UNIVERSITEIT GENT

FACULTEIT GENEESKUNDE EN GEZONDHEIDSWETENSCHAPPEN

Academiejaar 2010 - 2011

PROGNOSTIC AND PATHOBIOLOGIC RELEVANCE OF MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL MARKERS IN DUCTAL CARCINOMA IN SITU OF THE BREAST

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Onderzoeksrapport 4de master geneeskunde
Voor “STAGE Wetenschappelijk onderzoek voor de ziekenhuisarts”
in het kader van de opleiding tot
ARTS

“De auteur en de promotors geven de toelating dit onderzoeksrapport voor consultatie beschikbaar te stellen en delen ervan te kopiëren voor persoonlijk gebruik. Elk ander gebruik valt onder de beperkingen van het auteursrecht, in het bijzonder met betrekking tot de verplichting uitdrukkelijk de bron te vermelden bij het aanhalen van resultaten uit dit onderzoeksrapport.”

Datum

19 mei 2011

Prof. dr. Louis Libbrecht

Dr. Kathleen Lambein

Mieke Van Bockstal

Wouldn't it be easier if we lived in a binary world?
Everything would be either black or white, yes or no, 1 or 0.
And biopsy results would be either normal or cancer.

Welch G.H., Woloshin S. and Schwartz L.M.
Editorial, JNCI 2008, Vol. 100, Issue 4

Teachers can open the door, but you must enter by yourself.

A Chinese proverb

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1. ABSTRACT

BACKGROUND. The incidence of the pre-invasive lesion DCIS has increased since the introduction of screening mammography. The current treatment consists of mastectomy or breast conserving surgery (BCS), often with adjuvant radiotherapy. Mastectomy results in overtreatment of some patients, but on the other hand, breast conserving surgery results in tumour recurrence in about twenty percent of patients. Local recurrence might consist either of DCIS or invasive ductal carcinoma (IDC). Furthermore, breast irradiation might also represent overtreatment for a subset of patients.

Nowadays, the Van Nuys Prognostic Index (VNPI) is often used as a risk score to predict disease recurrence. Although the VNPI is a valuable tool, recurrence prediction is still not completely accurate and hence, it could be improved by the identification of additional prognostic markers. An ideal prognostic marker would permit to predict the biological behaviour of DCIS lesions, leading to a more individualized treatment of patients.

In addition, the exact mechanism of human breast cancer progression is still not completely understood. Currently, the mammary stromal compartment and its possible role in tumour progression is increasingly focused on. Investigating the biology of this mammary stroma and its suspected interactions with premalignant and malignant lesions might contribute to unravel the mechanisms of mammary tumour biology.

Amplification and overexpression of the *HER2* gene are known to be associated with a worse prognosis in invasive ductal carcinoma (IDC). However, little is known about the significance of *HER2* amplification in DCIS and its role in mammary tumour progression. Plenty of studies have analysed *HER2* status in both DCIS and IDC, but comprehensive information about the significance of *HER2* and *CEP17* copy number in DCIS is lacking, and its relations with HER2 protein expression have not been thoroughly investigated yet.

AIMS. The purpose of this study is to understand more about the pathobiology of DCIS and to identify new prognostic markers that might be used to estimate the biological behaviour of a DCIS lesion.

Our aim is to explore the correlation between various histopathological features and the stromal protein expression of several molecular markers. In addition, we want to explore how our findings correlate with recent publications about histopathological characteristics of DCIS. A third objective is to carry out a thorough evaluation of the relationships between histopathological features and the FISH and IHC analyses of HER2.

METHODS. In this study, H&E slides of a retrospective cohort of 61 DCIS cases were evaluated and various histopathological features were assessed. Immunohistochemistry was performed for estrogen receptor, progesterone receptor and HER2. Fluorescence-in-situ-hybridisation (FISH) was used to detect *HER2* amplification.
We also performed an immunohistochemical analysis to evaluate protein expression of CD34, alpha-smooth-muscle-actin (SMA), caveolin-1, caveolin-2, aquaporin-1, laminin-beta-2, necdin and decorin in the stromal compartment of DCIS. Because of lack of follow-up data, protein expression was correlated with pathological classification, which was used as a surrogate prognostic marker. Statistical analysis was used to study possible correlations between these variables.

RESULTS. In this cohort, high grade DCIS lesions, according to both the VNPI pathological classification and the Pinder classification, were significantly associated with the presence of myxoid stroma and a stromal inflammatory infiltrate.
Stromal expression of caveolin-1, SMA, CD34, CD10, necdin and aquaporin-1 did not correlate with pathological classification of DCIS cases. Reduced caveolin-2 expression was more often observed in association with intermediate grade DCIS. In contrast, high grade lesions were significantly correlated with reduced stromal protein expression of decorin and laminin-beta-2. Surprisingly, we also noted a remarkable correlation between reduced stromal decorin expression and *HER2* gene amplification.
Amplified DCIS cases manifested significantly more stromal inflammation than non-amplified DCIS. The amplification status based on dual-probe FISH was 95% concordant with the results of a simulated single-probe FISH for *HER2*, and the *HER2/CEP17* ratios for these discrepant cases were all near the valid cut-off value. The mean *HER2* and *CEP17* copy number correlated in non-amplified DCIS, likely because of cell cycling. A gene dosage effect was observed in amplified DCIS cases: an increase in the mean *HER2* copy number strongly correlated with a higher IHC score.

CONCLUSION. This stromal protein expression study, combined with the histopathologic assessment of the DCIS cases and the IHC and FISH analysis of HER2, has led to a better understanding of the pathobiology of DCIS and the role of peritumoural stroma and HER2 in breast cancer.
Since the presence of myxoid stroma and an extensive stromal inflammatory infiltrate are associated with high-grade lesions, the potential of these histopathologic features as prognostic markers warrants further investigation.

We demonstrated that reduced decorin expression significantly correlated with high-grade lesions, and we believe this protein is promising as a prognostic marker in DCIS. Stromal laminin-beta-2 expression is significantly reduced in high-grade lesions and this might relate to a laminin isoform shift. Furthermore, we have shown a high frequency of *HER2* gene amplification in DCIS, and amplification was remarkably often associated with the presence of clusters of *HER2* signals. Therefore, we assume *HER2* gene amplification might have a different biological role in DCIS than in IDC.

This study may eventually contribute to the identification of subsets of patients with DCIS who can be treated with less aggressive therapy, like breast conserving surgery without subsequent radiotherapy. Moreover, the stromal compartment might become a potential target of novel anti-cancer therapies in the future.

2. INTRODUCTION

2.1. Epidemiology of ductal carcinoma in situ (DCIS)

2.1.1. Definition of pure DCIS and DCIS with microinvasion

Ductal carcinoma in situ (DCIS) constitutes a morphologically and genetically heterogeneous group of lesions, and is considered to be a pre-invasive precursor of invasive ductal carcinoma of the breast. DCIS can be defined as an intraductal clonal proliferation of epithelial cells, without invasion of the basement membrane or the surrounding stroma (*1*). This definition implies that pure DCIS cannot occur with coexistent metastasis, since the presence of metastases requires disruption of the basement membrane and subsequent invasion, allowing tumour cells to spread through the body.

However, it is widely presumed that DCIS lesions can progress to invasive ductal carcinoma (IDC), and DCIS with microinvasion (DCIS-Mi) is regarded as an intermediate stage between pure DCIS and IDC. DCIS-Mi is defined as one or more foci of malignant epithelial cells extending beyond the basement membrane, into the (non-specialized) stroma surrounding the ducts. Each of these invasive foci measures no more than 0,1 cm in size (*1, 2*).

2.1.2. The consequences of screening mammography

In the seventies of the previous century, DCIS was a rather uncommon entity, representing less than two percent of all clinically diagnosed breast cancers (*3*). Usually, DCIS is a nonpalpable and asymptomatic lesion (*4*). Nowadays, most women, especially women aged 50 to 69, are diagnosed with DCIS due to screening. Following the widespread introduction of mammographic breast cancer screening, the incidence of DCIS has increased enormously, accounting for about 15-20% of all breast cancers diagnosed at present (*5, 6*).

For instance, in the southern part of the Netherlands, the incidence of mammary DCIS in women aged 50-69 years augmented from 3 per 100.000 in 1984 to almost 34 per 100.000 person-years in 2006, and the introduction of mass mammographic screening was suggested to be responsible for this increase (*6*). In the USA, the age-adjusted annual incidence of DCIS rose from 5,8 per 100.000 in 1975 to 32,5 per 100.000 women, and several population-based trials provide strong evidence that this increase can be attributed to screening mammography (*7*).

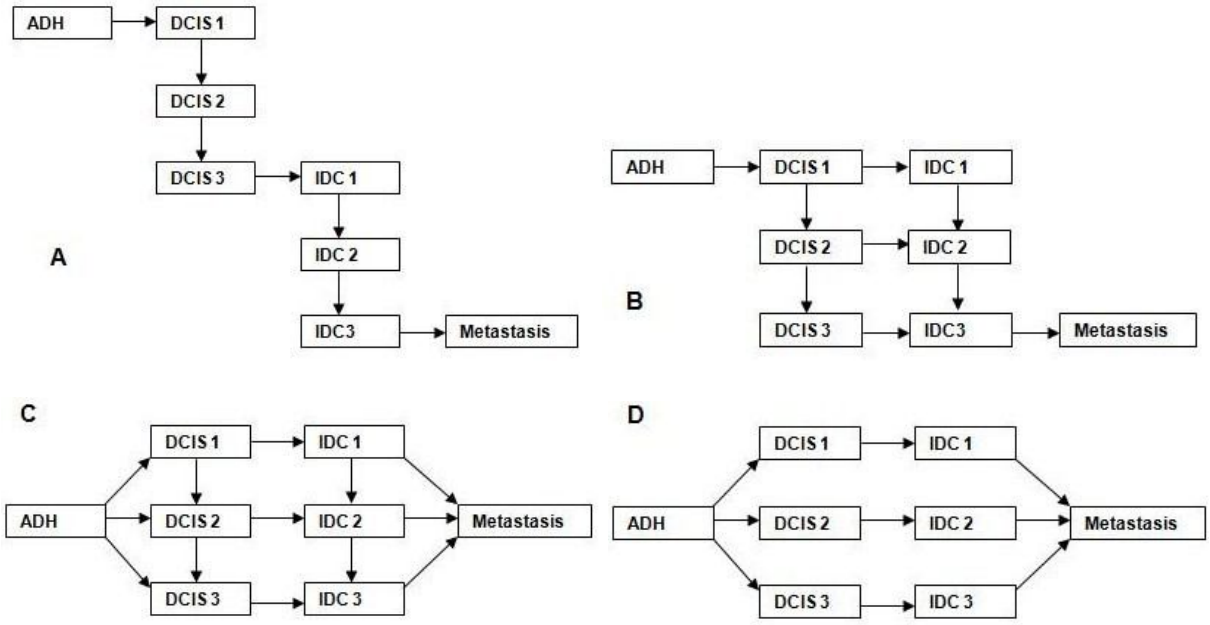
2.1.3. The natural history of DCIS: unravelling the enigma

In the past decades, more and more evidence pointed out that not all DCIS lesions progress to invasive ductal carcinoma. The probability of progression is still subject to debate, and since the standard treatment is based on surgical excision of the lesion, it cannot be directly observed (*8*). Retrospective studies about DCIS lesions that were originally misdiagnosed as benign at biopsy, were able to investigate the incidence of invasive breast carcinoma in these small series of untreated patients (*9-11*). Long-term follow-up in those studies shows that the risk of developing a subsequent invasive breast cancer ranges from 14 to 53%, if patients receive no further treatment after biopsy (*8-10*). This might be an underestimation as well, since low grade DCIS lesions are more likely to be misdiagnosed, possibly causing a downward bias of the progression rate (*8*).

