of patient characteristics with gene expression profiles versus patients without gene expression profiles. *An Odds ratio above 1 means that gene expression analysis is more likely in this patient group. ER denoted oestrogen receptor, HER2 ERBB2.*

Variable		Frequency	Odds ratio	95% CI	P-value
Age	<50	339	1.00		
	>=50	245	0.91	0.64 – 1.28	0.58
ER	ER Neg	218	1.00		
	ER Pos	366	1.19	0.81 – 1.74	0.38
HER2	Her2 Neg	440	1.00		
	Her2 Pos	144	1.20	0.81 – 1.80	0.36
Grade	Grade 1 & 2	336	1.00		
	Grade 3	248	2.56	1.77 – 3.71	**<0.01**
T-stage	T1/T2	408	1.00		
	T3/T4	176	0.94	0.65 – 1.35	0.73
N-stage	Neg	163	1.00		
	Pos	421	1.53	1.05 – 2.23	**0.03**

To investigate the influence of the various steps of sample selection for gene expression analysis in more detail, we compared the clinical variables between in- and excluded samples after each selection step (Table 3). Interestingly, in every selection step, we enrich for high-grade tumors: a high tumor grade is associated with larger biopsies, higher tumor percentage and high quality and quantity RNA. In addition, high quality and sufficient quantity of RNA is more often found in HER2+ tumors and node-positive tumors.

Chemotherapy response was not significantly different between GE+ and GE- tumors. However, we observed that tumors with high tissue quality of frozen biopsies more often achieved a pathological compete response (pCR) after treatment than samples with poor quality biopsies (p=0.02; Table 3). We did not observe a significant difference in recurrence free survival between samples included and excluded in gene expression analysis, although a trend was visible in triple negative patients (Fig. 2). A cox proportional hazard model did not indicate GE+ as a variable associated with survival (Supplementary, Table 2).

Results in the validation dataset

The RASTER data were used to validate our observations. This set includes 812 breast cancer patients enrolled between 2004 and 2006 (see Methods). Because node positive patients were excluded in this study for clinical reasons and therefore a gene expression profile was not performed, we analyzed the samples of node negative patients (n=585, see Supplementary Figure 1, for a flowchart). Therefore, we could not validate our association with nodal status in this set; however, most other clinical variables were available. Of these samples, 27% dropped out because of incorrect procedure or sample failure. Similar to the observations in the neoadjuvant cohort, gene expression profiling was more often possible in high-grade tumors (borderline significant; p=0.05, Table 4).

Table 3. Characteristics of patients excluded versus remaining group. Due to rounding, some percentages do not count up to 100%. *IDC denoted invasive ductal carcinoma, ILC invasive lobular carcinoma, ER oestrogen receptor, HER2 ERBB2, pCR pathological complete response. Association with treatment response and survival*

		Frozen tissue available			**Quality of frozen biopsy**			**Tumor%**			**RNA Quality and quantity**		
		NO (n=73)	YES (n=665)	p-value	Insufficient (n=78)	Sufficient (n=587)	p-value	<50% (n=126)	>=50% (n=461)	p-value	Insufficient (n=70)	Sufficient (n=391)	p-value
Age	<50	44 (62%)	382 (58%)	0.50	34 (45%)	348 (59%)	**0.02**	77 (61%)	271 (59%)	0.66	34 (49%)	237 (61%)	0.06
	>=50	27 (38%)	279 (42%)		41 (55%)	238 (41%)		49 (39%)	189 (41%)		36 (51%)	153 (39%)	
	Unknown	2	4		3	1		0	1		0	1	
Histology	IDC	58 (92%)	477 (88%)	0.31	49 (85%)	428 (88%)	0.43	88 (87%)	340 (88%)	0.74	49 (82%)	291 (90%)	0.08
	ILC	5 (8%)	67 (12%)		9 (16%)	58 (12%)		13 (13%)	45 (12%)		11 (18%)	34 (11%)	
	Unknown	10	121		20	101		25	76		10	66	
Subtype	ER+,Her2 -	33 (45%)	331 (50%)	0.62	46 (60%)	285 (49%)	0.18	62 (49%)	223 (48%)	0.79	41 (59%)	182 (47%)	**0.04**
	Her2+	20 (28%)	150 (23%)		14 (18%)	136 (23%)		31 (24%)	104 (23%)		8 (11%)	96 (25%)	
	Triple negative	20 (28%)	183 (27%)		17 (22%)	167 (28%)		33 (26%)	134 (29%)		21 (30%)	113 (29%)	
	Unknown	0	1		1	0		0	0		0	0	
ER	ER Neg	26 (36%)	245 (37%)	0.82	24 (32%)	221 (38%)	0.30	48 (38%)	173 (38%)	0.91	24 (34%)	149 (38%)	0.54
	ER Pos	47 (64%)	418 (63%)		52 (68%)	366 (62%)		78 (62%)	288 (63%)		46 (66%)	242 (62%)	
	Unknown	2	2		2	0		0	0		0	0	
Her2	Her2 Neg	52 (72%)	513 (78%)	0.31	63 (82%)	450 (77%)	0.33	95 (75%)	355 (77%)	0.65	62 (89%)	293 (75%)	**0.02**
	Her2 Pos	20 (28%)	149 (22%)		14 (18%)	135 (23%)		31 (25%)	104 (23%)		8 (11%)	96 (25%)	
	Unknown	1	3		1	2		0	2		0	2	
Grade	Grade 1 & 2	36 (72%)	306 (57%)	**0.03**	43 (72%)	263 (55%)	**0.01**	61 (66%)	202 (52%)	**0.02**	44 (72%)	158 (48%)	**<0.01**
	Grade 3	14 (28%)	236 (43%)		17 (28%)	219 (45%)		32 (34%)	187 (48%)		17 (28%)	170 (52%)	
	Unknown	23	123		18	105		33	72		9	63	
T-stage	T1/T2	43 (61%)	459 (70%)	0.11	58 (78%)	401 (69%)	0.09	84 (67%)	317 (69%)	0.59	47 (67%)	270 (70%)	0.68
	T3/T4	28 (39%)	199 (30%)		16 (22%)	183 (31%)		42 (33%)	141 (31%)		23 (33%)	118 (30%)	
	Unknown	2	7		4	3		0	3		0	3	
N-stage	neg	21 (29%)	177 (27%)	0.68	21 (28%)	156 (27%)	0.81	35 (28%)	121 (26%)	0.76	27 (39%)	94 (24%)	**0.01**
	pos	51 (71%)	482 (83%)		54 (72%)	428 (73%)		91 (72%)	337 (74%)		43 (61%)	294 (76%)	
	Unknown	1	6		3	3		0	3		0	3	
Response	No pCR	55 (76%)	508 (77%)	0.91	65 (88%)	443 (76%)	**0.02**	98 (78%)	345 (75%)	0.52	52 (74%)	293 (75%)	0.88
	pCR	17 (24%)	152 (23%)		9 (12%)	143 (24%)		28 (22%)	115 (25%)		18 (26%)	97 (25%)	
	Unknown	1	5		4	1		0	1		0	1	

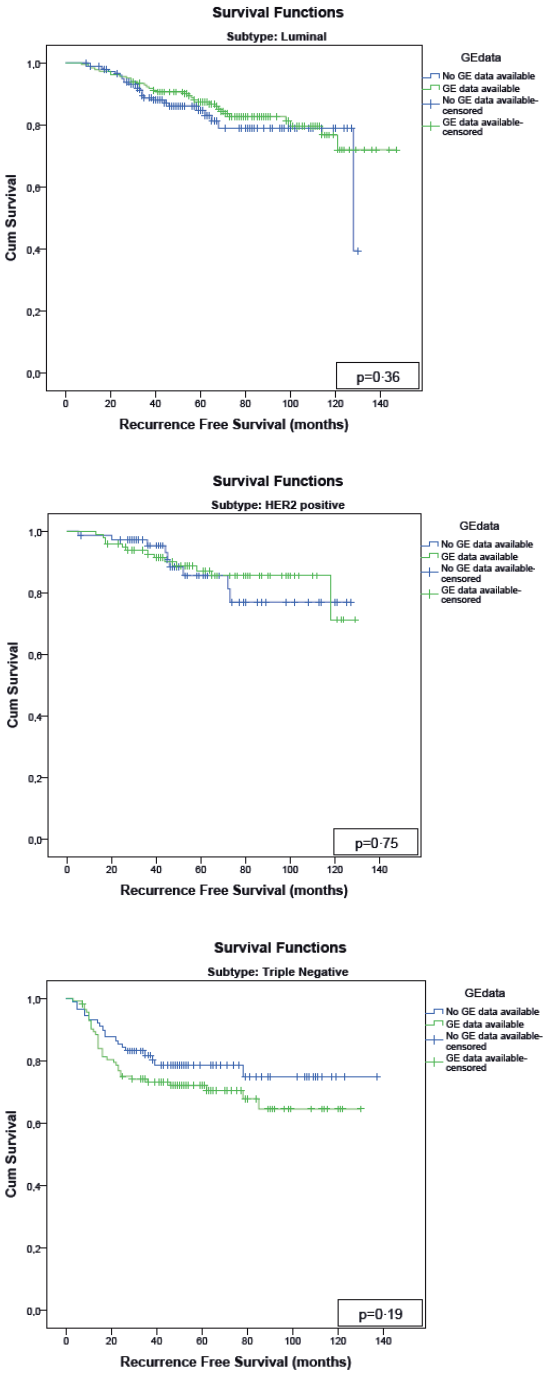


Figure 2. Kaplan-Meier curves of recurrence free survival to compare patients with and without gene expression profiles, stratified by subtype. *GE denoted gene expression*

Table 4. Patient characteristics in the validation dataset, split for gene expression status. *GE denoted gene expression, IDC invasive ductal carcinoma, ILC invasive lobular carcinoma, ER oestrogen receptor, HER2 ERBB2.*

		no GE data available(n=158)	GE data available (n=427)	p-value
Age	<50 yr	86 (54%)	251 (59%)	0.34
	≥50 yr	72 (46%)	176 (41%)	
	Unknown	0	0	
Histology	IDC	125 (79%)	345 (81%)	0.11
	ILC	12 (7%)	47 (11%)	
	Rest	21 (13%)	35 (8%)	
	Unknown	0	0	
Subtype	Luminal	121 (77%)	312 (73%)	0.49
	Her2	19 (12%)	48 (11%)	
	Triple negative	18 (11%)	65 (15%)	
	Unknown	0	2	
ER	ER Neg	23 (15%)	85 (20%)	0.14
	ER Pos	135 (85%)	342 (80%)	
	Unknown	0	0	
Her2	Her2 Neg	119 (75%)	358 (88%)	0.55
	Her2 Pos	19 (14%)	48 (1%)	
	unknown	20	21	
Grade	Grade 1 & 2	120 (76%)	291 (68%)	**0.05**
	Grade 3	37 (24%)	136 (32%)	
	Unknown	1	0	
T-stage	T1/T2	158 (100%)	426 (99%)	0.54
	T3/T4	0	1 (1%)	
	Unknown	0	0	

Discussion

In this study, we showed that patients of whom gene expression data were obtained had more often high grade tumors and lymph node metastasis, features associated with a worse prognosis. It is important to acknowledge that, due to the selection of tumors with good quality samples and a high cellularity for gene expression studies, we select for a certain subgroup of tumors. Most likely, this selection bias is present in the majority of published gene expression studies for breast cancer.

Our findings are indeed in line with published literature. Cremoux et al.[13] studied (pre-) analytical steps in tissue handling by comparing different institutes. As in our study, they found that tumors suitable for gene expression profiling were more often high grade and of ductal subtype. Mook et al. observed a 17% dropout and

concluded that the rejected samples were obtained from slightly smaller tumors[14]. Also Goetz et al. observed that women without expression data had more often a small tumor[15]. Together with the results of our validation dataset, there is substantial evidence towards the selection of larger and more aggressive tumors for gene expression studies.