Moreover, the linear multistep process of breast cancer progression has been questioned. For many years, breast cancer progression was compared to the well-known intestinal adenoma/carcinoma sequence, presuming that breast lesions proceed through consecutive stages. According to this linear model (Fig1A), flat epithelial atypia or atypical duct hyperplasia (ADH) progress to low grade DCIS, which becomes high-grade DCIS and subsequently progresses to invasive ductal carcinoma, which in turn further dedifferentiates (*8, 12-14*). Currently, this linear pathway has been opposed by many authors, since more and more evidence supports the existence of other pathways of breast cancer progression (*12, 14, 15*). Moreover, it is questioned whether DCIS is an obligate precursor lesion of IDC.

Sontag and Axelrod evaluated four possible breast tumour progression pathways, in order to identify a pathway which can best describe the relationship between the different grades of DCIS and IDC (*12*). Among these models are the aforementioned linear pathway, a non-linear pathway (Fig 1B), a branched pathway (Fig 1C) and a parallel pathway. The linear, non-linear and branched pathways regard DCIS as a precursor of IDC (*12*). In contrast, the parallel pathway considers DCIS and IDC as two different entities, originating from a common progenitor. According to the non-linear pathway, ADH can only give rise to grade 1 DCIS, and not to high-grade DCIS. Moreover, high-grade IDC would be the only type of IDC which is able to metastasize. Both the linear and non-linear pathway did not reproduce the clinical observations very well (*12*). The branched pathway resembles the non-linear pathway but takes more possible transitions into account. More specifically, ADH can give rise to each grade of DCIS, and each grade of IDC can cause distant metastasis (*12*).

Figure 1 (A) Schematic view of the linear pathway, according to Sontag and Axelrod (*12*); (B) Non-linear pathway, as designed by Sontag and Axelrod (*12*); (C) Schematic view of the branched pathway, according to Sontag and Axelrod (*12*); (D) Drawing of the 'committed' pathway.



In the parallel pathway (Fig 2), both DCIS and IDC progress in parallel, in a linear way from low-grade to high-grade, but transitions from DCIS to IDC do not occur (*12*). This model would explain why some patients have coexistent DCIS and IDC, why not all patients with DCIS lesions develop IDC, and in reverse, why not all patients with IDC present DCIS lesions in coexistence with their invasive tumour. However, the existence of DCIS with the presence of microinvasion can be regarded as a plea against the parallel pathway theory, since this kind of DCIS lesion is an apparent intermediate stage between DCIS and plain IDC. Interestingly, the observed frequency of DCIS-Mi is rather low, accounting for approximately 1% of all breast cancers (*16*). If DCIS would be an obligatory precursor of IDC, one would expect to observe much more DCIS-Mi than is currently diagnosed.

Although Sontag and Axelrod described the parallel pathway as the model that fits best with the clinically observed data, even this 'best' model differs significantly from the underlying unknown pathway that caused the lesions (*15*). Lin performed an analysis of possible combinations of the aforementioned four pathways (*15*). When the Van Nuys system was applied, a combination of the non-linear and the parallel pathway fitted the observed clinical data best, whereas a combination of the linear and parallel pathway was the best fit when the system of Holland et al. was used (*15*).

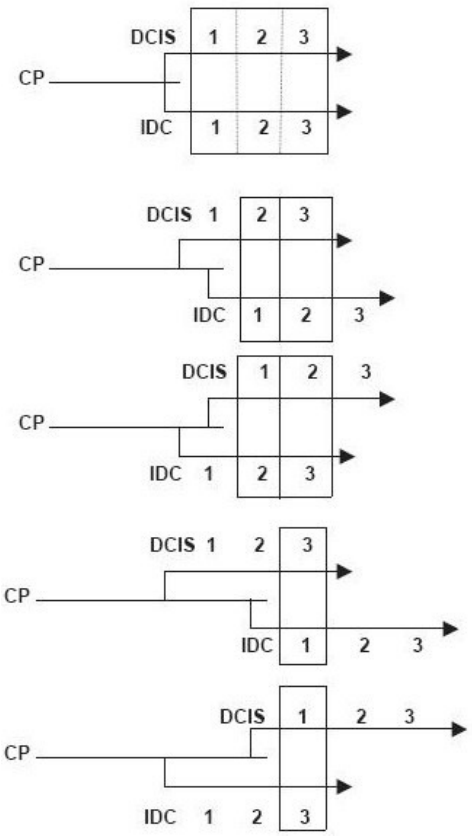


Figure 2 The parallel pathway of breast cancer progression, as proposed in Sontag and Axelrod (*12*). The boxes present DCIS and IDC stages which coexist. CP: common progenitor

Both Lin and Sontag and Axelrod omitted to assess a fifth pathway, which can be described as a 'commitment' pathway (Fig 1D), in which low-grade DCIS progress to low-grade IDC, and high-grade DCIS progress to high-grade IDC, without dedifferentiation from low-grade to high-grade (*13, 14*). This commitment model also regards DCIS as an obligate precursor of IDC, and is based on the 'commitment' of a DCIS lesion to progress to IDC with the corresponding grade.

Many studies provide sufficient evidence to support this theory (*17-21*). For example, in the study of Steinman et al., expression of molecular markers significantly differed between high-grade and non-high-grade carcinomas, supporting the view that they are molecularly distinct entities (*20*). Moreover, the authors demonstrated co-expression of molecular markers in co-existent DCIS/IDC cases (*20*). Another example is the gene expression profile study of Ma et al., in which the gene expression patterns in DCIS and IDC lesions appeared to be highly similar to each other, but the gene expression profile in DCIS differed enormously from that in normal breast epithelium (*17*).

Still, these observations not only match the commitment theory, but they often match the parallel pathway as presented by Sontag and Axelrod as well. Alas, it is currently not possible to determine with certainty which pathway or combination of pathways can fully explain mammary tumour biology.

2.2. Treatment of DCIS: from radical mastectomy to current policy

2.2.1. Mastectomy versus breast conserving surgery

Until the early eighties, a total mastectomy with low axillary dissection or a modified radical mastectomy was propagated, because DCIS was considered to be of a more aggressive nature and to have a poorer prognosis than LCIS (*3*). During the past decades, it has become clear that not every patient diagnosed with DCIS needs to undergo a mastectomy. As not every DCIS lesion will progress to invasive ductal carcinoma (IDC), performing a mastectomy in every DCIS patient would result in overtreatment (*22*). Overtreatment in this context means, that patients undergo surgery of which they cannot benefit because their DCIS lesion will never progress to IDC and, consequently, it will not become a life-threatening disorder (*4*). Hence, performing this treatment can only be potentially harmful to the patient. After all, we should keep in mind the old adage 'primum non nocere'.

Possibilities to determine the exact incidence of invasive and non-invasive recurrence after breast conserving treatment for DCIS were limited in the era of the 'golden standard of care' mastectomy. Nowadays, we know that invasive local recurrence occurs less often after mastectomy, but despite the higher recurrence rate after breast conserving surgery (BCS), the breast-cancer specific survival is similar in both groups (*22, 23*). Silverstein et al. reported a 10-year disease free survival of 98% in patients who underwent mastectomy, whereas BCS with adjuvant radiotherapy resulted in a 81% 10-year disease free survival (*23*). Nevertheless, there were no differences in both the overall survival and breast-cancer specific survival in the two treatment groups (*23*). Lee et al. described an invasive recurrence rate of 0.5% after mastectomy, and 12% after breast preservation (*22*). BCS and mastectomy appeared to be associated with a 12-year probability of breast cancer-specific mortality of 1.0% and 0.8%, respectively. Even if patients developed invasive recurrence, the 12-year mortality due to metastatis amounted 12%, which implies they still had a quite good long-term prognosis (*22*).

2.2.2. Breast conserving surgery: with or without adjuvant radiotherapy?

Several studies have been performed to determine whether patients benefit from undergoing breast irradiation after BCS. A Cochrane review, analysing the results of four randomized trials (yielding 3925 patients), reported a reduction of the overall recurrence risk in the treated breast by 51% because of adjuvant radiotherapy (*24*). Despite this significant benefit, no differences in survival were noted in comparison with excision alone (*24*).

A meta-analysis, examining the results of four randomized trials (yielding 3665 women), concluded that adding radiotherapy to the treatment of DCIS reduced the overall recurrence risk with about sixty percent (*25*). No benefit for overall survival was seen, compared with lumpectomy alone (*25*). In an overview written by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), radiotherapy was found to reduce the 10-year overall recurrence rate in the ipsilateral breast by 15%, but there was no effect on breast cancer-specific or overall mortality (*26*). Furthermore, radiotherapy appeared to be effective regardless of margin status, tumour size, tumour grade, the concomitant use of tamoxifen and patient age at the moment of diagnosis (*26*).