In a systematic literature search that we performed prior to this study (Supplementary Figure 2), finally resulting in 110 articles, 39% of the studies were indistinct about exclusion criteria and associated dropout rates, indicating unawareness. The remaining 61% did mention about exclusion criteria and numbers. These studies show a variety of dropout rates (1-83%, average 21%). Most gene expression profiles had been developed on frozen sample collections available at the biobank of the respective institute. These samples are a selection of relatively large and easily accessible tumors. Also, only samples that met the strict criteria of tumor RNA quality and quantity were used. Nowadays FFPE material from all laboratories (both biopsies and resection material), with very different protocols, can be measured by commercial available platforms, such as Mammaprint and OncotypeDx, and tumor percentage can be as low as 30% [16]. This is possible due to advances in the technique. However, we should be aware that such assays were originally not developed on samples with comparable characteristics, and validation on small samples with lower tumor cell percentage is warranted. In addition, also for research purposes, it is important to acknowledge that due to analytical requirements high-grade tumors might be overrepresented in GE datasets.

This study has some limitations. First, fresh frozen tumor samples were used. In general it is more difficult to obtain fresh frozen material than FFPE tumor material, resulting in a higher dropout rate. Second, this study was done on pre-treatment biopsies, which yield smaller quantities of tumor material than resection specimens. Third, this study was performed in the neoadjuvant setting, which results in the selection of locally advanced tumors. Consequently, we could not look at stage I or stage IV tumors. Strong points of our study are that samples were obtained from a consecutive cohort of neoadjuvant treated patients, and not on a highly selected clinical trial population. Furthermore, we had an independent cohort for validation purposes that corroborated our findings. Although there were some differences in the way the samples were collected (resections versus biopsies, mailed transport versus snap frozen), the validation cohort consisted of early stage breast cancer samples and had information on grade, enabling us to validate our main finding in an independent cohort. Finally, all samples were from one institute and handled by one dedicated technician to preclude variability in centre or in lab handling.

In conclusion, we showed that breast cancers for which gene expression data were successfully obtained were associated with a higher grade and with lymph node

metastasis, due to the selection of samples with a high tumor percentage and good quality RNA. These tumors have, on average, a more aggressive phenotype and a relatively poor prognosis. In general, when interpreting test results, it is important to realize that patient populations for which GE profiles are used, often differ substantially from the ones in which they were originally developed, particularly when using a development cohort consisting of frozen tumor tissue and a test cohort consisting of FFPE samples. At this point, it is uncertain what the impact might be on treatment decisions in the clinic.

Additional information

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval and informed consent

Specific approval and informed consent for the current study was not required, because the current study comprises a retrospective analysis of residual tissue samples. According to the policy of The Netherlands Cancer Institute residual tissue may be used for scientific purposes unless a patient explicitly objects.

Consent for publication

Not applicable.

Authors' contributions

EL, JW designed the study. MS, AM did the literature search. LW, SR, GS, CD provided clinical data. CL provided radiological data. EL, LM prepared the samples. EL, JW, MS, AM interpreted the data and wrote the manuscript. AM, MS analyzed the data. MS, AM, LM, MH, SR, GS, JW, EL discussed the data. All authors reviewed the manuscript and approved the final version.

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Supplementary files

Supplementary Table 1. Characteristics of patients with gene expression profiles versus patients without gene expression profiles separated by subtype *GE denoted gene expression, IDC invasive ductal carcinoma, ILC invasive lobular carcinoma, ER estrogen receptor, HER2 ERBB2, T-stage tumor stadium, N-stage N stadium, SUV standardized uptake value*

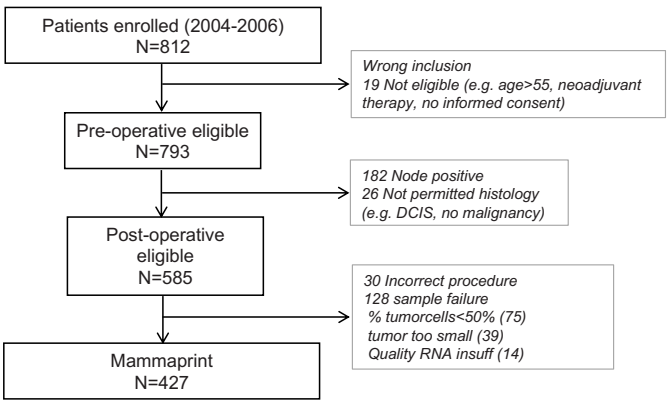
Luminal				
		no GE data available (n=182)	GE data available (n=182)	p-value
Age	<50 yr	95 (53%)	104 (57%)	0.44
	>=50 yr	84 (47%)	78 (43%)	
	Unknown	3	0	
Histology	IDC	135 (80%)	139 (82%)	0.74
	ILC	33 (20%)	31 (18%)	
	Unknown	14	12	
Grade	Grade 1 & 2	122 (88%)	95 (65%)	**<0.001**
	Grade 3	16 (12%)	52 (35 %)	
	Unknown	44	35	
T-stage	T1/T2	120 (67%)	120 (66%)	0.88
	T3/T4	60 (33%)	62(34%)	
	Unknown	2	0	
N-stage	neg	44 (24%)	37 (20%)	0.35
	pos	136 (76%)	145 (80%)	
	Unknown	2	0	
Response (breast)	NR or PR	143 (79%)	147 (81%)	0.75
	(n)pCR	37 (21 %)	35 (19%)	
	Unknown	2	0	
Response (breast and lymph node)	No pCR	168 (93 %)	174 (96%)	0.34
	pCR	12 (7%)	8 (4%)	
	Unknown	2	0	
Ki-67	>15%	94 (67%)	93 (68%)	0.76
	≤15%	47 (33%)	43 (32%)	
	Unknown	41	46	
Mass (MRI)	Non-mass	29 (60%)	27 (68%)	0.49
	Mass	19 (40%)	13 (33%)	
	Unknown	134	142	
Maximal SUV uptake mean (sd)		6.12 (2.99)	7.85 (4.17)	**0.03**
Unknown		133	142	

HER2				
		no GE data available (n=73)	GE data available (n=96)	p-value
Age	<50 yr	37 (51%)	54 (57%)	0.43
	>=50 yr	36 (49%)	41 (43%)	
	Unknown	0	1	
Histology	IDC	45 (94%)	68 (97%)	0.37
	ILC	3 (6%)	2 (3%)	
	Unknown	25	26	
Grade	Grade 1 & 2	34 (60%)	40 (44%)	0.07
	Grade 3	23 (40%)	50 (56%)	
	Unknown	16	6	
T-stage	T1/T2	45 (62%)	65 (69%)	0.31
	T3/T4	28 (38%)	29 (31%)	
	Unknown	0	2	
N-stage	neg	18 (25%)	17 (18%)	0.30
	pos	55 (75%)	77 (82%)	
	Unknown	0	2	
Response (breast)	NR or PR	22 (31%)	28 (30%)	0.92
	(n)pCR	50 (69%)	66 (70%)	
	Unknown	1	2	
Response (breast and lymph node)	No pCR	41 (56%)	53 (56%)	0.96
	pCR	32 (44%)	42 (44%)	
	Unknown	0	1	
Ki-67	>15%	7 (25%)	5 (21%)	0.72
	≤15%	21 (75%)	19 (79%)	
	Unknown	45	72	
Mass (MRI)	Non-mass	13 (68%)	14 (74%)	0.72
	Mass	6 (32%)	5 (26%)	
	Unknown	54	77	
Maximal SUV uptake mean (sd)		5·75 (2·80)	7·1 (2·52)	0.12
Unknown		53	76	

Triple negative				
		no GE data available (n=91)	GE data available (n=113)	p-value
Age	<50 yr	56 (63%)	79 (70%)	0.30
	>=50 yr	33 (37%)	34 (30%)	
	Unknown	2	0	
Histology	IDC	64 (97%)	84 (99%)	0.42
	ILC	2 (3%)	1 (1%)	
	Unknown	25	28	
Grade	Grade 1 & 2	28 (41%)	23 (25%)	**0.04**
	Grade 3	41 (59%)	68 (75%)	
	Unknown	22	22	
T-stage	T1/T2	67 (76%)	85 (76%)	0.97
	T3/T4	21 (24%)	27 (24%)	
	Unknown	3	1	
N-stage	neg	41 (46%)	40 (36%)	0.14
	pos	48 (54%)	72 (64%)	
	Unknown	2	1	
Response (breast)	NR or PR	37 (42%)	47 (42%)	0.95
	(n)pCR	51 (58%)	66 (58%)	
	Unknown	3	0	
Response (breast and lymph node)	No pCR	61 (69%)	66 (58%)	0.14
	pCR	28 (32%)	47 (42%)	
	Unknown	2	0	
Ki-67	>15%	2 (5%)	0 (0%)	0.15
	≤15%	39 (95%)	41 (100%)	
	Unknown	50	72	
Mass (MRI)	Non-mass	4 (22%)	12 (32%)	0.43
	Mass	14 (78%)	25 (68%)	
	Unknown	73	76	
Maximal SUV uptake mean (sd)		11.71 (6.63)	13.97 (8.65)	0.33
Unknown		73	76	

Supplementary Table 2. Multivariate cox proportional hazard analysis of the risk of recurrence (recurrence free survival) for clinical markers and a succesful gene expression profile. *GE denoted gene expression, IDC invasive ductal carcinoma, ILC invasive lobular carcinoma, ER estrogen receptor, HER2 ERBB2, T-stage tumor stadium, N-stage N stadium*

Variable		event/total n	HR (95% CI)	p-value
Age	<50 year	72/426	1.00	0.15
	>= 50 year	62/306	1.33 (0.90-1.96)	
ER	Neg	58/269	1.00	**0.03**
	Pos	77/463	0.62 (0.40-0.94)	
HER	Neg	112/561	1.00	**0.001**
	Pos	23/169	0.41 (0.24-0.69)	
Grade	Grade 1 or 2	55/341	1.00	0.23
	Grade 3	56/250	1.30 (0.85-1.96)	
T_stage	T1/T2	85/502	1.00	0.22
	T3/T4	48/227	1.28 (0.86-1.90)	
N_stage	Neg	19/198	1.00	**<0.001**
	Pos	115/533	3.10 (1.76-5.48)	
GEdata	No GE data available	55/343	1.00	0.33
	GE data available	80/391	1.23 (0.82-1.85)	



Supplementary Figure 1. Flow diagram of included patients in validation dataset (RASTER study). DCIS denoted ductal carcinoma in situ



Chapter 6

Prognostic value of residual disease after neoadjuvant therapy in HER2-positive breast cancer evaluated by Residual Cancer Burden, Neoadjuvant Response Index & Neo-Bioscore

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ABSTRACT

Purpose

In breast cancer, pathologic complete response (pCR) to neoadjuvant systemic therapy (NST) is associated with favorable long-term outcome. Trastuzumab-emtansine as additional adjuvant therapy improves recurrence-free survival of patients with HER2-positive breast cancer without pCR, but it is uncertain whether all patients without pCR need additional therapy. We evaluated the prognostic value of residual disease after trastuzumab-based NST in patients with HER2-positive breast cancer using Residual Cancer Burden (RCB), Neoadjuvant Response Index (NRI) and Neo-Bioscore.

Experimental Design

We included patients with stage II or III HER2-positive breast cancer, treated with trastuzumab-based NST and surgery at the Netherlands Cancer Institute between 2004 and 2016. RCB, NRI and Neo-Bioscore were determined. Primary endpoint was 5-year recurrence-free interval (RFI). A 3% difference compared with the pCR-group was considered acceptable as noninferiority margin on the 5-year RFI estimate, based on a proportional hazards model, and its lower 95% confidence boundary.

Results

A total of 283 women were included. Median follow-up was 67 months (interquartile range 44–100). A total of 157 patients (56%) with pCR (breast and axilla) had a 5-year RFI of 92% (95%CI, 88-97); patients without pCR had a 5-year RFI of 80% (95%CI, 72-88). Patients with an RCB=1 (N=40, 15%), an NRI-score between 0.75-0.99 (N=30, 11%), or a Neo-Bioscore of 0 to 1 (without pCR; N=28, 11%) have a 5-year RFI that falls within a pre-defined noninferiority margin of 3% compared to patients with pCR.