2.2.3. Adjuvant systemic therapy with tamoxifen

In the NSABP B24 randomized trial, patients who were treated with BCS and radiotherapy, showed benefit when tamoxifen was added to their treatment (*27*). A significantly lower 5-year cumulative incidence in overall recurrence was seen in the tamoxifen treated group, both in the ipsilateral and contralateral breast. This was mainly caused by a decreased incidence of ipsilateral invasive recurrence and contralateral non-invasive recurrence (*27*). These findings contrast with the results of the UKCCCR/ANZ trial, in which tamoxifen was shown to reduce the incidence of contralateral but not ipsilateral invasive disease, although the overall recurrence of DCIS was significantly decreased (*28, 29*).

A recent meta-analysis of the two aforementioned studies concludes that adjuvant tamoxifen does not influence overall survival (*30*). However, patients do benefit from tamoxifen, because it reduces the risk of contralateral DCIS and ipsilateral IDC, independent of patient age (*30*).

2.2.4. Treatment of DCIS: current policy

Despite the fact that breast preserving treatment is associated with an increased risk of local disease recurrence, either as DCIS or as IDC, there is no difference in breast cancer-specific mortality between BCS and mastectomy (*22, 23*). Although radiotherapy does not affect overall survival of patients treated with BCS, adjuvant irradiation has been shown to considerably reduce the overall local recurrence risk (*24-26*). If patients are treated with adjuvant radiotherapy, only the ipsilateral breast benefits of the irradiation. In contrast, adjuvant tamoxifen treats the contralateral breast as well, causing both ipsilateral and contralateral decreased recurrence rates (*27-29*).

This ‘tamoxifen effect’ highlights the fact that DCIS confers a general risk: DCIS is not only a risk factor for developing a local invasive recurrence, but there is an increased risk for developing an invasive carcinoma elsewhere as well, either in the ipsilateral or contralateral breast (*4*).

In conclusion, DCIS, being a pre-invasive lesion, has a favorable prognosis. Therefore, management of DCIS lesions should be based on optimizing local control, using the least aggressive surgical treatment (*31*). Currently, the recommended therapy of choice is breast conserving surgery with adjuvant radiotherapy. Generally, mastectomy is reserved for extensive DCIS lesions. In case of estrogen-receptor positive DCIS tamoxifen should be added as well (*30*).

2.3. Classification of DCIS and its prognostic relevance

2.3.1. DCIS classification systems

Originally, DCIS used to be classified according to architectural patterns and the presence or absence of necrosis. Since one DCIS lesion often contains more than one specific growth pattern, this architecture-based classification system has been abandoned (*32, 33*).

In the nineties, Holland et al. proposed a new classification system, primarily based on cytonuclear differentiation, but also taking into account the cellular polarisation as a marker for architectural differentiation (*33*). These characteristics were chosen because they are more uniform throughout an individual DCIS case. According to this system, DCIS can be classified in three categories: well, intermediately and poorly differentiated (table 1) (*33*). Given the fact that this system is solely based on morphologic characteristics of DCIS lesions, the lack of correlation with clinical outcome in DCIS patient populations is a major drawback.

Table 1 Classification system for ductal carcinoma in situ, according to Holland et al. (*33*). The primary defining features are the cytonuclear characteristics, i.e. the shape of the nuclei, their chromatin and nucleoli, and the presence of mitoses. Architectural differentiation, i.e. the polarisation of cells, is a secondary defining feature.

Features	Well-differentiated	Intermediately differentiated	Poorly differentiated
Nuclei	Monomorphic nuclei, uniform size, regular outline and spacing	Moderate pleomorphic nuclei, with some variation in size, outline and spacing	Strong pleomorphic nuclei, variation in size, irregular outline and spacing
Chromatin	Uniform, fine	Fine to coarse	Coarse, clumped
Nucleoli	Insignificant	Evident	Prominent
Mitoses	Rare	Occasionally	Often present
Polarisation of cells	Marked	Present	Absent or minimal

In contrast, the pathological classification as a part of the Van Nuys Prognostic Index (VNPI) has been proved to significantly correlate with patient outcome, or more specifically, with local recurrence-free survival in patients receiving BCS with or without radiotherapy (*34*). The VNPI pathologic classification is based on the degree of nuclear atypia, and the presence of necrosis, resulting in three categories (table 2) (*34*). The VNPI is calculated based on four predictors: 1) the size of the DCIS lesion; 2) margin width; 3) patient age at the moment of diagnosis; and 4) pathologic classification (table 2). Each of these parameters has been shown to significantly correlate with local recurrence-free survival (*34*).

The VNPI was developed to predict local recurrence risk. Patients with the minimum score of 4 were least likely to develop recurrent disease, and the patients the most likely to recur had the maximum score of 12. This predicted recurrence risk enables clinicians to select subgroups of high risk patients who would benefit from mastectomy, or to select low risk subgroups of patients who do not need adjuvant irradiation in case of BCS (*34*).

Table 2 The USC/Van Nuys Prognostic Index scoring system. Four predictors of local breast recurrence are scored. All scores are added up to a VNPI of at least 4 and maximum 12 (according to Silverstein et al. (*34*)).

Score	1	2	3
Size (mm)	≤ 15	16-40	≥ 41
Margin width (mm)	≥ 10	1-9	< 1
Pathologic classification	Non-high grade without necrosis (nuclear grades 1 or 2)	Non-high grade with necrosis (nuclear grades 1 or 2)	High grade with or without necrosis (nuclear grade 3)
Age (year)	> 60	40-60	< 40

Recently, a new pathological classification system was proposed by Pinder et al., based on a central pathologic review of DCIS cases entered in the UKCCCR/ANZ trial (*35*). Their classification is mainly based on cytonuclear grade, but in contrast with former classification systems, it consists of three alternative categories. Lesions with low and intermediate cytonuclear grade are grouped together, and the group of high cytonuclear grade DCIS is subdivided according to two criteria into a 'high risk' and a 'very high risk' group, depending on the absence or presence of >50% solid architecture and >50% ducts bearing comedo necrosis (table 3) (*35*). This alternative classification relies on the correlation with recurrence risk in patients with locally excised DCIS (*35*). Just like the VNPI and its pathologic classification, the Pinder classification aims to predict the ipsilateral recurrence risk in order to guide therapy. Regarding recurrence rate, little difference was seen between the low and intermediate risk group, which explains why these two groups were merged (*35*). Patients in this mixed low/intermediate group have an ipsilateral recurrence rate of 6.1%, but in the high risk and very high risk group, this risk amounted 10.9% and 18.2%, respectively (*35*).

Table 3 Pathological classification for grading DCIS (according to Pinder et al.(*35*)).

Low/intermediate	High	Very high
DCIS with low or intermediate cytonuclear grade	High cytonuclear grade DCIS, not pure comedo (i.e. < 50% of ducts show necrosis, OR the growth pattern is not predominantly solid)	High cytonuclear grade DCIS AND > 50% solid architecture AND > 50% of the affected ducts show comedo necrosis

2.3.2. The need for prognostic factors

None of the recommended therapies is harmless. Surgery can be disfiguring, irradiation has its short-term and long-term consequences, and also systemic therapy with tamoxifen is not without side effects. Currently, it is not possible to identify a subgroup of patients with DCIS who can be treated with wide excision alone. Actually, we don't even know for sure whether there exists such a subgroup.

Although the VNPI is a useful tool in assessing the recurrence risk in an individual DCIS patient, a recent study provided evidence that even in the 'low recurrence risk' group (VNPI score 4-6), 8% patients still developed recurrent disease, in comparison with 9% of patients in the 'intermediate risk' group (VNPI score 7-9) (*36*). Moreover, the current morphologic and immunohistochemical markers are not adequate enough to predict the recurrence risk and the biological behaviour of the tumour in patients with DCIS, irrespective of their treatment (BCS or mastectomy) (*36*). Such prognostic factors provide useful information, but these factors are not able to predict what kind of recurrent disease (DCIS or IDC) a single patient will develop. Hence, the search for accurate prognostic factors continues, aiming to improve the quality of risk assessment in a specific patient. Maybe such predictors will enable us one day to predict the biological behaviour of DCIS lesions and to identify patients who benefit of a less aggressive therapy.

2.4. The stromal compartment in breast cancer progression

2.4.1.DCIS and the surrounding stroma: enemies or allies?

Generally, the stromal compartment has long been regarded as an innocent bystander in tumour biology, and research used to be confined to the malignant epithelial cells. Nowadays, the peritumoural stroma is increasingly focussed on, in both DCIS and IDC (*17, 37*). It has become clear that tumour progression is the result of a complex interaction between the cells of a tumoural lesion and the neighbouring 'normal' non-tumoural stromal cells, such as fibroblasts and inflammatory cells (*17*). To date, little is known about the exact role of stromal fibroblasts and stromal genes in the biology of DCIS lesions, and what role the periductal stroma plays in the progression to invasion.

Atypical tumour-stromal fibroblasts have been described in invasive ductal carcinoma of the breast (*38*). Such atypical fibroblasts were in particular present in stromal fibrotic foci, and they were significantly associated with tumour recurrence and breast cancer-specific mortality (*38*). Finak et al. developed a 26-gene stroma-derived prognostic predictor, based on differentially expressed genes in normal stroma and the stromal compartment of IDC and invasive lobular carcinomas (*39*). The authors demonstrated that tumour-associated changes in stromal gene-expression can be used to predict disease progression and patient outcome for invasive breast cancer (*39*).

In another gene expression profile study, specifically concerning the tumour microenvironment during breast cancer progression, not only normal breast tissue and peritumoural stroma of IDC was examined, but also periductal stroma in DCIS lesions (*17*). It was observed that the tumoural stroma exhibited a clear gene expression signature correlating with the histological tumour grade (*17*). Moreover, the mammary stroma appeared to undergo more extensive gene expression alterations during the transition from normal mammary breast tissue to DCIS, than during the transition from DCIS to IDC (*17*).
During the progression to the invasive stage, upregulation of genes coding for matrix metallo-proteinases (MMP) was observed, suggesting that cell-cell communication between the two compartments plays a major role during progression to IDC (*17*). Apparently, the malignant cells alter the composition of the extracellular matrix by activating MMP production by stromal fibroblasts, thus preparing their microenvironment for subsequent invasion and turning the stroma into an ally.

On the other hand, several stromal molecular markers appear to be associated with a better outcome, which means the stromal compartment might act as an enemy of the tumour. Proof of such stromal behaviour can be found in various studies. For instance, a T helper type 1 immune response was demonstrated to associate with a better outcome in IDC (*39*).