Conclusions

The RCB, NRI, and Neo-Bioscore can identify patients with HER2-positive breast cancer patients with minimal residual disease (i.e., RCB=1, NRI ≥0.75 or Neo-Bioscore=0-1) after NST who have similar 5-year RFI compared with patients with pCR.

Introduction

Neoadjuvant systemic therapy (NST) is increasingly used in patients with high-risk breast cancer, in particular in case of HER2-positive disease. NST increases rates of breast-conserving surgery and enables response monitoring during therapy. In addition, the pathological response after therapy is increasingly recognized as prognostic indicator to guide further treatment. The recently published Katherine-study used non-pathologic complete response (non-pCR) to select patients for additional adjuvant therapy with the trastuzumab drug-conjugate trastuzumab-emtansine (T-DM1). T-DM1 reduced the relative risk of recurrence of invasive breast cancer or death with 50% and the risk of distant recurrence with 40% compared with trastuzumab alone(1). The effect was consistent in all subgroups. HR for patients with ypT0, ypT1a, ypT1b, ypT1mic, ypTis, ypT1 or ypT1c, ypT2, and ypT3 were 0.66 (95% confidence interval (CI), 0.44-1.00), 0.34 (95% CI, 0.19-0.62), 0.50 (95% CI, 0.31-0.82), and 0.40 (95% CI, 0.18-0.88), respectively. As patients' recurrence risk is related to the extent of residual disease after NST, adjuvant therapy might be adapted according to an individual patients' risk.

A pCR is associated with favorable long-term outcome, in particular for triple-negative and HER2-positive breast cancer (2–5). However, with the binary outcome of pCR, valuable response information is lost. Therefore, other response indices that quantify the amount of residual disease were developed including the Residual Cancer Burden (RCB), Neoadjuvant Response Index (NRI), and Neo-Bioscore. The RCB uses the diameter of residual disease, percentage of vital tumor cells, and diameter of largest involved lymph node to quantify residual disease (6). The quantification of residual disease based on the RCB is prognostic for long-term survival after neoadjuvant chemotherapy in hormone receptor–positive/HER2-negative, HER2-positive, and triple-negative subgroups of breast cancer (6). The NRI is a semicontinuous score between 0 and 1 in which the extent of downstaging of the primary tumor and involved axillary lymph nodes is measured (7). It has been shown to better reflect efficacy of NST than the binary pCR classification in breast cancer. Its value to predict recurrence-free survival was validated in an independent cohort of patients with triple negative breast cancer (7,8). The Neo-Bioscore uses clinical stage, pathological stage after NST, estrogen receptor (ER) and HER2-status, and nuclear grade to create seven response categories (9,10). The final score provides a more refined stratification for disease-specific survival than pretreatment clinical stage or final pathologic stage alone across breast cancer subtypes (9,10).

We compared the RCB, NRI, and Neo-Bioscore and established the long-term prognosis based on various categories of residual disease in HER2-positive breast cancer.

Statement of translational relevance

The prognostic value of minimal residual disease after neo-adjuvant breast cancer treatment has become increasingly important as it can aid decision making for additional adjuvant therapy. Non-pathological complete response was used as selection criterion in the recently published Katherine study that showed improved long-term outcome with adjuvant T-DM1; however, offering T-DM1 to all patients with non-pathological complete response may result in significant overtreatment if patients with minimal residual disease have a similarly good prognosis as those with no residual disease. In this study we used Residual Cancer Burden, Neoadjuvant Response Index and Neo-Bioscore to evaluate prognostic value of residual diseases after trastuzumab-based neoadjuvant therapy. We think that using these response indices could help to decide if patients need additional systemic therapy and therefore should (after validation) be incorporated in clinical practice.

Materials and Methods

Patients and data collection

All patients with primary invasive noninflammatory HER2-positive stage II or III breast cancer who received trastuzumab-based neoadjuvant therapy at the Netherlands Cancer Institute between November 2004 and February 2016 were included. Patients with bilateral breast cancer, those who did not undergo surgery for other reasons than inoperability, patients with progressive disease prior to surgery, and those with prior breast cancer <25 years ago were excluded.

Patients were identified from the Netherlands Cancer Institute's tumor registry. Patient, tumor, and treatment characteristics were extracted from the medical records. All patients received one full year of trastuzumab according to Dutch national guidelines, unless precluded by toxicity. Adjuvant chemotherapy in case of non-pCR was not in our institute's guideline. Endocrine therapy was given for 5 to 10 years adjuvantly according to up-to-date guidelines. HER2 positivity was defined as a score of 3+ by immunohistochemistry or gene amplification by *in situ* hybridization (11,12). ER and progesterone receptor (PR) positivity was defined as nuclear staining of ≥10% based on European and Dutch guidelines (12,13). Clinical and pathologic staging was based on the tumor-node-metastasis (TNM) classification, American Joint Committee on Cancer (AJCC) stage 6th and 7th based on year of diagnosis. According to these guidelines, the presence of isolated tumor cells (<0.2mm) in the lymph nodes was classified as pN0.

Clinical nodal staging was based on all available information from imaging and results of the sentinel node procedure. The subclassification of a positive nodal stage in N1, N2, or N3 was based on the number and localization of positive lymph nodes, similar to the pathologic nodal staging system of the TNM classification. This adapted counting of positive lymph nodes based on radiology results was used because we could not distill from the patients' records whether the palpable lymph nodes were movable or fixed, and it may better reflect current practice.

This study was approved by the Review Board of the Netherlands Cancer Institute and conducted in accordance with the Declaration of Helsinki.

Response indices

pCR

Pathologic responses were assessed by breast pathologists at the Netherlands Cancer Institute and extracted from original reports. PCR was defined as no residual invasive tumor in breast and axilla (ypT0/is, ypN0).

RCB

The RCB quantifies the extent of residual disease after NST for patients into four categories. RCB=0 is equal to pCR for breast and axilla, RCB=1 indicates minimal residual disease, RCB=2 indicates intermediate residual disease and RCB=3 extensive residual disease (9). To calculate RCB scores, all surgical specimens (breast and axilla tissue) of patients without pCR were reviewed and scored (MvS) as described previously (6). In case of uncertainty of extent of residual disease slides were discussed with another breast cancer specialized pathologist (JW).

NRI

The NRI is a score between 0 and 1 and uses a ratio of pre-NST and post-NST information to classify patients. A score of 1 represents pCR in breast and axilla and a score of 0 indicates no downstaging (or progression). The NRI calculation was based on original pathology reports as described previously (7). In brief, the NRI is the sum of a breast and a nodal response score divided by the maximum achievable score, which is based on the clinical tumor and nodal stage. For our analysis we used a slightly adapted version of the nodal response score (described above) to make it more suitable for current practice. The exact calculation and adapted allocation of points are summarized in Supplementary Table S1.

Neo-Bioscore

The Neo-Bioscore was calculated for each patient based on information from the medical records according to the previous reported staging system, with the exception that clinical nodal staging was performed as described above (9,10). The Neo-Bioscore gives points for higher clinical stages (higher than IIB), higher

pathologic stages (II and III), ER-negativity, grade 3 and HER2-negativity. A higher score represents more unfavorable prognostic characteristics. The maximum Neo-Bioscore in HER2-positive patients is 6, as none receives a point for HER2 negativity. Please note that a score of 0 does not represent pCR.

Statistical analyses

Descriptive statistics were used for baseline and surgery characteristics. For all patients and for the subgroup of patients without a pCR the median NRI was calculated.

Recurrence-free interval (RFI) was calculated as time from breast cancer diagnosis until locoregional or distant recurrence or death due to breast cancer, whichever came first (14). Patients without distant metastases at last follow-up or death due to other or unknown causes were censored at the corresponding dates. Breast-cancer-specific survival (BCSS) was defined as date of diagnosis until date of death due to breast cancer. Patients alive at last follow-up or who died due to other or unknown causes were censored at the respective dates. Database cutoff was set on October 2, 2018.

Follow-up time was calculated with the reverse Kaplan-Meier method. Cox proportional hazards models were used to provide hazard ratios (HR) and estimate the RFI-probabilities at five years with their corresponding 95% confidence interval (CI). In order to allow for nonlinearity of their effects, the NRI and Neo-Bioscore were entered as a continuous variable with a restricted cubic-spline transformation. Four knots were chosen so that the resulting model would have approximately 10 events per degree of freedom. For the NRI however it was not possible to place 4 knots so 3 were placed instead. It was not possible to place even 3 knots in a meaningful way for the RCB, because it has only 4 categories. Therefore, a quadratic polynomial model was used instead of a spline-curve for RCB.

A 3% difference in RFI was defined as noninferiority margin. The 3% margin is internationally used in treatment decisions whether to add chemotherapy (15). The cutoff of the NRI score was chosen such that the 5-year RFI-estimate at the cutoff and the lower bound of the 95% CI were within a margin of 3% from the estimate and lower 95% CI bound of the pCR group. For the RCB and Neo-Bioscore we used the predefined categories (6,9,10). The number of patients identified in this way as a percentage of the non-pCR patients, was compared across the three methods with Fisher exact test.

P-values <0.05 were considered statistically significant; all tests were two sided. Statistical analyses were performed using R version 3.5.2.

Results

Patients

We identified 303 patients who were treated with neoadjuvant trastuzumab-based therapy between November 2004 and February 2016, at The Netherlands Cancer Institute. Of them, 283 met the inclusion criteria and were included in the analyses. Figure 1 summarizes numbers and reasons for exclusion. Baseline characteristics, treatment regimens, and surgery are summarized in Table 1.

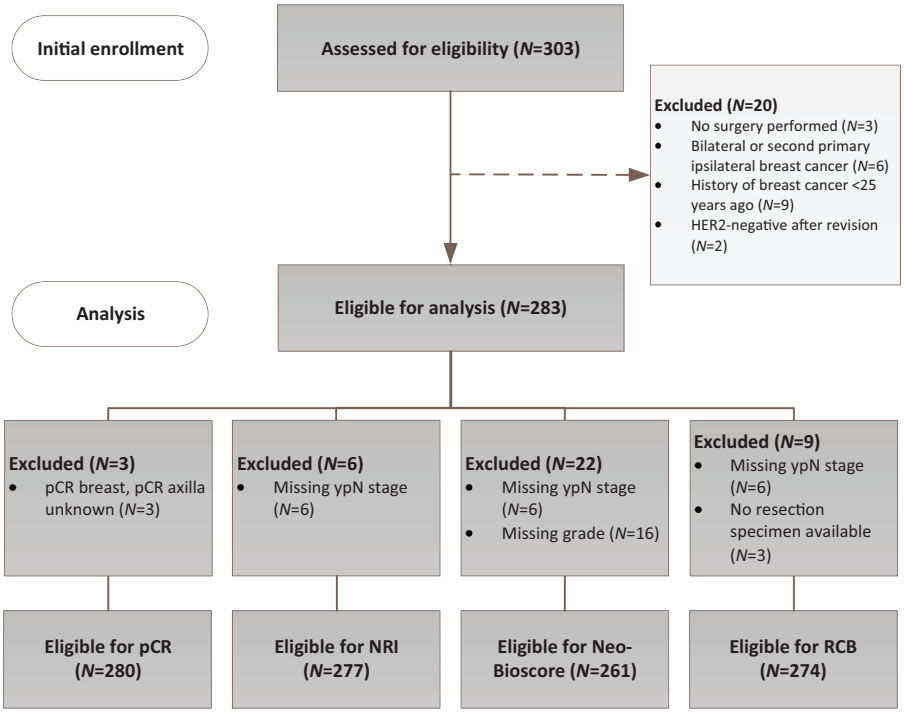


Figure 1. Consort flow diagram of included and excluded patients for analysis.
N, number of patients; pCR, pathologic complete response; NRI, neoadjuvant response index; RCB, residual cancer burden; ypN, pathological nodal stage.