Moreover, a downregulated expression of several stromal proteins was associated with worse patient outcome in both IDC and DCIS. A decrease in stromal decorin expression correlated with a poor prognosis in lymph node negative IDC (*40*). Absence of stromal caveolin-1 expression correlated with early tumour recurrence and a poor prognosis in invasive breast cancer (*41*). Conversely, high stromal caveolin-1 levels were associated with a reduced risk of metastasis and improved patient survival in invasive breast cancer (*42*). Moreover, reduced stromal caveolin-1 levels also correlated with early transition from DCIS to IDC (*43*). These observations led to the assumption that caveolin-1 might function as a tumour suppressor in the stromal compartment (*43*).
In comparison with moderately differentiated DCIS lesions, high grade DCIS cases were found to be associated with a decrease of CD34 positive and an increase in SMA positive fibroblasts (*44, 45*). Elevated stromal CD10 expression was associated with early DCIS recurrence (*46*), and it also correlated with higher tumour grade and reduced survival in invasive breast cancer (*47*).
These observations confirm that the tumoural microenvironment plays a major role in mammary tumour progression (*37*). However, more research will be acquired to unravel the exact mechanisms that control the transition from DCIS to IDC (assuming DCIS is an obligate precursor of IDC!).

2.4.2. Selection of molecular markers at the protein level

A recent gene expression profile study investigated the gene expression and the subsequent clustering profile of various primary epithelial tumours. Among them were 353 primary breast tumours (*48*). The obtained breast cluster was subdivided according to histology, into a mixed lobular-ductal cluster and a mainly ductal cluster (*48*). The mixed lobular-ductal cluster contained a lobular carcinoma group and a group comprising grade 1 and grade 2 IDC. The mainly ductal cluster contained predominantly grade 3 IDC, and was subdivided in a triple negative IDC group and a group of tumours with comparable receptor status as the IDC in the mixed lobular-ductal cluster (*48*).

A signature, composed of genes that were differentially expressed between the lobular cluster and the triple negative IDC cluster, appeared to be prognostic in three external breast cancer data sets (*48*). Genes that were overexpressed in the lobular tumour group were associated with a good prognosis, and often coded for secreted proteins or proteins situated in the extracellular matrix (*48*). Moreover, some of these differentially expressed genes, like *CAV1*, *CAV2* and *DCN* (coding for caveolin-1, caveolin-2 and decorin, respectively) had already been investigated and their reduced stromal expression was found to be associated with poor outcome in breast cancer (*40-43, 49*).

In the present study, we selected six genes out of 'additional file 4' of the taxonomy study of Gevaert et al. (*48*). Since all six genes were upregulated in the lobular cluster when compared to the triple negative IDC cluster, we expected these genes to be upregulated in well-differentiated DCIS lesions and downregulated in poorly differentiated DCIS lesions. In the taxonomy study, gene expression profiles were based on whole-tumour derived data (*48*), which highlights the robustness of the six molecular markers we selected. Even in whole-tumour samples, gene expression confined to the tumoural stroma contributed to the gene expression profiles, and the consequential prognostic signature is expected to be even more demonstrable when applied in samples solely consisting of stroma adjacent to mammary tumours.

Because of this suspected strong correlation with tumour grade, we wanted to evaluate the stromal expression of the six chosen genes at the protein level in our cohort of DCIS patients, and evaluated a possible correlation between this stromal protein expression and two different pathologic classification systems, which were shown to correlate with patient outcome (*34, 35*).

2.5. HER2 in DCIS: still a relatively unexplored territory

Patients with invasive breast cancer and demonstrated HER2 overexpression are treated with trastuzumab and lapatinib (*50*). Although HER2-positive breast cancer has a worse prognosis, treatment with trastuzumab and lapatinib is associated with improved clinical outcomes. Notwithstanding the amount of evidence suggesting the efficacy of anti-HER2 treatment in IDC, we do not know whether patients with HER2-positive DCIS are eligible for these therapies, e.g. for slowing down the progression of DCIS to IDC.

In fact, we hardly know anything about the role of HER2 in DCIS and in mammary tumour progression, despite the results of various studies, in which an immunohistochemical analysis of HER2 was performed (*18, 36, 51-53*). Even a simple fact as the prevalence of HER2 protein overexpression in DCIS differs among these studies, as the methods for assessing HER2 expression are not fixed. Although HER2 overexpression is known to be more common in DCIS than in IDC, this discrepancy has not been elucidated yet.

Fewer studies have analysed *HER2* gene amplification status by FISH (*19, 53*), but none of them supplies information about the presence of clusters of *HER2* signals in DCIS. To our knowledge, no studies have been published in which *HER2/CEP17* ratio, and *HER2* and *CEP17* copy numbers have been assessed in DCIS. Since we have previously shown the existence of a remarkable *HER2* gene dosage effect in invasive breast cancer (*54*), we wanted to explore whether a similar relation between *HER2* copy number and protein expression existed in DCIS.

3. GOALS OF THE RESEARCH

The purpose of this study is to understand more about the pathobiology of DCIS and to identify new prognostic markers that might be used to estimate the biological behaviour of a DCIS lesion, leading to a more individualized treatment of patients diagnosed with DCIS.
Our aim is to explore the correlation between the assessed histopathological features and the stromal protein expression of several molecular markers. In addition, we want to explore how our findings correlate with recent publications about histopathological characteristics of DCIS, such as the study of Pinder et al. (*35*) concerning a new pathological grading system for DCIS. A third objective is to carry out a thorough evaluation of the relationships between histopathological features and the FISH and IHC results for HER2, as already has been performed for invasive breast cancer by Lambein et al. (*54*). This will enable us to compare the pathobiological features of HER2 in DCIS and in invasive breast cancer.

In a cohort of 61 DCIS cases, we evaluated various histopathological features and applied different systems of categorization (*34, 35*), to evaluate a possible correlation with stromal expression of various molecular markers at the protein level. These molecular markers (caveolin-1, caveolin-2, aquaporin-1, laminin-beta-2, necdin and decorin) were selected out of a list of stromal genes associated with prognosis in invasive breast tumours. The selected molecular markers were expected to be downregulated in the stroma of high grade DCIS lesions (additional file 4) (*48*). The selection of these genes was mainly influenced by the availability of antibodies with known effectiveness when applied in immunohistochemistry on paraffin embedded tissue. Other proteins (CD34, SMA and CD10) with known association with tumoural stromal fibroblasts were used to complete this selection, although they do not belong to the list with identified stromal genes (additional file 4) (*48*). Unlike the molecular markers selected out of the taxonomy study by Gevaert et al. (*48*), stromal expression of SMA and CD10 is not inversely correlated with DCIS histopathological grade (*44-46*).
Beside an immunohistochemical analysis of the selected stromal molecular markers on the protein level, we also performed an immunohistochemical analysis of the hormone receptor status and HER2 protein expression in these DCIS cases. Since tamoxifen is currently recommended for treating DCIS, we wanted to explore the hormone receptor status of the lesions in this patient cohort. In addition, fluorescence-in-situ-hybridisation was used to determine the rate of *HER2* amplification.

4. METHODS

4.1. Patients and tissue samples

A cohort of 61 ductal carcinoma in situ cases was examined. This cohort comprised 55 cases (90%) of pure DCIS (i.e. without associated invasive ductal carcinoma or microinvasion), and six cases (10%) of DCIS with associated microinvasion. Microinvasion was defined as foci of malignant epithelial cells extending beyond the basement membrane, into the (non-specialized) stroma around the ducts, with each focus measuring no more than 0,1 cm in size (*2*). Hematoxylin and eosin-stained (H&E) slides of each patient were obtained from the archives of the pathology department (N. Goormaghtigh Instituut voor Pathologische Anatomie) of the Ghent University Hospital (Belgium). All available H&E slides were reviewed by an experienced breast pathologist (KL) and a trainee in pathology (MVB) using a multihead light microscope. One representative slide, with presence of both ducts affected by DCIS and surrounding specialized stroma, was selected for each case. According formalin-fixed paraffin-embedded tissue blocks were obtained from the aforementioned archives.

All tissue specimens originate from 61 female patients who underwent a lumpectomy and/or mastectomy in the period from January 2007 till February 2011. Patients were retrospectively selected, based on a search in the electronic histopathologic reports. Patients who underwent only a needle biopsy and got further treatment in a hospital elsewhere, were excluded. Macroscopic examination was performed according to the ASCO/CAP guidelines (*55*). The surface of every lumpectomy or mastectomy specimen was inked, often using multiple colours to permit microscopic identification of margins of the resection. Subsequently, every specimen was cut in slices of approximately 0,5 cm and each suspicious area was sampled. All tissue specimens were fixed in 4% formalin during at least 6 hours and maximum 48 hours, before being embedded in paraffin. Tissue sections of 3.5 µm were cut and mounted on Superfrost slides (Menzel-Gläser, Braunschweig, Germany).

Two patients underwent axillary lymph node dissection and 23 patients underwent a sentinel node procedure. In the case of a sentinel procedure, the lymph node(s) were cut into slices of 2 mm. Sections stained with H&E and a broad-spectrum keratin antibody were performed at 3 levels at 100 µm intervals. Status of the resected lymph nodes was recorded retrospectively using the electronic reports. None of the lymph nodes showed micro- or macrometastasis, nor the presence of isolated tumour cells, as defined by the TNM classification (*2*).

4.2. Histopathological evaluation of DCIS

4.2.1. Architectural patterns

The architectural features of each case of DCIS were assessed and classified in four types: cribriform, micropapillary, papillary or solid growth pattern, as described by Pinder et al. (*35*). Several cases exhibited different architectural patterns, so for each case, the main growth pattern was also determined. We did not consider 'comedo' as a distinct growth pattern, but scored the presence or absence of comedo necrosis as a separate feature, since necrosis could be found in the presence of all four aforementioned architectural patterns.

4.2.2. Nuclear atypia

Nuclear grade was classified in three categories: low (grade I), intermediate (grade II) and high grade (grade III), following recommendations of the CAP (*55*). Six morphologic characteristics were used to determine the nuclear grade: size, pleomorphism, presence of nucleoli and mitoses, chromatin distribution and orientation of the nuclei (table 4) (*55*). For each DCIS case, the main nuclear grade was assessed and presence of more than one grade (heterogeneity) was noted.

Tabel 4 Morphologic features to assess nuclear grade in DCIS (as described by Lester et al. (*55*)).