Table 1. Patient and treatment characteristics (*N*=283)

		N	(%)
Age	Median age in years (range)	48	(24-82)
Clinical tumor stage	TX	1	(<1)
	T1	32	(11)
	T2	178	(63)
	T3	70	(25)
	T4	2	(1)
Clinical nodal stage	N0	75	(27)
	N1mi	2	(1)
	N1	123	(43)
	N2	36	(13)
	N3	47	(17)
Clinical stage	IIA	77	(27)
	IIB	87	(31)
	IIIA	70	(25)
	IIIB	2	(1)
	IIIC	47	(17)
ER-status	Negative	135	(48)
	Positive	150	(52)
PR-status	Negative	194	(69)
	Positive	88	(31)
Tumor grade	1-2	127	(45)
	3	140	(49)
	Unknown	16	(6)
Histology	Ductal	261	(92)
	Lobular	13	(5)
	Other	9	(3)
Neoadjuvant therapy regimen			
Taxane-based	PTCb	176	(62)
	PTCb-Ptz	40	(14)
	PTCb → Vinorelbine/T	2	(<1)
Anthracycline/taxane	PTCb → FECT	13	(5)
	AC → PTCb	8	(3)
	AC → PT	2	(1)
	EC → PT	1	(<1)
	AC → PTCb-Ptz	1	(<1)
	FECT-Ptz → PTCb-Ptz	39	(14)
Other	Vinorelbine/T	1	(<1)
Neoadjuvant pertuzumab	No	203	(72)
	Yes	80	(28)
Surgical treatment			
Type of breast surgery	Breast conserving surgery	166	(59)
	Mastectomy (directly or later)	116	(41)
	No breast surgery[a]	1	(<1)
Axillary node dissection	No	142	(50)
	Yes	141	(50)

Table 1. Continued.

		N	(%)
Adjuvant treatment			
Adjuvant 1 year of trastuzumab-therapy completed	No	10[b]	(4)
	Yes	273	(96)
Adjuvant endocrine therapy in case of ER-positive tumor	No	3[c]	(2)
	Yes	147	(98)

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; PTCb, paclitaxel, trastuzumab, carboplatin; Ptz, pertuzumab; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; AC, doxorubicin, cyclophosphamide; PT, paclitaxel, trastuzumab; EC, epirubicin, cyclophosphamide.

[a] One patient had an occult breast cancer

[b] Five patients discontinued adjuvant trastuzumab treatment because of toxicity during neoadjuvant or adjuvant trastuzumab treatment, three patients declined adjuvant trastuzumab treatment, two patients did not start with adjuvant trastuzumab treatment for unknown reasons.

[c] Three patients declined endocrine therapy.

Response indices and 5-year RFI per category

The median follow-up was 67 months (interquartile range (IQR) = 44–101). In total, there were 37 patients (13%) who experienced an RFI-event: 5 patients had a locoregional recurrence and 32 patients had distant metastases as first RFI-event. The 5-year RFI for all patients was 87% (95% CI, 82–91).

One-hundred and fifty-seven patients (56%) achieved pCR. The pCR rate was significantly higher in ER-negative compared with ER-positive tumors (74% versus 40%, $P<0.001$). The 5-year RFI was 92% (95% CI, 88-97) for patients with pCR and 80% (95% CI, 72-88) for patients without pCR. As we defined the noninferiority margin as a maximum of 3% decrease in RFI, the extra patients with residual disease should have a 5-year RFI of minimum 89%, with a 95% lower bound CI of at least 85%.

One-hundred and sixty-one patients (59%) were classified as RCB=0. In the group with residual disease, 40 patients (15%) were classified as RCB=1, 61 (22%) as RCB=2, and 12 (4%) as RCB=3 (Fig. 2A). RCB was significant for RFI prognosis ($p<0.0001$) when modeled with a polynomial shape, although the test for nonlinearity was not significant ($p=0.18$). Relative hazard rates per RCB score and estimated 5-year RFI per class with corresponding 95% CI are shown in Fig. 3A and B and Supplementary Table S2. As can be distilled from the table, patients with an RCB=1 ($N=40$), 35% of patients without pCR meet the noninferiority margin of 89% 5-year RFI, and thus have a similar good prognosis as the pCR patients.

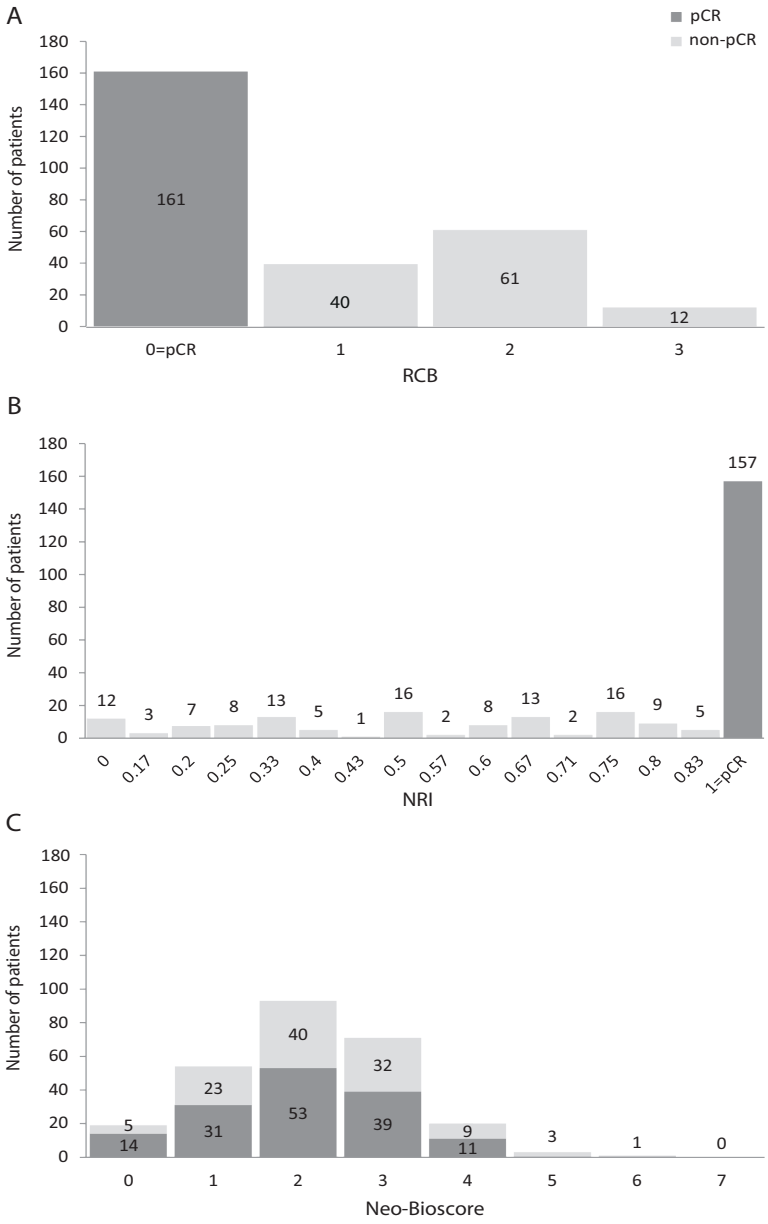


Figure 2. Distribution of patients in the different response indices.
A, distribution of RCB scores; B, distribution of NRI scores; C, distribution of Neo-Bioscore scores.
Legend: dark grey = pCR, light grey = residual disease. RCB, residual cancer burden; NRI, neoadjuvant response index; pCR, pathological complete response.

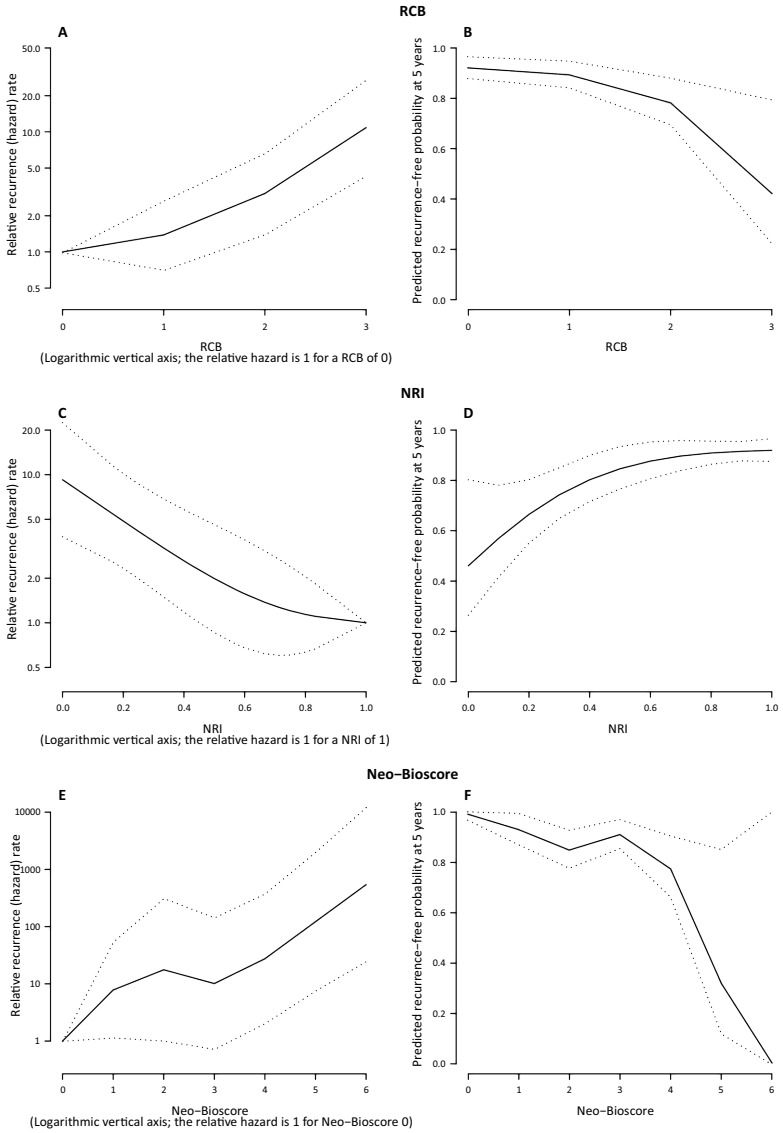


Figure 3. Relative hazard rates for the 5-year RFI prediction for RCB, NRI en Neo-Bioscore
A, Relative hazard rate for predicted 5-year recurrence-free-interval per RCB score in a quadratic polynomial model. The relative hazard is 1 for RCB=0; B, Five-year RFI estimates per RCB score. The 95%CI is shown with the dotted lines. C, Relative hazard rate for predicted 5-RFI per NRI score in a proportional hazards model treating NRI as continuous variable. The relative hazard is 1 for NRI=1; D, Five-year RFI estimates per NRI score. The 95%CI is shown with dotted lines. E, Relative hazard rate for predicted 5-year RFI per Neo-Bioscore score in a proportional hazards model treating Neo-Bioscore as continuous variable. F, Five-year RFI estimates per Neo-Bioscore. The 95%CI is shown with dotted lines.
RCB, residual cancer burden; NRI, neoadjuvant response index.

The median NRI was 1.00 (IQR= 0.60-1.00) in all patients and 0.50 (IQR =0.31-0.75) in the subgroup of patients who did not achieve pCR. The distribution of all NRI scores is shown in Fig. 2B. NRI was significant for RFI prognosis ($p<0.0001$) when modeled with a restricted cubic spline with 3 knots, although the test for nonlinearity was not significant ($p=0.30$). The relative hazard rates per NRI score are shown in Fig. 3C. Five-year RFI predictions per NRI score are shown in Fig. 3D and Supplementary Table S3. For NRI scores ≥0.75-0.99 the corresponding 5-year RFI is higher than 90% (95% CI, 85-96; Supplementary Table S3) and meet the noninferiority margin. As a result, 30 patients without pCR (25% of patients without pCR) were identified by NRI with similar good prognosis as pCR, this was not significantly different from the number identified by the RCB ($p=0.09$).

The distribution of the Neo-Bioscore in the overall cohort is summarized in Fig. 2C. Neo-Bioscore was significant for RFI prognosis ($p<0.0001$) when modeled with a spline curve with 4 knots, and the test for nonlinearity was significant ($p=0.008$). Relative hazard rates per Neo-Bioscore score are shown in Fig. 3E. Five-year RFI-estimates with 95% CI per score are given in Fig. 3F and Supplementary Table S4. Patients with Neo-Bioscores of 0 or 1 have a higher estimated 5-year RFI compared with patients achieving pCR; 99% (95% CI, 97-100) and 93% (95% CI, 87-99), respectively. These categories jointly comprise 72 patients, of whom 45 patients achieved pCR and 28 patients did not. As a result 28 patients (10%) without pCR were identified by Neo-Bioscore with similar good prognosis as pCR (not significantly different from the RCB, $p=0.11$).

Overlap of patients classified in the categories that meets the noninferiority margin by each response measure is shown in Supplementary Fig. S1. The difference as a percentage of non-pCR patients was not significantly different across the 3 methods ($p=0.14$).

Discussion

We evaluated the prognostic value of residual disease using the RCB, NRI, and Neo-Bioscore in a HER2-positive breast cancer patient cohort to select a subgroup with minimal residual disease after NST but similar long-term outcome as patients achieving pCR. Indeed, the RCB, NRI and Neo-Bioscore were all able to identify a group of patients within the 3% noninferiority margin of the 5-year RFI as the pCR-group, that is 92% (95% CI, 88-97).

Our findings underline the clinical importance of response indices that accurately predict long-term outcome of patients after neoadjuvant systemic therapy. An adequate neoadjuvant response measure serves at least two purposes. First, a response index with demonstrated prognostic value may aid selecting patients

for more, less or no additional adjuvant therapy. Second, the more accurate the magnitude of response can be assessed, the better we can evaluate the true effect of new treatments in neoadjuvant trials. We showed that all response measures give more prognostic information than the binary pCR index and thereby select a subgroup that could be considered similar to patients achieving pCR.

In our cohort, the 5-year RFI for all patients was 87%, patients who achieved pCR had an estimated 5-year RFI of 92%. This is comparable with 5-year follow-up data in studies evaluating trastuzumab in HER2-positive breast cancer. In the BCIRG-006 study 5-year disease-free survival of the two groups of patients who received trastuzumab was 82% and 84% (16). Recurrence-free survival of the subgroup with HER2-positive breast cancer, who achieved pCR according to Symmans and colleagues (17) was 95%. In the NeoSphere study, all treatment groups combined, 5-year disease-free survival was 85% for patients achieving pCR (18). However, these 5-year survival data leave room for improvement in the treatment of HER2-positive breast cancer. The recently published Katherine-study for patients who did not achieve pCR showed an improvement in invasive-disease-free survival and distant-recurrence-free survival for patients who received T-DM1 compared with patients who received trastuzumab-monotherapy adjuvantly (HR 0.50; 95% CI, 0.39-0.64). Overall survival results were not mature yet. The improvement was seen regardless of the size of the residual tumor, with some suggestion of a stronger effect in case of more extensive residual disease (1). Masuda and colleagues (19) showed benefit in overall survival from adjuvant capecitabine therapy after NST in patients with triple negative breast cancer who did not achieve pCR. Two trials are currently evaluating the effect of adjuvant chemotherapy in patients not achieving pCR after NST; one adds capecitabine (NCT03684863) in patients with HER2-positive breast cancer and the other is open for all breast cancer subtypes and adds eribulin (NCT01401959). Aside from optional additional adjuvant chemotherapy, all patients with HER2-positive breast cancer receive a total of 1-year trastuzumab, partly adjuvant. The optimal duration of adjuvant trastuzumab is questioned and three studies evaluated noninferiority of 6 months trastuzumab to the arbitrary set 12 months that is considered standard. Studies that compared 6 versus 12 months adjuvant trastuzumab all showed similar survival curves for shorter of longer duration, although noninferiority was not shown in all studies (20–22). Patients with an excellent prognosis based on a pCR or near pCR may be suitable candidates to further pursue a strategy to reduce the duration of trastuzumab-treatment adjuvantly. In order to select patients for additional adjuvant therapy and optimize the balance between improving outcome and forego overtreatment, adequate response measures are crucial.

The RCB, NRI and Neo-Bioscore definitions of minimal residual disease do not identify the exact same patient populations. Discrepancies appear due to unequal

weighing of tumor features, including lymph node status, ER, HER2 and grade. The RCB grants relatively high value to positive lymph nodes compared with NRI and Neo-Bioscore. Neo-Bioscore uses ER/HER2-status and tumor grade additional to downstage calculation, whereas NRI purely uses downstaging. To the best of our knowledge it is not known whether downstaging or extent of residual disease is more important in terms of prognosis. Bianchini and colleagues analyzed PAM50 scores at baseline and in residual disease after neoadjuvant treatment in the NeoSphere study (22) and showed that PAM50 scores at surgery are more informative for prognosis than baseline scores. They also noticed an increase in Luminal A subtype and a decrease in Luminal B and HER2-enriched subtypes at surgery compared to baseline, reflecting the dynamic modulation of tumors to evolve or select a clone under pressure of therapy (23). One could argue that based on the dynamic modulation of tumors under pressure of treatment (23), the extent of residual is more informative for prognosis than the extent of downstaging. In contrast, downstaging may better reflect treatment effectiveness, when evaluating new treatments.

In daily clinical practice, local preference decides which evaluation method is used. To our knowledge, guidelines do not determine how NST should be evaluated. To calculate the NRI, no additional information is needed to the standard TNM classification. This makes this response index easy to incorporate in clinical practice. As we showed, use of different methods could give different prognostic information for individual patients. It is important to be aware of that when used in clinical setting. Additionally, the number of patients with non-pCR that is re-classified as low risk may depend on the method. In our cohort, the RCB seemed to identify most patients without pCR (*35%)* who meet the noninferiority margin, although the difference with the NRI (25%) was not statistically significant (p=0.09).

Although we were able to answer the clinically relevant question about the prognostic value of minimal residual disease after NST in HER2-positive breast cancer, our study has some limitations. First, our cohort is too small to draw conclusions from subgroups of patients with ER-positive versus ER-negative disease. Some ER-positive tumors might derive more benefit from the adjuvant endocrine therapy, which is reflected in the 5-year RFI but not in the response score. Small numbers also precluded subgroup analysis of patients treated with both trastuzumab and pertuzumab as neoadjuvant therapy separately. However, we think that the type of therapy needed to accomplish tumor downstaging is less important than the fact that it is accomplished.
Second, we used information from pathology reports to calculate the NRI and Neo-Bioscore and reviewed surgical specimens from the non-pCR group to score the RCB. This resulted in four patients classified as RCB=0 without pCR in the original pathology report. Furthermore, the RCB is sometimes hard to assess retrospectively, especially when the macroscopic information is incomplete. Consequently, the

scores contain a level of uncertainty. However the 5-year RFI-estimates per RCB group correspond well with previously described results (17), and therefore we think our results are reliable. Third, nonlinearity of the association of 5-year RFI with Neo-Bioscore was significant in our cohort, but not with RCB or NRI. However, it seems unreasonable to assume that these associations are linear, which is why we modeled them with a nonlinear shape nevertheless. In fact, it seems reasonable to believe that with 37 events, the test for nonlinearity had low power. Therefore, the shape of the curve is somewhat uncertain and that is why we defined our noninferiority criterion in terms of the lower bound of the 95% CI. Ideally, our results should be validated in an independent cohort. Actually, conclusive proof of non-inferiority requires a randomized trial.
Despite the limitations, we think that our study reflects daily clinical practice, which makes these response indices suitable to use in clinical practice and make these outcomes relevant.

To conclude, the RCB, NRI, and Neo-Bioscore are able to select a group of HER2-positive breast-cancer patients with minimal residual disease that have a similar good prognosis as patients with pCR. These patients may not benefit from adjuvant therapy with T-DM1, trastuzumab, pertuzumab, neratinib or additional chemotherapy. Validation of our outcomes is needed before these response measures can be incorporated into clinical practice and help to identify which patients may or may not benefit from additional adjuvant systemic therapy.

Additional information

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Disclosures of potential conflicts of interests

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Supplementary files

Supplementary Table S1. NRI calculation

NRI calculation				
NRI[a]	= (a - b + c) + (d - e) / (a + 1) + d	= Breast response score + Nodal response score / Sum of maximal achievable response score		
Breast response score				
a	Clinical T stage (cT)			
b	Pathological T stage (ypT)[b]			
c	Response based on pathology			
	Assignment of points:	no pCR = 0 points near pCR[c] (<0.5cm) = 1 point pCR = 2 points		
Nodal response score				
d	Clinical N stage (cN)			
e	Pathological N stage (ypN)			
Detailed scoring system for nodal response score				
Baseline clinical stage		**d**	**Post-therapy pathological stage**	**e**	**d-e**
cN0, clinically negative or FNA/SNP negative		0	ypN0/no ALND/no SNP	0	0
			ypN1(mi) (≤3 micro/macrometastases)		0
cN1[d], on imaging		1	ypN0	0	1
(≤3 LNNs) and FNA/SNP+			ypN1(mi) (≤3 micro/macrometastases)	1	0
cN2[d], on imaging		2	ypN0	0	2
(4-9 LNNs) and FNA/SNP+			ypN1(mi) (≤3 micro/macrometastases)	1	1
			ypN2 (4-9 macrometastases)	2	0
cN3[d], on imaging		3	ypN0	0	3
(≥10 LNNs and/or infra-/ supraclavicular/ parasternal) and FNA/SNP+			ypN1(mi) (≤3 micro/macrometastases)	1	2
			ypN2 (4-9 macrometastases)	2	1
			ypN3 (≥10 metastases and/or supra-/infraclavicular)	3	0

[a] In case of progressive disease NRI=0

[b] b cannot be <1

[c] The presence of only isolated tumor cells (<0.2mm) in the lymph nodes was classified as ypN0.

[d] In case of cN1-3 without pathological nodal staging after neoadjuvant therapy (ypNx), NRI cannot be calculated

Supplementary Table S2. RCB-scores and 5-year RFI-estimates

RCB	Number of patients (%)		5-year RFI	(95% CI)	
0	161	(59)	92%	(88-97)	
1	40	(15)	90%	(85-95)	*Non-inferiority*
2	61	(22)	79%	(70-89)	*margin*
3	12	(4)	43%	(23-80)	

Estimated from a proportional hazards model using RCB in a quadratic polynomial model.