Feature	Grade I	Grade II	Grade III
Pleomorphism	Monotonous (monomorphic)	Intermediate	Markedly pleomorphic
Size	1,5 x to 2 x the size of a normal RBC or a normal duct epithelial cell nucleus	Intermediate	> 2,5 x the size of a normal RBC or a normal duct epithelial cell nucleus
Chromatin	Usually diffuse, finally dispersed chromatin	Intermediate	Usually vesicular with irregular chromatin distribution
Nucleoli	Only occasional	Intermediate	Prominent, often multiple
Mitoses	Only occasional	Intermediate	May be frequent
Orientation	Polarized towards luminal spaces	Intermediate	Usually not polarized towards luminal spaces

4.2.3. Stromal morphology and inflammation

The morphology of the specialized stroma around the involved ducti was assessed and classified as sclerotic or myxoid. Some cases displayed both sclerotic and myxoid stromal morphology; and for each case the predominant type was determined. The amount of peri-ductal chronic inflammation was also assessed. In view of discrepancies of the definition of stromal inflammation, we scored the infiltration of the specialized stroma by lymphoid cells in a semi-quantitative way as absent, mild, moderate or extensive.

4.2.4. Comedo necrosis and calcifications

The presence of calcifications was assessed and classified in two categories (absent or present), as described by Pinder et al. (*35*). Because of several discrepancies of the definition of necrosis, the presence of necrosis was subdivided in four categories: absent necrosis, single cell necrosis, focal comedo necrosis and extensive comedo necrosis. Single cell necrosis was determined as the presence of a few necrotic cells in the lumen of the ducts affected by DCIS, which could only be detected at higher magnification. Focal and extensive comedo necrosis were defined as the presence of necrotic debris in less or more than fifty percent of the affected ducts, respectively. Both focal and extensive comedo necrosis are easily detected at low magnification.

4.2.5. Extent of DCIS lesions

The size of DCIS lesions was determined retrospectively by reviewing the electronic histopathological records. In three cases (5%), the extent of a DCIS lesion was not mentioned in the electronic report, and size was estimated by multiplying the number of blocks involved by DCIS with 0,4 (i.e. the approximate width of a tissue section), as described by Lester et al. (*55*). In twenty patients, the lactiferous ducts were also surgically removed, sampled during macroscopic pathologic examination and histologically evaluated on H&E slides.

4.2.6. Margin width after excision

Tumour-free margin width was defined as the smallest distance measured between the border of the DCIS lesion and the inked resection margin. Margin width of every lumpectomy or mastectomy specimen was determined retrospectively by reviewing the electronic histopathological records. In five cases (8%), tumour-free margin width was not mentioned in the original report. In these cases, margin width was measured on the slides.

4.2.7. Van Nuys Prognostic Index and pathological classification

After evaluating the aforementioned histopathological features of this retrospective DCIS cohort, the pathologic classification (table 2) as well as the modified Van Nuys Prognostic Index (VNPI, table 2) were recalculated for every DCIS case (*34*). Pathologic classification is a part of the Van Nuys Prognostic Index and comprises three categories: 1) non-high grade without necrosis; 2) non-high grade with necrosis; and 3) high-grade with or without necrosis. Besides pathological classification, three other independent predictors of local recurrence are determined to calculate the VNPI. These predictors include patient age, the size of the DCIS lesion and the tumour-free margin width (*34*).

4.2.8. Pinder pathological classification for DCIS

After the assessment of the aforementioned histopathological features of this retrospective DCIS cohort, we also applied the three-tiered pathological classification developed by Pinder et al. (*35*). This system comprises three categories: a group of low and intermediate cytonuclear grade DCIS, and a group of high cytonuclear grade DCIS which is subdivided according to two criteria (the absence or presence of >50% solid architecture and >50% ducts bearing comedo necrosis) into a 'high risk' and a 'very high risk' group (table 3) (*35*).

Both the VNPI pathologic classification and the Pinder classification have clearly been shown to correlate with disease recurrence and are developed as a prognostic tool. Since follow-up data of the patients included in this study are not available, we used both the VNPI pathologic classification and the Pinder classification as surrogate prognostic markers.

4.3. Immunohistochemistry

4.3.1. Hormone receptor status

Immunohistochemical stained slides for nuclear estrogen receptor (ER) and nuclear progesterone receptor (PR) were available in the archives for a subset of 46 patients. These stainings needed to be performed for the fifteen remaining patients. Slides of all 61 cases were also stained and scored for membrane expression of HER2/Neu.

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections of 3,5 µm in thickness, using a Ventana Automated Slide Stainer (Benchmark XT, Ventana Medical Systems Inc., Arizona, USA) according to manufacturer's instructions. The following primary antibodies were used: CONFIRM™ anti-Estrogen Receptor, a monoclonal rabbit antibody to human estrogen receptor alpha (clone SP1, ready-to-use, Ventana Medical Systems Inc., Arizona, USA), CONFIRM™ anti-Progesterone Receptor, a monoclonal rabbit antibody to human progesterone receptor A and B (clone 1E2, ready-to-use, Ventana Medical Systems Inc., Arizona, USA), and PATHWAY™ HER-2/Neu, a monoclonal rabbit antibody directed against the internal domain of the c-erbB-2 oncoprotein (HER2) (clone 4B5, ready-to-use, Ventana Medical Systems Inc., Arizona, USA).

Heat-induced epitope retrieval was carried out, using Cell Conditioning 1 for ER and PR, and using Cell Conditioning 2 for HER2 (Ventana Medical Systems Inc., Arizona, USA). For all antibodies, visualisation was achieved with the ultraView™ Universal DAB Detection Kit (Ventana Medical Systems Inc., Arizona, USA), according to manufacturer's instructions. Dehydratation of the tissue sections and applying coverslips was carried out using an automated coverslipper (Tissue-Tek Film Coverslipper, Sakura, Alphen aan den Rijn, The Netherlands).

Expression of ER and PR was evaluated by light microscopy and scored using the Allred scoring system as described by Harvey et al. (*56*). The proportion of nuclear positive-staining tumour cells in the DCIS lesions, and the mean intensity of positive-staining nuclei was assessed and scored as shown in table 5. Both proportion and intensity score were added, which resulted in a total 'Allred score' or quick score ranging from 0 to 8. An Allred score of 2 or more was considered as positive, and an Allred score of 0 was considered as negative (*56*).

Table 5 Allred Quick Score. The average intensity and the estimated proportion of positive staining tumour cells is assessed and scored semi-quantitatively. Both scores are totalled, ranging from 0 to 8 (Harvey et al. (*56*)).

Intensity score	**0**	**1**	**2**	**3**		
Intensity	none	weak	intermediate	strong		
Proportion score	**0**	**1**	**2**	**3**	**4**	**5**
Proportion	none	< 1/100	1/100-1/10	1/10-1/3	1/3- 2/3	> 2/3

Membrane expression of human epidermal growth factor receptor 2 (HER2) was evaluated by light microscopy and scored using an adaptation of the four-point scale of the ASCO/CAP guidelines for HER2 testing in invasive breast cancer (*57*), as performed by Altintas et al. (*36*):

3+ : uniform, intense membrane staining of >30% of the intraductal tumour cells was considered as a positive result.

2+ : complete non-uniform or weak membrane staining but with clear circumferential distribution in >10% of the cells, or intense complete staining in <30% of intraductal tumour cells was considered as an equivocal result.

1+ : weak, incomplete membrane staining in any proportion of the cells, or weak, complete staining in <10 % of intraductal tumour cells was regarded as negative.

0 : a negative result was defined as complete absence of staining.

4.3.2. Immunohistochemical evaluation of stromal proteins.

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections of 3,5 µm in thickness, using a Ventana Automated Slide Stainer (Benchmark XT, Ventana Medical Systems Inc., Arizona, USA) according to manufacturer's instructions.

The following primary antibodies were used: mouse monoclonal antibody to alpha smooth muscle actin (α-SMA) (clone 1A4, dilution 1/400, BioGenex, Den Haag, The Netherlands), a monoclonal mouse antibody to the transmembrane phosphoglycoprotein CD34 (clone QBEnd10, ready-to-use, Beckman-Coulter Inc., Marseille, France), a monoclonal mouse antibody to the cell surface enzyme CD10/CALLA (Common Acute Lymphocytic Leukemia Antigen) (clone 56C6, ready-to-use, Thermo Fisher Scientific, Astmoor, Cheshire, UK), a monoclonal mouse antibody to the transmembrane protein caveolin-1 (clone C060, dilution 1/400, BD Biosciences, San Jose, CA, USA), a monoclonal mouse antibody to the transmembrane protein caveolin-2 (clone 65/caveolin2, dilution 1/100, BD Biosciences, San Jose, CA, USA), a monoclonal mouse antibody to the transmembrane protein aquaporin-1

(ab9566, dilution 1/50, Abcam, Cambridge, UK), a polyclonal rabbit antibody to laminin-beta-2 (dilution 1/50, Novus Biologicals, Littleton, CO, USA), a monoclonal mouse antibody to necdin (ab55501, dilution 1/200, Abcam, Cambridge, UK) and a monoclonal mouse antibody to decorin (ab54728, dilution 1/500, Abcam, Cambridge, UK). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (Ventana Medical Systems Inc., Arizona, USA) during staining for CD10, CD34 and SMA. No pre-treatment was used during the staining procedure for decorin. Heat-induced antigen retrieval was carried out using Cell Conditioning 2 (Ventana Medical Systems Inc., Arizona, USA) during staining for caveolin-1, caveolin-2, aquaporin-1, laminin-beta-2 and necdin. For all antibodies, visualisation was achieved with the ultraView^{TM} Universal DAB Detection Kit (Ventana Medical Systems Inc., Arizona, USA), according to manufacturer's instructions. Dehydratation of the tissue sections and applying coverslips was carried out using an automated coverslipper (Tissue-Tek Film Coverslipper, Sakura, Alphen aan den Rijn, The Netherlands).

Expression of all proteins was evaluated by light microscopy. The stromal expression of SMA, CD34, caveolin-1, necdin and aquaporin-1 was scored semi-quantitatively as absent (0; defined as no staining or weak staining in <10% of fibroblasts), weak (1; either diffuse weak staining or strong staining in <1/3 of stromal fibroblasts), moderate (2; either diffuse moderate staining or strong staining in >1/3 and <2/3 of stromal fibroblasts) and strong (3; diffuse strong staining in >2/3 of stromal fibroblasts).
The stromal staining for CD10, caveolin-2 and laminin-beta-2 was scored semi-quantitatively as absent (0; defined as no staining or weak staining in <10% of fibroblasts), low (1; either diffuse weak staining or strong staining in <1/3 of stromal fibroblasts) and high (2; defined as strong staining in >1/3 of stromal fibroblasts). The stromal decorin expression was scored semi-quantitatively as strong (0; defined as complete absence of stromal sparing around >90% of the affected ducts), moderate (1; defined as focal stromal sparing around >10% and <2/3 of the affected ducts) and weak (2; defined as stromal sparing around >2/3 of the affected ducts).