Supplementary Table S3. NRI scores and 5-year RFI-estimates

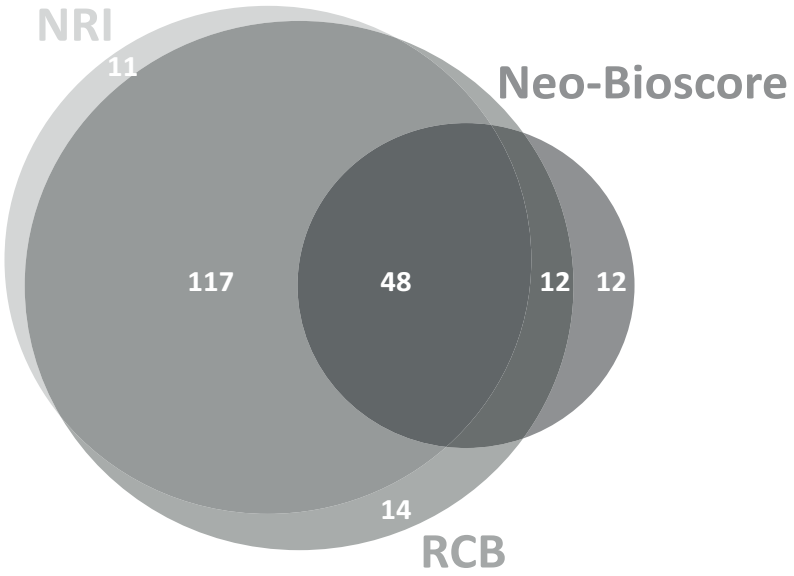
NRI	Number of patients (%)		5-year RFI	(95% CI)	
0	12	(4)	46%	(26-80)	
0.16	3	(1)	64%	(51-79)	
0.20	7	(3)	67%	(55-80)	
0.25	8	(3)	71%	(61-83)	
0.33	13	(5)	77%	(67-87)	
0.40	5	(2)	80%	(72-90)	
0.43	1	(<1)	82%	(73-91)	
0.50	16	(6)	85%	(77-94)	
0.57	2	(1)	87%	(80-95)	
0.60	8	(3)	88%	(81-95)	
0.67	13	(5)	89%	(83-96)	
0.71	2	(1)	90%	(84-96)	*Non-inferiority*
0.75	16	(6)	90%	(85-96)	*margin*
0.8	9	(3)	91%	(86-96)	
0.83	5	(2)	91%	(87-96)	
1	157	(57)	92%	(88-97)	

Estimated from a proportional hazards model treating NRI as continuous variable.

Supplementary Table S4. Neo-Bioscore scores and 5-year RFI-estimates

Neo-Bioscore	Number of patients (%)		5-year RFI	(95% CI)	
0	19	(7)	99%	(97-100)	
1	54	(21)	93%	(87-99)	*Non-inferiority*
2	93	(36)	85%	(78-93)	*margin*
3	71	(27)	91%	(85-97)	
4	20	(8)	77%	(66-91)	
5	3	(1)	32%	(12-85)	
6	1	(<1)	0.6%	(0-1)	
7	n.a.		n.a.	n.a.	

Estimated from a proportional hazards model treating Neo-Bioscore as continuous variable.



Supplementary Figure S1. Overlap and discrepancies in patients with 5-year estimated RFI ≥89% (non-inferiority margin lower bound of 95% CI ≥85%)
NRI, neoadjuvant response index; RCB, residual cancer burden



Chapter 7

Summarizing discussion and future perspectives

In this thesis, we evaluated selected aspects of diagnostic test accuracy and prognostics for Ductal Carcinoma In Situ (DCIS) and for invasive breast cancer (IBC). Below, we summarize the main conclusions, put them into clinical context and discuss future perspectives.

Summary of main findings

Ductal carcinoma in situ: diagnostic accuracy and prognosis

Chapter two introduced our PREvent ductal Carcinoma In Situ Invasive Now (PRECISION)-initiative that aims to prevent the burden of DCIS overtreatment. The PRECISION-initiative included a broad range of research with the ability of collecting large data and tissue sample sets. These are being used for epidemiology studies, genomic characterization and validation in *in vitro* and *in vivo* models. We also reviewed literature regarding DCIS incidence, current treatment strategies, and molecular aspects of DCIS progression. Furthermore, we discussed the gaps in knowledge regarding DCIS and how the PRECISION-initiative can contribute to bridge these.

Within the PRECISION DCIS data cohorts, we observed a range in the distribution of DCIS histological grade. As the histological grade is a debatable prognostic factor for DCIS[1–3] but is nevertheless used to guide treatment decisions and to determine eligibility for inclusion in clinical trials, we aimed to achieve more consistency in grading of DCIS. We set up an interobserver study and compared DCIS histology among nine pathologists based in the Netherlands, the United Kingdom and the United States (**chapter three**). The majority opinion was used as a reference to determine 'the truth'. Histological grade showed a moderate association between the observers. Furthermore, in a subgroup analysis we showed that adding immunohistochemical stains of estrogen receptor (ER) expression and HER2 protein receptor amplification supported conformity in distinguishing high grade (poorly differentiated) DCIS from non-high-grade DCIS.

In **chapter four** we evaluated the progression risks of DCIS in a population-based Dutch cohort with women diagnosed with DCIS between 1989-2004 with follow-up till 2017. We showed lower risks for any ipsilateral breast event in women treated with breast conserving surgery (BCS) supplemented with radiotherapy (RT) compared to women treated with BCS alone. After ten years of follow-up the incidence of newly reported invasive lesions was approximately similar between the two treatment groups indicating that the effect of RT as adjunct to BCS is primarily seen in the first ten years since primary DCIS diagnosis. Furthermore, we demonstrated that after ten years DCIS rarely recurs as ipsilateral DCIS while incidence of subsequent ipsilateral invasive lesions continued.

Invasive breast cancer: diagnostic accuracy and prognosis

In **chapter five** we investigated how quality control criteria for gene expression assays could lead to sample selection bias. Gene expression assays are increasingly used as prognostic tests to support decisions in treatment strategy. We established that when information from the assays is obtained, tumors were more often associated with larger size and lymph node metastasis; features corresponding with on average a more aggressive phenotype. Literature is sparse about sample dropout while performing gene expression assays; however, this information is important for accurate interpretation and utility of these upcoming clinical tests.

Lastly, **chapter six** focused on the evaluation of resection specimens after systemic treatment followed by surgery in HER2 positive breast cancer. We showed that currently used evaluation systems are able to select a group of patients with minimal residual disease that have a similar good prognosis as pCR, the best achievable outcome. This group of patients might be eligible for de-escalation of adjuvant systemic treatment.

Clinical implications

Diagnostic test accuracy

In this thesis we investigated diagnostic test accuracy mainly in two chapters, one regarding DCIS and one regarding invasive breast cancer. In **chapter three** we showed that the same slide was interpreted differently by different pathologists and in **chapter six** that the prognosis of the same resection specimen has been predicted differently by using various classification systems. This has consequences for individual patients, as different judgment of the same patients' tissue might lead to other treatment decisions. Additionally, it is very hard to understand from a patient's perspective that the same tissue would be differently scored depending on the observer or the used system. In general, the aim of classification is to determine prognosis for optimal treatment of an individual patient. Categories are predominately defined by analyzing groups of patients and reporting average outcomes[4,5]. Some patients are in the grey zone between categories, for example due to heterogeneity, and their classification is therefore dependent on the diagnostic distinction of the observer.

For the evaluated tissues in both **chapters three and six,** the followed guidelines differ not only internationally, but also nationally and sometimes even within the same hospital[6,7]. In general, using one international guideline would contribute to achieve more conformity. Van Bockstal et al.[8] showed that using a two-tier system instead of a three-tier system also improved concordance in grading DCIS, although our study did not demonstrate improvement with a binary grading system

(**chapter three**). Regarding evaluation of NST, RCB is increasingly used mainly in the United States, because of low interobserver variability and the good correlation to prognosis[9]. Disadvantage of the use of RCB is the time needed to correctly process the specimen in the gross room. Consequently, RCB is not commonly applied in daily diagnostics yet.

In **chapter three**, we showed that ER and HER2 expression could support conformity in distinguishing high grade (grade 3) from low grade (grade 1 and 2) DCIS. Currently, ER and HER2 expression is not routinely performed at DCIS cases in Europe and validation of these markers in an independent cohort is necessary. However, these markers for DCIS would be easy to introduce in daily diagnostics as they are routinely used for invasive breast cancer. Until specific biomarkers to distinguish between high and low risk DCIS based on outcome are available, ER and HER2 could be introduced in DCIS diagnostics, as the markers seem to ably distinguish high from low grade DCIS.

Limitations of diagnostic tests depend on the context of application of the test[10]. The molecular tests discussed in **chapter five** seemed to be accurate, but, according to our systematic review, 20% of the studies often ignored sample dropout. Hence, the molecular test is not applicable to all types of tumors and suggests performing suboptimal in certain subgroups of patients, i.e. with small sized tumors or histological subtypes with low tumor cell density. When interpreting the test results, it is important to realize that patient populations for which these gene expression data is used might differ from the ones in which they were originally developed.

De-escalation of therapy

De-escalation of therapy is only safe for an eligible subgroup if the prognosis for de-escalation is similar to that of standard treatment. In general, survival is the ultimate outcome to investigate prognosis in clinical studies, however, **chapter four and six** used progression as primary endpoint. **Chapter four** included a cohort of DCIS patients in which risk of progression is commonly used as end point, because survival of precursor lesions is assumed to be similar to the normal population. Because of the low event rate of DCIS a large number of patients and/or long-term follow-up is needed to observe differences in outcome upon different treatments. Additionally, a complicating factor is that treatment strategies are changing over time. We found that younger women, particularly diagnosed between 1989-1998 had the least benefit of radiotherapy and therefore might seem candidates for omitting radiotherapy. However, we should be careful in interpretating these results because we have no information regarding the reasons for administering a specific treatment. Like the DCIS study, the study described in **chapter six** used progression of disease (recurrence free interval; RFI) as primary outcome instead of survival. This study included HER2-positive breast cancer patients of whom 13% experienced

a recurrence. Recurrence free and overall survival of HER2-positive breast cancer patients have improved in the last decades mainly due to the introduction of HER2-directed therapies. Despite that the 5-year survival data could still be improved, the event rate is quite low with only 37 events (13%). As a consequence, we were not able to assign one of the classification systems as preferable. Obviously, the low number of events in these two chapters are a result of the high chance of recovery for patients, which is the ultimate aim in the end.

Future perspectives

Diagnostic test accuracy

To improve conformity in grading DCIS and ultimately distinguish indolent from hazardous DCIS, artificial intelligence (AI) algorithms might support pathologists in the (near) future. Currently, deep learning methods using convolutional neural networks (CNNs) are investigated for application in pathology[11]. CNNs are a type of algorithm that comprises multiple layers, the machine is fed with raw data and the algorithm learns itself representations needed for pattern recognition[12]. In prostate cancer biopsies, it was already proven that the performance of an algorithm was at least similar to grading by a group of 15 pathologists[13]. The key is to collect more data and to choose the outcome wisely, preferable survival or progression of disease. The pitfall is that we will create an extra observer, while we aim on merely supporting the pathologist with the additional diagnostic value of the computer. We are planning to use primary DCIS slides from patients with and without recurrences as an input to predict prognosis.

De-escalation of therapy in DCIS

Several studies demonstrated that adding radiotherapy does not contribute to improved survival risk for DCIS patients[14,15] and, as a result, studies to de-escalate (radio)therapy are ongoing. Currently, low risk DCIS defined by clinico-pathological features[16] and molecular assays as the DCIS OncotypeDX[17] are used to select low risk patients who are potentially eligible for omitting radiotherapy[18]. Given our results that the risk of recurrence varied overtime, long-term follow-up is needed to validate these de-escalated treatment strategies. Furthermore, within our PRECISION consortium, studies are investigating associations of mammographic patterns and genomic profiles with DCIS progression risk to search for low risk patients' subgroups. Lastly, three ongoing trials[19–21] randomize between standard treatment (surgery with or without radiotherapy) and active surveillance to omit therapy for women with low risk DCIS. Although we do not expect results within five years, this will lead to better understanding of the natural history of DCIS.

De-escalation of therapy in NST

Chapter six showed that various classification systems are able to select a group of patients with a similar prognosis as pCR. Recently, a meta-analysis was published which demonstrated that additional adjuvant chemotherapy did not further improve outcomes after pCR in triple negative and HER2 positive breast cancer[22] confirming current treatment guidelines[23]. Nowadays, patients with residual disease are switched to ado-trastuzumab ametansine independent of the amount of residual disease[23,24]. Ongoing clinical trials (NCT01401959 and NCT03684863) investigate the beneficial value of adjuvant systemic treatment in patients with non-pCR. Furthermore, a meta-analysis by Deng et al. including five randomized clinical trials showed a slight advantage for patients treated with 12 months HER2 directed therapy over patients treated for6 months. However, more frequent and severe cardiac toxicities were observed[25]. Our study in **chapter six** assigned a group of patients that might be treated as pCR patients to forego additional toxicities without compensating prognosis. Our data is merged with similar data from different centers around the world to perform analysis with more statistical power. Unfortunately, in contrary to our data, preliminary conclusions did not show similar good prognosis of RCB I to pCR[26]. Perhaps a specific subgroup within the RCB I group might be candidate for omitting additional therapy. Moreover, a debate has started to omit surgery completely in patients with an pCR determined on imaging and biopsy[27,28]. These treatment strategies are already applied in rectal and esophageal cancer. Although patient groups for omitting surgery will exist, finding them is still a challenge and well-designed trials are necessary to enable accurate identification of eligible patient groups.