Since 66% (40/61) of our patient cohort underwent mastectomy instead of BCS, and these patients were all rather recently diagnosed with DCIS (January 2007 till January 2011), clinical follow-up data on DCIS recurrence and DCIS progression to invasive breast cancer were not available. Therefore, we used the aforementioned 2-tier VNPI pathologic classification and the 3-tier Pinder classification as surrogate prognostic markers, and correlated stromal protein expression with these two pathologic classification systems.

4.4. HER2 fluorescence-in-situ-hybridisation (FISH)

Fluorescence-in-situ-hybridisation (FISH) was used to assess the rate of *HER2/Neu* gene amplification in sixty DCIS cases in our cohort. FISH could not be performed on one case due to insufficient DCIS-containing tissue available in the selected paraffin block after tissue sections were cut for immunohistochemistry, and no other tissue blocks of this case contained ducts affected by DCIS.

All resected specimens were fixed in a 4% formaldehyde solution for 6 to 48 hours and embedded in paraffin. Tissue sections of 3,5 µm in thickness were cut and mounted on Superfrost slides (Menzel-Gläser, Braunschweig, Germany). Depending on the tissue size, 7-10 µl of the LSI *HER2/Neu* and *CEP17* probe (PathVysion *HER2* DNA Probe Kit; Abott, Illinois, USA) was applied. After denaturation for 5 minutes at 75°C in a water bath, the sections were hybridised overnight in an incubator at 37°C. Afterwards, 10 µl of DAPI (4', 6-diamidino-2-phenylindole) was added, and a coverslip was applied.

According to ASCO/CAP guidelines (*57*), red (for *HER2*) and green (for *CEP17*) non-overlapping signals were counted in twenty nuclei, using a fluorescence microscope equipped with appropriate filters (Olympus BX40, Tokyo, Japan). Nuclei without signals, or nuclei with signals of only one colour were excluded. The presence of clusters of *HER2* signals was assessed. The mean *HER2* and *CEP17* copy number was calculated by dividing the total number of signals by the number of counted nuclei. *HER2* gene amplification was defined as a *HER2/CEP17* ratio of > 2.2, and a *HER2/CEP17* ratio < 1.8 was considered as non-amplified. When the ratio was $\geq$ 1.8 and $\leq$ 2.2 (equivocal result), additional cells were counted and a ratio of $\geq$ 2.0 was used as cut-off for amplification (*57*).

4.5. Statistical analysis

Data were analysed using SPSS 16.0 software (Chicago, IL, USA). Spearman's correlation, Fisher's Exact test, Mann-Whitney U test, Kruskal-Wallis test or ANOVA were performed when appropriate. All mentioned p-values are two-sided, and $p<0.05$ was considered statistically significant.

5. RESULTS

5.1. Patient characteristics

All 61 patients were Caucasian women, diagnosed with pure DCIS (55 cases; 90%) or DCIS with microinvasion (six cases; 10%). Mean age at the moment of the first surgical procedure was 53 (range 33-81; median 52). Nine women (15%) underwent a lumpectomy (i.e. breast conserving surgery). Because of involvement of the surgical resection margin in the original lumpectomy specimen, twelve patients (20%) underwent a re-excision and seven patients (11%) underwent a secondary mastectomy. An initial mastectomy was performed in 33 women (54%).

None of these patients was known with a history of invasive ductal carcinoma in the ipsilateral breast. One patient had a history of invasive ductal adenocarcinoma in the contralateral breast. Three patients (5%) exhibited pure DCIS lesions in one breast, with a simultaneous invasive ductal adenocarcinoma (two patients) or a spindle cell tumour (a desmoid tumour, one patient) in the contralateral breast.

A sentinel node procedure was performed in 23 patients (38%) and two patients (3%) underwent an initial axillary lymph node dissection. The mean number of resected lymph nodes was 3,2 (range 1-15) after sentinel procedure, and was 8,5 after axillary lymph node dissection. None of the resected lymph nodes displayed signs of metastasis (including isolated tumour cells, micro- and macrometastasis). Table 6 shows the distribution of patient characteristics and histopathological features in this cohort.

5.2. Histopathological features of DCIS cases

5.2.1. Architectural patterns

Twelve cases (19%) presented with a single type of growth pattern, which implies that the majority of DCIS cases displayed architectural heterogeneity. Twenty-one (34%) cases displayed two kinds of architectural patterns, three different architectural patterns were present in 24 (39%) cases, and all four different growth patterns were present in four cases (7%). For each case, the main growth pattern was determined. Nine cases (15%) displayed a predominantly micropapillary architectural pattern (Fig 3B, 5B), thirty cases (49%) were mainly solid (Fig 3A), 19 cases (31%) were mainly cribriform (Fig 4A) and three cases (5%) displayed a predominantly papillary growth pattern (Fig 4B), as shown in table 6.

5.2.2. Nuclear atypia

A single grade of nuclear atypia was present in 28 cases (46%) and 33 cases (54%) presented with two grades. In these cases with heterogeneous cytonuclear differentiation, three cases (5%) had both grade I and grade II atypia, and thirty cases (49%) displayed both grade II and grade III nuclear atypia. For each case, the main nuclear grade was also determined. One case (2%) displayed grade I nuclear atypia; 37 cases (61%) presented with grade II nuclear atypia and the remaining 23 cases (38%) displayed grade III nuclear atypia (Fig 5B).

5.2.3. Comedo necrosis and calcifications

Two cases (3%) showed no necrosis (Fig 3A) and three cases (5%) displayed single cell necrosis (Fig 4A). Focal comedo necrosis was present in 36 cases (69%) and twenty cases (33%) manifested extensive comedo necrosis (Fig 4A). Calcifications were frequently found within the intraluminal necrotic debris or within intraluminal secretions. Fifteen cases (25%) presented without, and 46 cases (75%) presented with calcifications (Fig 5A).

5.2.4. Stromal morphology and inflammation

Nine cases (15%) presented with mainly myxoid stroma (Fig 5B) and 52 cases (85%) exhibited mainly sclerotic stroma (Fig 3A). Both myxoid and sclerotic stroma (mixed type) was present in 27 cases (44%). Three cases (5%) displayed no stromal inflammation (Fig 3A). Mild periductal inflammation was present in 27 cases (44%) (Fig 4A). Twenty cases (33%) presented with moderate (Fig 5A) and eleven cases (18%) with extensive lymphocytic infiltration of the specialized stroma (Fig 3B), as shown in table 6.

5.2.5. Extent of DCIS lesions

The DCIS lesions had a mean size of 26 mm (range 5-100 mm, median 20 mm). In twenty patients (33%), the lactiferous ducts were evaluated histologically on H&E slides. Six of these patients (10% of the total cohort) displayed involvement of the lactiferous ducts by DCIS, and the mean size of these lesions measured 37.5 mm.

5.2.6. Margin width after excision

In twenty patients (33%), margin width measured less than 1 mm. In 24 patients (39%), margin width was 1 to 9 mm and in seventeen patients (28%), margin width measured 10 mm or more (table 6).

Table 6 Distribution of patient characteristics and histopathological features in this cohort of 61 DCIS cases.

Feature	n (%)	Feature	n (%)
Nuclear atypia (main grade)		Surgical intervention	
Grade I	1 (2)	Lumpectomy	9 (15)
Grade II	37 (61)	Lumpectomy + re-excision	12 (20)
Grade III	23 (38)	Mastectomy	33 (54)
Heterogeneity in nuclear grade		Lumpectomy + mastectomy	7 (11)
Single type of grade	28 (46)	Lymph node examination	
Mixed (two types)	33 (54)	Sentinel procedure	23 (38)
Architectural pattern		Axillary dissection	2 (3)
Micropapillary	9 (15)	Absence or presence of microinvasion	
Solid	30 (49)	Pure DCIS	55 (90)
Cribriform	19 (31)	DCIS with microinvasion	6 (10)
Papillary	3 (5)	VNPI age groups (years)	
Architectural heterogeneity (number of		<40	4 (7)
growth patterns present in one case)		40-60	42 (69)
1 type	12 (19)	>60	15 (25)
2 types	21 (34)	VNPI size groups (mm)	
3 types	24 (39)	≤15	24 (39)
4 types	4 (7)	16-40	30 (49)
Necrosis		≥41	7 (11)
Absent necrosis	2 (3)	VNPI margin width groups (mm)	
Single cell necrosis	3 (5)	<1	20 (33)
Focal comedo necrosis	36 (49)	1-9	24 (39)
Extensive comedo necrosis	20 (33)	≥10	17 (38)
Calcifications		VNPI pathologic classification	
Absent	15 (25)	Low	1 (2)
Present	46 (75)	Intermediate	37 (61)
Stromal morphology		High	23 (28)
Sclerotic	9 (15)	VNPI score	
Myxoid	52 (85)	4-5-6	9 (15)
Heterogeneity in stromal morphology		7-8-9	42 (69)
Single type	34 (56)	10-11-12	10 (16)
Mixed (myxoid + sclerotic)	27 (44)	Pinder pathologic classification	
Stromal inflammation		Low	1 (2)
Absent	3 (5)	Intermediate	37 (61)
Mild	27 (44)	High	17 (28)
Moderate	20 (33)	Very high	6 (10)
Extensive	11 (18)		

Figure 3 Hematoxylin and eosin (H&E) staining with original magnification 10x objective. (A) Solid DCIS lesion with grade 2 nuclear atypia, no necrosis and sclerotic stroma without stromal inflammation. (B) Micropapillary DCIS lesion with grade 3 nuclear atypia, extensive comedo necrosis, mixed sclerotic and myxoid stroma, and extensive stromal inflammation (black arrow).

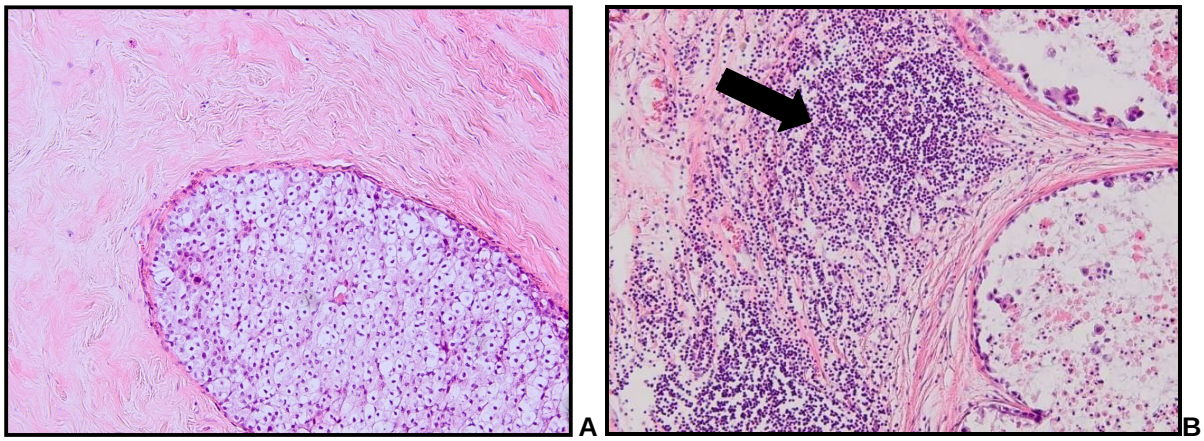


Figure 4 H&E staining with original magnification 10x objective. (A) Cribriform DCIS lesion with extensive comedo necrosis (black arrow) without calcifications, sclerotic stroma and mild stromal inflammation (red arrow). (B) Papillary DCIS lesion with grade 2 nuclear atypia.

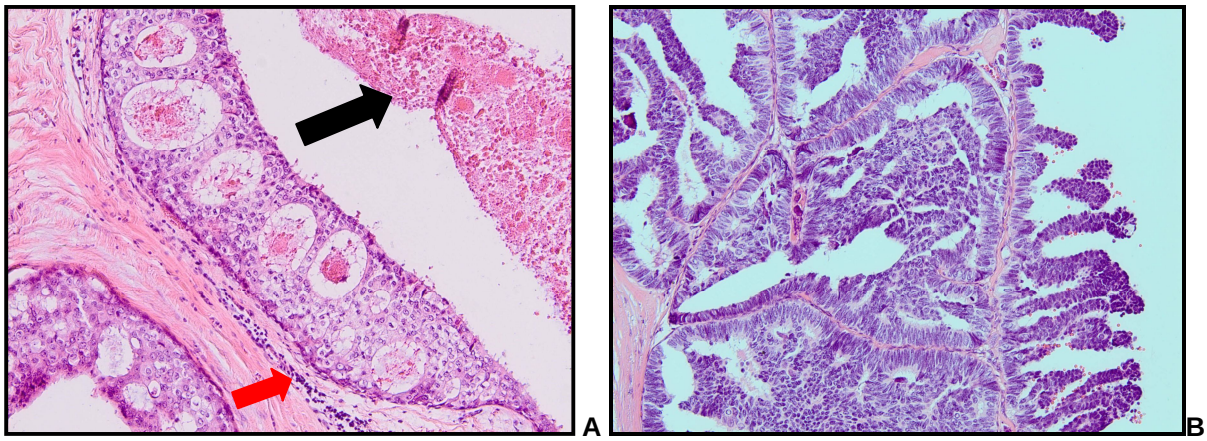
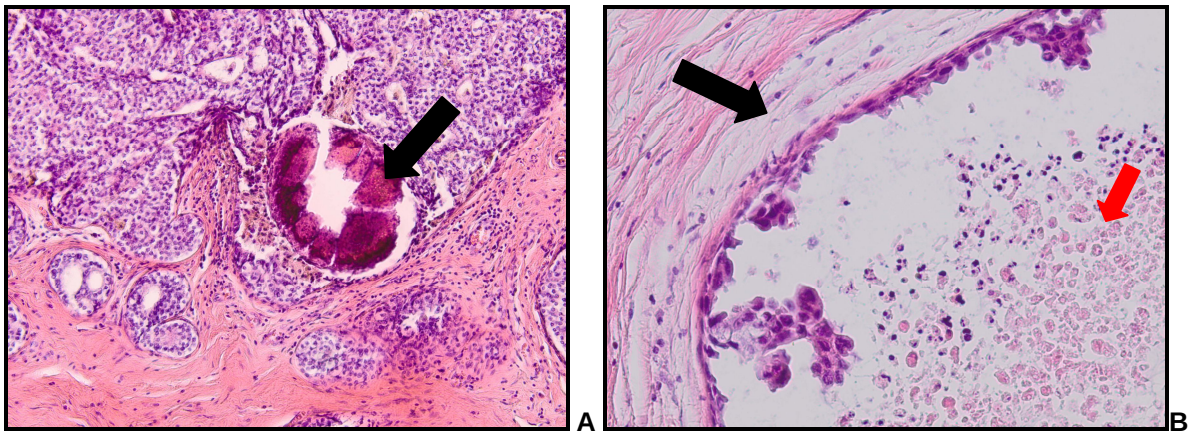


Figure 5 H&E staining with original magnification 10x objective. (A) Solid DCIS lesion with single cell necrosis and presence of intraductal calcifications (black arrow). (B) Micropapillary DCIS lesion with grade 3 nuclear atypia, extensive comedo necrosis (red arrow) and myxoid stroma (black arrow).



5.2.7. Van Nuys Prognostic Index, VNPI pathologic classification and their relation with patient features and histopathological characteristics

The VNPI pathologic grade and the Van Nuys Prognostic Index were calculated for al cases as described by Silverstein et al. (table 2) (*34*). Only one DCIS case (2%) was classified as non-high grade without necrosis; 37 cases (61%) were classified as non-high grade with necrosis and 23 cases (38%) as high grade with or without necrosis.

Although the VNPI was originally developed as a tool to guide treatment decisions and to predict disease recurrence in patients with pure DCIS treated with breast preservation (*34*), we calculated the VNPI for every patient in this cohort, even if the patient was treated with mastectomy. Median VNPI for the total cohort was 8 (range 5-11). In the total cohort, nine patients (15%) had a VNPI score of 5 or 6, and 42 patients (69%) had a score of 7, 8 or 9. A score of 10 or 11 was found in ten patients (16%) (table 6-7).

Table 7 Van Nuys Prognostic Index (VNPI) and its four predictors in relation to the surgical intervention (lumpectomy group versus mastectomy group). The 'intermediate' group in the 2-tier VNPI pathologic classification also has a single case with grade I nuclear atypia. All p-values are calculated with Fisher's Exact Test or Mann-Whitney U test.

	Lumpectomy, n (%) or mean ± SD	**Mastectomy, n (%) or mean ± SD**	**p-value**
Age (years)	57.12±9.91	51.45±9.41	0.020
Age (years) VNPI groups			0.182
<40	1 (5)	3 (8)	
40-60	12 (57)	30 (75)	
>60	8 (38)	7 (18)	
Size (mm)	14.43±9.36	32.42±25.08	<0.001
Size (mm) VNPI groups			0.001
≤15	15 (71)	9 (23)	
16-40	6 (29)	24 (60)	
≥41	0 (0)	7 (18)	
Margin width (mm)	5.41±4.57	4.91±5.86	0.630
Margin width (mm) groups			0.342
<1	7 (33)	13 (32)	
1-9	6 (29)	18 (45)	
≥10	8 (38)	9 (23)	
Pathological classification VNPI			0.728
Intermediate	14 (67)	24 (60)	
High	7 (33)	16 (40)	
VNPI (total score)	7.24±1.61	8.32±1.25	0.006

Mean VNPI for the patients who underwent lumpectomy (with or without additional re-excision) was 7.24 (range 5-11), and it amounted 8.32 (range 6-11) for the patients who underwent mastectomy (with or without previous lumpectomy). Although this seems to be only a small difference, the total VNPI score correlated significantly with the type of surgical intervention (p=0.006, table 7).

The VNPI is not necessarily low in patients who undergo mastectomy. This could be caused by young age of the patient, or by narrow resection margins in the mastectomy specimen, but the most important reason in this cohort was the extent of the DCIS lesions (p<0.001). The mean size of the lesions in the lumpectomy group amounted 14 mm (range 5,5-40 mm), whereas the mean size amounted 32 mm (range 5-100 mm) in the mastectomy group. The mean margin width was 5 mm in both the lumpectomy and mastectomy group, and thus could not explain the difference in total VNPI score between these two groups (p=0.630, table 7). The mean age was 57 (range 38-81) in the lumpectomy group and was 51 (range 33-70) in the mastectomy group. When patient age was regarded as a continuous variable, it correlated with the type of surgical intervention (p=0.020), although the VNPI age groups did not (p=0.182), as is shown in table 7. Since mastectomy was associated with younger age, and because DCIS size was significantly higher in the mastectomy group, we analysed whether there was an association between patient age and size. Both the VNPI age groups and age as a continuous variable did not correlate with the VNPI size groups (p=0.474 and p=0.300, respectively).

Pathological classification was not significantly different between the lumpectomy and mastectomy group (p=0.728, table 7), but if all four types of surgical interventions were analysed, a significant correlation with the VNPI pathological classification was noted (p=0.021, table 8). This is mainly due to the fact that none of the patients who underwent a lumpectomy without re-excision or secondary mastectomy showed to have a DCIS lesion which was classified as high grade. When patient age was considered as a continuous variable, it did not correlate with the VNPI pathological classification (p=0.113), although the VNPI age groups did (p=0.013, table 8). In this cohort, only four patients were younger than forty, but they all appeared to have a high grade DCIS lesion.

No correlation was found between VNPI pathological classification and size of the DCIS lesions, presence of microinvasion, the involvement of lactiferous ducts, extensive comedo necrosis, calcifications or architectural pattern (p-values, table 8). Strikingly, high grade lesions presented with more chronic stromal inflammation (p=0.007), and they also displayed a myxoid periductal stroma more frequently (p=0.001).

Table 8 Histopathological characteristics and patient age in relation to the 2-tier VNPI pathologic classification and the 3-tier Pinder classification. Values are n (%) or mean ± SD. The 'intermediate' group (intermed.) in both the 3-tier classification system of Pinder et al. and the 2-tier VNPI pathologic classification also contains a single case with grade I nuclear atypia. All p-values are calculated with Fisher's Exact, Kruskal-Wallis test or Mann-Whitney U test.