Conclusion

This thesis demonstrates that diagnostic testing in pathology is subject to substantial variation regarding DCIS grading and response evaluation of HER2-positive breast cancer. This variation can be attributed to subjectivity and/or the use of different diagnostic guidelines for these assessments. We also defined a way to improve the robustness of DCIS grading. In addition, our data regarding treatment response evaluation will be used in a larger set to validate and extend our results. Furthermore, one should be aware that molecular tests were developed on high grade tumors and application on all tumors might provide biased results. Lastly, we provided insights in the recurrence risks of treated DCIS in an unselective cohort. All these studies contributed to optimize clinical tests and prediction of prognosis within DCIS and invasive breast cancer management.

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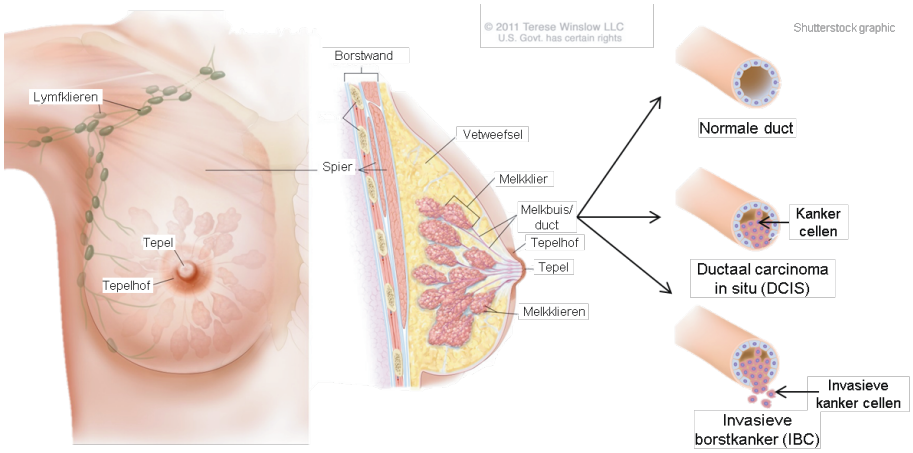
Appendices

List of abbreviations
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List of abbreviations

ADH	Atypical ductal hyperplasia
BCS	Breast conserving surgery
CI	Confidence interval
CRUK	Cancer research United Kingdom
DCIS	Ductal carcinoma in situ
DUMC	Duke University Medical Center
ER	Estrogen receptor
FFPE	Formalin fixed paraffin embedded
GE	Gene expression
GLMM	Generalized linear mixed models
H&E	Hematoxylin and eosin
HR	Hazard ratio
IBC	Invasive breast cancer
iDCIS	ipsilateral ductal carcinoma in situ
IHC	Immunohistochemistry
iIBC	ipsilateral invasive breast cancer
KC	Kings College London
κma	Chance-corrected kappa for association
KWF	Dutch cancer society
MDACC	MDAnderson Cancer Center
NKI	Netherlands Cancer Institute
NL	Netherlands
NRI	Neoadjuvant resonse index
NST	Neoadjuvant systemic treatment
PALGA	Pahologisch Anatomisch Landelijk Geautomatiseerd Archief (Dutch nationwide registry of pathology reports)
pCR	Pathological complete response
PR	Progesteron receptor
PRECISION	PREvent ductal Carcinoma In Situ Invasive Overtreatment Now
QC	Quality Control
RCB	Residual cancer burden
RT	Radiotherapy
SISH	Silver in situ hybridization
TIL	Tumor infiltrating lymphocytes
TMA	Tissue micro array
UK	United Kingdom
USA	United States of America
WP	Work package

Nederlandse samenvatting



Figuur 1. Anatomie van de vrouwelijke borst.
Aangepaste versie van:https://www.cancer.gov/types/breast/patient/breast-prevention-pdq en https://www.shutterstock.com/nl/image-illustration/breast-cancer-ductal-carcinoma-147789416

Algemene introductie ductaal carcinoma in situ en borstkanker

De borst van de volwassen vrouw bestaat grotendeels uit melkklieren en vet. Vanaf de melkklieren monden de melkbuizen uit in de tepel (figuur 1). De melkklieren produceren melk, die tijdens de borstvoeding via de buizen getransporteerd wordt naar de tepel. Kanker is ongeremde celgroei, waarbij door foutjes in het DNA te veel weefsel, een tumor, ontstaat. Bij ductaal carcinoma in situ (DCIS) blijft deze ongeremde celgroei beperkt tot de melkbuizen. Zodra deze ongeremde kankercellen door de buis heen groeien en daarbij het vetweefsel betreden, wordt het (invasieve) borstkanker ('invasive breast cancer', IBC). DCIS wordt beschouwd als een voorloper van borstkanker en kan zelf niet uitzaaien naar op afstand gelegen delen van de borst of andere organen. De meerderheid van de DCIS-afwijkingen groeit niet uit tot borstkanker en is dus op zich niet schadelijk. Een minderheid kan wel uitgroeien tot borstkanker en is daarom ook potentieel levensbedreigend. Op dit moment kunnen we geen onderscheid maken tussen DCIS welke niet zal uitgroeien tot borstkanker en welke een grote kans hebben om wel door te groeien tot (kwaadaardig) borstkanker.

DCIS: diagnostiek en behandeling

DCIS wordt meestal gevonden op een borstfoto (mammogram) gemaakt bij de nationale borstkankerscreening. Hiervoor werden alle vrouwen tussen de 50 en 75

jaar oud en woonachtig in Nederland, om het jaar, uitgenodigd. Sinds eind 2020 worden deze vrouwen eens in de drie jaar uitgenodigd voor deze screening. Als het mammogram afwijkend is en verder onderzoek behoeft, wordt een stukje weefsel door middel van een biopt weggenomen. De patholoog beoordeelt of het weefsel afwijkend is, bijvoorbeeld DCIS bevat, en bepaalt in dat geval ook de mate waarin de cellen nog lijken op normale cellen of al op kankercellen, graderen genaamd. De schaal van graad bestaat uit 3 stappen, van laaggradig waarin de cellen nog veel lijken op normale cellen naar hooggradig waarbij de cellen er sterk afwijkend uitzien. Indien de diagnose DCIS is, wordt de vrouw chirurgisch behandeld met een borstsparende operatie of een borstamputatie. Het geopereerde weefsel gaat weer naar de patholoog om te zien of de afwijking helemaal verwijderd is en de graad wordt opnieuw beoordeeld. In geval van een borstsparende operatie volgt vrijwel altijd ook bestraling (radiotherapie).

Invasieve borstkanker: diagnostiek en behandeling

Indien niet de diagnose DCIS maar de diagnose borstkanker gesteld wordt, wordt er een risico inschatting van de patiënt gemaakt. Kenmerken van de patiënt, zoals leeftijd, en eigenschappen van de tumor, zoals graad, spelen hierin een rol. Ook de biologische eigenschappen van de tumor worden betrokken in het risicoprofiel. De aanwezigheid van hormoonreceptoren, de oestrogeenreceptor (ER) en de progesteronreceptor (PR), en de aanwezigheid van de groeifactorreceptor HER2, dat een rol speelt bij celgroei van de tumor, zijn mogelijke indicaties voor therapieën gericht op deze receptoren. We kunnen steeds beter meten welke processen aan en uit staan in een tumor. Hiervoor kijken we naar de activiteit van genen, een zogenaamd 'genexpressieprofiel'. Een bekend voorbeeld van zo'n genexpressietest is de MammaPrint®. Na de risico inschatting wordt een behandeling geadviseerd. Indien bijvoorbeeld chemotherapie nodig is, kan de chemotherapie vooraf aan de operatie plaatsvinden, genaamd neoadjuvante chemotherapie, of na de operatie (adjuvante chemotherapie). Het maakt voor de overleving van de patiënt niet uit of de chemotherapie voor of na de operatie wordt gegeven. Het voordeel van neoadjuvante chemotherapie is dat de tumor voorafgaand aan de operatie al kleiner wordt en dus mogelijk kan leiden tot een kleiner operatiegebied. Verder kan na de operatie de respons van de tumor op de chemotherapie worden bepaald door vast te stellen hoeveel levende tumorcellen er nog over zijn. Als alle tumorcellen vernietigd zijn, heeft de chemotherapie goed gewerkt en is de prognose voor de patiënt gunstig.

Introductie proefschrift

Borstkanker is de meest voorkomende kankersoort bij vrouwen. Volgens de Nederlandse kankerregistratie krijgt 1 op de 7 Nederlandse vrouwen in haar leven borstkanker. In 2019 werd de diagnose borstkanker 14.808 gesteld en ter vergelijking:

de diagnose DCIS 2.229 keer. In het algemeen staan medische onderzoeken aan de basis van goede gezondheidszorg. Binnen de borstkankerzorg beogen medische onderzoeken de volgende doelen: i) aantonen of uitsluiten van een verdachte afwijking in zowel het bevolkingsonderzoek als op indicatie (verdenking op borstkanker), ii) wanneer de afwijking is bevestigd, deze te classificeren en de prognose te bepalen en iii) de ziekte te monitoren na adjuvante behandeling of het effect van de therapie op de borstkanker te meten na neo-adjuvante behandeling. Deze medische testen hebben voor- en nadelen uitgedrukt in sensitiviteit (gevoeligheid), specificiteit en accuraatheid (nauwkeurigheid), waarbij de context van de uitgevoerde test medebepalend is voor hoe belangrijk deze maten zijn. Een test kan heel accuraat zijn met een juiste diagnose, maar als deze diagnose nooit tot klachten of overlijden leidt, noemen we dit overdiagnose en de bijbehorende behandeling overbehandeling. Daarom is informatie van de context waarin een test afgenomen wordt belangrijk om rekening mee te houden in de interpretatie van de testresultaten. Belangrijk is de verhouding tussen het nut van de behandeling ('benefit') ten opzichte van de aangedane schade door de behandeling ('harm'), dit geldt zowel voor de individuele patiënt als op populatieniveau. Epidemiologie ondersteunt het berekenen van de risico's van ziekte op populatieniveau en deze kennis is dus onontbeerlijk in de interpretatie van medische testen.

Doel van het onderzoek

In dit proefschrift hebben we verschillende klinische testen onderzocht, die gebruikt worden bij het diagnosticeren en classificeren van DCIS en borstkanker. Het doel was om voor- en nadelen van deze testen in kaart te brengen om in de toekomst beter DCIS en borstkanker te kunnen identificeren, classificeren, vervolgen en bijbehorende risico's te kunnen voorspellen. Het proefschrift is in twee delen gesplitst, in het eerste deel wordt gefocust op DCIS, in het tweede deel op borstkanker.

Overzicht van de beschreven onderzoeken

DCIS: nauwkeurigheid van diagnostische onderzoeken en prognose

In **hoofdstuk 2** hebben we met behulp van beschikbare literatuur een overzicht gegeven over het onderwerp DCIS. We hebben de risico's op progressie, huidige behandelingsmogelijkheden, theorieën van natuurlijk beloop en huidige moleculaire kennis besproken. Tevens introduceerden wij het 'PREvent ductal Carcinoma In Situ Invasive Overtreatment Now' (PRECISION-consortium) waarin het ultieme doel is om onderscheid te maken tussen onschadelijk DCIS en potentieel gevaarlijk DCIS, DCIS dat zich kan ontwikkelen tot borstkanker.

In **hoofdstuk 3** hebben we gekeken naar de variatie tussen pathologen in het beoordelen van DCIS-weefsel met de nadruk op de gradering. Drie pathologen uit

het Verenigd Koninkrijk, drie uit de Verenigde Staten en drie pathologen uit Nederland hebben elk 425 digitale coupes (glaasjes met plakjes weefsel) beoordeeld. We vonden een matige overeenstemming tussen de pathologen in de gradering van deze coupes. Dat betekent voor een patiënt dat de gradering van de DCIS afhankelijk is van welke patholoog naar het weefsel kijkt en dit kan een verschillende behandeling tot gevolg hebben. We hebben in een subgroep van de coupes kunnen onderzoeken dat hooggradig DCIS beter van laaggradig DCIS kon worden onderscheiden als ER en HER2 expressie toegevoegd werd aan de gradering. Om dit beter uit te zoeken en daadwerkelijk te implementeren in de klinische praktijk, zou dit bevestigd moeten worden in een groep met andere pathologen en andere coupes. Aangezien ER- en HER2- expressie al toegepast wordt bij borstkanker is het relatief eenvoudig om dit te implementeren in de klinische praktijk voor het graderen van DCIS.

In **hoofdstuk 4** hebben we de absolute risico's (cumulatieve incidentie) en de relatieve risico's (hazard ratios) op de progressie van DCIS berekend en onderzocht in hoeverre de risico's afhingen van het type behandeling dat de vrouwen initieel gehad hadden. Van totaal 10.045 vrouwen in Nederland waarbij DCIS was gediagnosticeerd tussen 1989 en 2004 was bekend of in dezelfde borst opnieuw DCIS of borstkanker was ontstaan in de periode vanaf DCIS diagnose tot en met 31 december 2016. We hebben gevonden dat tien jaar na diagnose bijna geen nieuwe DCIS meer ontstaat in dezelfde borst als waar de initiële DCIS was gevonden. Verder hebben we gevonden dat de kans op een nieuwe tumor, DCIS of borstkanker, het laagste is na borstsparende operatie aangevuld met radiotherapie vergeleken met alleen borstsparende operatie. Voor borstkanker geldt dat het voordelige effect van radiotherapie ontstaat in de eerste tien jaar na initiële DCIS diagnose. Daarna was het aantal nieuwe borstkankers in zowel de groep alleen behandeld met borstsparende operatie als in de groep die ook radiotherapie kreeg, gelijk. Deze studie is uniek in vergelijking met andere studies, omdat het alle patiënten in Nederland omvat bij wie tussen 1989 en 2004 DCIS gevonden was en een zeer lange follow-up heeft. Dit onderzoek toonde daarmee inzicht in de kansen op het opnieuw krijgen van DCIS en het ontwikkelen van borstkanker na DCIS op de lange termijn.

Borstkanker: nauwkeurigheid van diagnostische onderzoeken en prognose

In **hoofdstuk 5** keken we naar moleculaire testen, in dit geval genexpressie profielen, die samen met kenmerken van de patiënt en van de tumor worden gebruikt voor een risico inschatting (zie ook hierboven in algemene introductie). Voordat zo'n moleculaire test uitgevoerd kan worden, wordt het tumorweefsel eerst getest om te beoordelen of voldoende tumorweefsel aanwezig is voor zo'n analyse en daarna of de kwaliteit daarvan een voldoende betrouwbaar resultaat oplevert. Dat betekent dat als er te weinig tumorweefsel is of de kwaliteit onvoldoende is, er geen betrouwbare testuitslag mogelijk is. In de studie beschreven in hoofdstuk 5 zijn kenmerken van de tumorweefsels die niet voldeden aan de genoemde criteria (hoeveelheid en

kwaliteit) vergeleken met kenmerken van weefsels waarop de moleculaire test wel op betrouwbare wijze verricht kon worden. Het bleek dat vaker met succes een genexpressie profiel verkregen kon worden van hooggradige, meer agressief borstkankertumorweefsel dan van minder agressieve, veelal meer laaggradige borstkankertumoren. Ook was vaker sprake van uitzaaiing naar de lymfklieren in de groep waarvan wel succesvol een genexpressieprofiel verkregen kon worden vergeleken met de groep waarbij de bepaling van het genexpressieprofiel niet succesvol was. Dit wekt de suggestie dat het slagen van de test een aanwijzing is voor een agressievere tumor. Literatuur over het ontwikkelen van deze moleculaire testen zegt vaak niks over deze uitval van de tumorweefsels en waarschijnlijk zijn deze testen ontwikkeld op een selectieve groep van tumoren die kan verschillen van de groep waar ze nu op worden toegepast. Bij het ontwikkelen van genexpressie testen is het dus voor onderzoekers heel belangrijk om zich te realiseren dat de test groep mogelijk anders kan zijn dan de groep patiënten waar de test uiteindelijk voor bedoeld is.

Tenslotte hebben we in **hoofdstuk 6** gekeken naar de beoordeling van operatie-preparaten van HER2 positieve borstkanker na neoadjuvante chemotherapie door de patholoog. Daarbij wordt de respons van de chemotherapie geëvalueerd en de prognose van de patiënt bepaald. De prognose van de patiënt is het meest gunstig als alle tumorcellen ten gronde zijn gegaan en de patiënt dus een volledige respons op de therapie heeft. Als het verwijderde borstweefsel (operatie preparaat) van de patiënt nog vitale tumorcellen bevat, bestaan er verschillende classificatiesystemen om de achtergebleven tumorcellen te meten en de prognose te bepalen. Deze studie heeft gekeken naar verschillende classificatiesystemen om uit te zoeken of de aanwezigheid van weinig tumorcellen net zo'n goede prognose heeft als helemaal geen vitale tumorcellen in het operatiepreparaat. Er werd gevonden dat de verschillende classificatiesystemen allen in staat zijn om patiënten te identificeren die weliswaar nog vitale tumorcellen in het operatiepreparaat hebben, maar waarbij de prognose even gunstig is als patiënten waarbij alle tumorcellen ten gronde waren gegaan. Verder bleek dat de classificatiesystemen niet precies dezelfde groep van patiënten met achtergebleven tumorcellen identificeerden met een even gunstige prognose. De groep van patiënten met een even gunstige prognose zou misschien in aanmerking kunnen komen voor het geven van minder (chemo)therapie, maar dan zou beter onderzocht moet worden welke patiënten daadwerkelijk in aanmerking zouden komen.

Klinische implicaties

De nauwkeurigheid van een diagnostische test

In dit proefschrift werd in hoofdstuk 3 en 6 de nauwkeurigheid van een diagnostische test bekeken. In hoofdstuk 3 bleek dat verschillende pathologen DCIS verschillend

graderen en daarmee de behandeling voor dezelfde patiënt kan verschillen bij een beoordeling door een andere patholoog. In hoofdstuk 6 gaven andere classificatiesystemen andere voorspellingen voor de prognose van dezelfde patiënt. Voor patiënten is lastig te begrijpen dat behandeling afhangt van een verschillende beoordeling. Ook werd in deze hoofdstukken duidelijk dat internationaal, maar ook nationaal en zelfs binnen hetzelfde ziekenhuis verschillende classificatiesystemen gebruikt worden. In het algemeen zou de overeenstemming toenemen als hier duidelijkere afspraken over gemaakt worden.

Beperkingen van een test hangen af van de context waarin testen gebruikt worden. In hoofdstuk 5 toonden we aan dat tumorweefsels van borstkanker soms niet voor aanvullende moleculaire testen in aanmerking komen en dat de reden van uitval al iets zegt over het tumorweefsel zelf. Bij het interpreteren van de uitslag is het essentieel dat men zich bewust is van deze selectie van veelal meer agressieve, deels reeds naar de lymfklieren uitgezaaide tumoren.

De-escalatie van therapie

Er wordt veel onderzoek gedaan om patiënten minder therapie te kunnen geven, de-escaleren van therapie. Dit is alleen mogelijk als de uiteindelijke uitkomst voor de patiënt, op zowel de korte als de lange termijn, niet nadelig wordt beïnvloed. In hoofdstuk 4 keken we naar het risico van het terugkeren van een DCIS of borstkanker in dezelfde borst als de oorspronkelijke DCIS-afwijking. We vonden onder andere dat vrouwen onder de 50 jaar oud en gediagnosticeerd tussen 1989-1998 een minder uitgesproken voordeel leken te hebben van radiotherapie als je kijkt naar ontwikkelen van borstkanker in dezelfde borst. Omdat we geen informatie hebben over waarom bepaalde vrouwen wel of geen radiotherapie kregen, moeten we voorzichtig zijn met het interpreteren van deze resultaten en kunnen we niet zomaar zeggen dat deze groep vrouwen veilig minder radiotherapie zou kunnen krijgen. In hoofdstuk 6 werd onderzocht of een subgroep van neoadjuvant behandelde patiënten met Her2 positieve borstkanker kon worden geïdentificeerd, die weliswaar overgebleven tumorcellen hadden, maar eenzelfde (gunstige) prognose als patiënten met een complete respons op neoadjuvante therapie. Alle classificatiesystemen die werden onderzocht, identificeerden zo'n subgroep. Deze patiënten zouden mogelijk in aanmerking kunnen komen voor de-escalatie van therapie. Echter, ten eerste was het aantal patiënten dat progressie van ziekte toonde (aantal events) erg laag, wat deze conclusie (nog) niet heel solide maakt. Ten tweede toonden de verschillende classificatiesystemen niet precies dezelfde patiënten in de subgroep. Voordat de-escalatie van therapie kan worden overwogen, is validatie in een onafhankelijke patiëntengroep zeer belangrijk.

Conclusie

Dit proefschrift laat zien dat diagnostische testen in de pathologie subjectieve variatie laten zien in het graderen van DCIS en in de respons evaluatie van chemotherapie voorafgaand aan de operatie. Deze variatie ontstaat door subjectiviteit van beoordelaars (i.e. pathologen) en gebruik van verschillende richtlijnen. We lieten zien hoe wellicht ER- en HER2-expressie het graderen van DCIS in de toekomst kan ondersteunen. Onze resultaten van respons evaluatie zullen gebruikt worden in een grotere vergelijkbare set om ze te kunnen controleren. Verder lieten we zien dat bij het ontwikkelen van een moleculaire test het belangrijk is om de juiste patiëntenpopulatie te selecteren vergelijkbaar aan een populatie waarop de test uiteindelijk zal worden toegepast. Sommige testen stellen hoge eisen aan het tumorweefsel, waardoor niet iedere tumor geschikt is voor zo een type test. Ook gaven we inzicht in het risico van vele duizenden niet geselecteerde vrouwen die ooit waren gediagnosticeerd met en behandeld waren voor DCIS op terugkeer van DCIS of borstkanker. Al deze studies droegen bij aan het optimaliseren van klinische testen en gaven inzicht in de prognose bij patiënten met DCIS en borstkanker.

About the author

Maartje van Seijen was born in Rotterdam on May the 23rd, 1985. After graduating from the Hugo Grotius gymnasium, she started her studies in Bio-Pharmaceutical Sciences at the university of Leiden. One year later, in 2004, she also enrolled in Medicine at the same university. In 2009 she got her bachelor degree for Bio-Pharmaceutical Sciences. At that point she started her clinical rotations and in 2012 she graduated Medicine as well. To get clinical experience she started working as a resident on the emergency room at the "Langeland Ziekenhuis" hospital in Zoetermeer and later on the surgery department at the "Alrijne" hospital in Leiderdorp. In 2014, she was hired by the VUMC to start her training in pathology. As part of her breast pathology rotation she visited the Netherlands Cancer Institute (NKI) to examine slides under supervision of Jelle Wesseling. Because of her interest in the diagnostics of DCIS she was invited to do a research internship in breast pathology at the Wesseling research group. Afterwards she continued the three months internship in a full-time Phd for three years which started July 1st ,2017. In 2020 Maartje resumed her training at the VUMC to become a pathologist.

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