	VNPI pathologic classification			**Classification Pinder et al.**			
	Inter-mediate	**High**	**p value**	**Inter-mediate**	**High**	**Very high**	**p value**
Age (years)	55.4±9.6	50.1±9.7	0.113	55.4±9.6	50.5±9.9	49.0±9.9	0.273
VNPI age groups			0.013				0.040
<40	0 (0)	4 (17)		0 (0)	3 (18)	1 (17)	
40-60	26 (68)	16 (70)		26 (68)	12 (71)	4 (67)	
>60	12 (32)	3 (13)		12 (32)	2 (12)	1 (17)	
Surgical intervention			0.021				0.115
Lumpectomy	9 (24)	0 (0)		9 (24)	0 (0)	0 (0)	
Lump. + re-excis.[a]	5 (13)	7 (30)		5 (13)	5 (29)	2 (33)	
Mastectomy	21 (55)	12 (52)		21 (55)	9 (53)	3 (50)	
Lump. + Mastect.[b]	3 (8)	4 (17)		3 (8)	3 (18)	1 (17)	
Microinvasion			0.664				0.530
Absent	35 (92)	20 (87)		35 (92)	15 (88)	5 (83)	
Present	3 (8)	3 (13)		3 (8)	2 (12)	1 (17)	
Size (mm)	27.5±21.2	24.2±25.2	0.219	27.5±21.2	26.6±28.6	17.5±10.2	0.416
VNPI size groups			0.647				0.861
≤15	13 (34)	11 (48)		13 (34)	8 (47)	3 (50)	
16-40	20 (53)	10 (44)		20 (53)	7 (41)	3 (50)	
≥41	5 (13)	2 (9)		5 (13)	2 (12)	0 (0)	
Lactiferous ducts			0.303				0.354
Not affected	11 (79)	3 (50)		11 (79)	3 (60)	0 (0)	
Affected	3 (21)	3 (50)		3 (21)	2 (40)	6 (100)	
Extensive comedonecrosis			0.090				0.002
Absent	29 (76)	12 (52)		29 (76)	12 (71)	0 (0)	
Present	9 (24)	11 (48)		9 (24)	5 (29)	6 (100)	
Calcifications			1.000				0.904
Absent	9 (24)	6 (26)		9 (24)	5 (29)	1 (17)	
Present	29 (76)	17 (74)		29 (76)	12 (71)	5 (83)	
Main growth pattern			0.376				0.112
Micropapillary	4 (11)	5 (22)		4 (11)	5 (29)	0 (0)	
Solid	18 (47)	12 (52)		18 (47)	6 (35)	6 (100)	
Cribriform	13 (34)	6 (26)		13 (34)	6 (35)	0 (0)	
Papillary	3 (8)	0 (0)		3 (8)	0 (0)	0 (0)	
Solid vs. non-solid growth			0.795				0.021
Non-solid	20 (53)	11 (48)		20 (53)	11 (65)	0 (0)	
Solid	18 (47)	12 (52)		18 (47)	6 (35)	6 (100)	
Stromal inflammation			0.007				0.014
Absent	3 (8)	0 (0)		3 (8)	0 (0)	0 (0)	
Mild	22 (58)	5 (22)		22 (58)	5 (29)	0 (0)	
Moderate	9 (24)	11 (48)		9 (24)	8 (47)	3 (50)	
Extensive	4 (11)	7 (30)		4 (11)	4 (24)	3 (50)	
Stromal morphology			0.001				0.001
Slerotic	37 (97)	15 (65)		37 (97)	11 (65)	4 (67)	
Myxoid	1 (3)	8 (35)		1 (3)	6 (35)	2 (33)	

[a] Lumpectomy followed by re-excision
[b] Lumpectomy followed by mastectomy

5.2.8. Pinder pathological classification for DCIS

The pathological classification according to Pinder et al. (*35*) is a new system for grading DCIS, adding a new 'very high risk' group. A central pathological review of 1224 cases entered into the UKCCCR/ANZ DCIS trial revealed a subgroup of patients with a very poor prognosis. This ‘very high risk’ group comprises DCIS lesions characterized by the presence of extensive comedo necrosis (>50% of ducts), high cytonuclear grade and predominantly (>50%) solid growth pattern (table3) (*35*). Patients who were classified in this group, had an ipsilateral recurrence rate of DCIS or invasive disease of 18,2%, whereas the low/intermediate risk group and the high risk group had an ipsilateral recurrence rate of 6,1% and 10,9%, respectively (*35*).

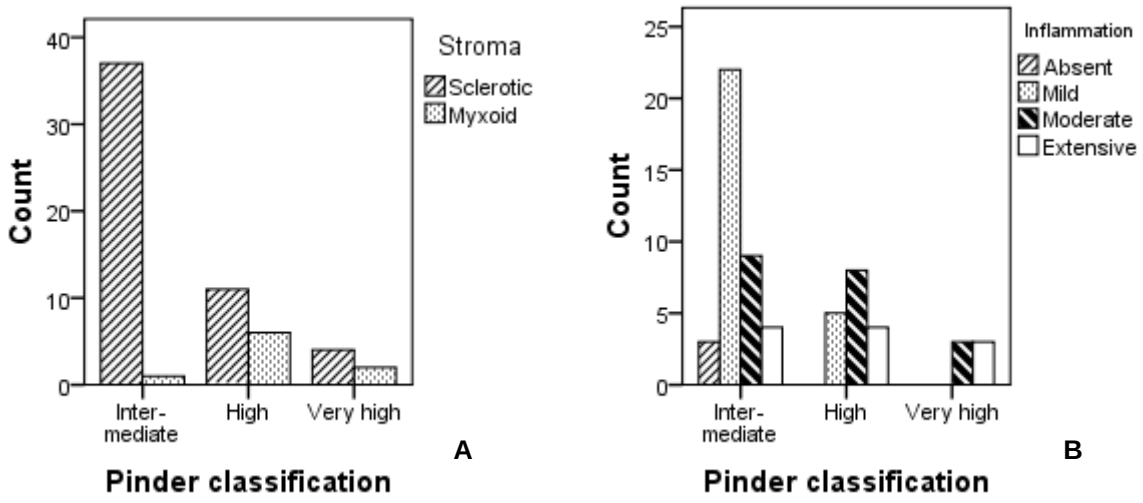
In our cohort, 38 patients (62%) were classified in the low/intermediate risk group (which is exactly the same group as the low/intermediate risk group in the VNPI pathologic classification), seventeen patients (28%) belonged to the high risk group and six patients (10%) were classified in the very high risk group. Since this cohort comprises only one DCIS case with low grade nuclear atypia, this single case was grouped together with all intermediate grade cases as one ‘intermediate risk’ group. We applied a 3-tier classification according to Pinder et al. (*35*).

When patient age was considered as a continuous variable, it did not correlate with the Pinder classification (p=0.273), although the VNPI age groups did (p=0.040, table 8). Patients older than sixty more often exhibited low grade DCIS lesions. Unlike the VNPI pathological classification, the Pinder classification did not correlate with the type of surgical intervention (p=0.115). No association was found between the Pinder classification and the presence of microinvasion, size of the DCIS lesions, involvement of lactiferous ducts and the presence of calcifications (p-values, table 8).

When all four types of growth patterns were regarded, no correlation was found with the Pinder classification (p=0.112). However, when the architectural patterns were grouped as solid and non-solid growth, a significant association with the Pinder classification was noted (p=0.021). The presence of extensive comedo necrosis also correlated with the Pinder classification (p=0.002). This is not surprising, taking into account that predominantly solid growth and extensive comedo necrosis are two required conditions for a DCIS lesion to be classified as ‘very high risk’.

The presence of a moderate or extensive inflammatory infiltrate in the periductal stroma was associated with lesions of a higher grade, and absent or mild stromal inflammation was mainly noted in the 'intermediate' risk group (p=0.014, Fig 6). Not only stromal inflammation, but also stromal morphology correlated with the Pinder classification (p=0.001). In this cohort, only nine cases presented with a predominantly myxoid periductal stroma, but six of them were graded as 'high risk', and two were graded as 'very high risk' (table 8).

Figure 6 (A) This clustered bar chart illustrates the correlation between the Pinder classification and the morphology of the periductal stroma. (B) Association between chronic stromal inflammation and the Pinder classification. All cases classified in the 'very high' risk group, present with a moderate or extensive stromal inflammatory infiltrate. P-values are shown in table 8.



5.3. Immunohistochemistry

5.3.1. Hormone receptor status

Expression of estrogen receptor (ER) and progesterone receptor (PR) was scored using the Allred scoring system as described by Harvey et al.(*56*). An Allred score of 0 was considered negative, and an Allred score of > 2 positive. Eight DCIS cases (13%) were ER negative and 53 cases (87%) were ER positive (Fig 7B). The mean Allred score for ER was 6 (range 0-8). ER expression correlated with the 2-tier VNPI pathologic classification (p=0.044). Ninety-five percent of the intermediate grade lesions were ER positive, while only 74 percent of the high grade lesions were positive (table 9). The same correlation was found with the 3-tier Pinder classification (p=0.043). Only five percent of the intermediate grade cases were ER negative, whereas one in four high grade cases and one in three very high grade cases presented no ER expression (table 9).

Twelve DCIS cases (20%) were PR negative and 49 cases (80%) were PR positive. The mean Allred score for PR was 5 (range 0-8). PR expression correlated with the VNPI pathologic classification, but not with the Pinder classification (p=0.039 and p=0.051, respectively). In the VNPI intermediate grade group, ninety percent of DCIS appeared to be PR positive, while only 65 percent of the VNPI high grade group displayed PR positivity (table 9).

Membranous staining for human epidermal growth factor receptor 2 (HER2) was scored using an adaptation of the ASCO/CAP guidelines for HER2 testing in invasive breast cancer (*57*), as described under 'Methods' section. None of the DCIS cases was assigned 0 as a HER2 IHC score. Fifteen cases (25%) were scored negative (1+), ten cases (16%) were equivocal (2+) and 36 cases (59%) were positive (3+, Fig 7A).

Figure 7 (A) Membranous 3+ HER2 immunostaining in cribriform DCIS, 20x objective (B) Estrogen receptor immune-staining (Allred score = 8) in a solid DCIS lesion with central comedo necrosis, 10x objective.

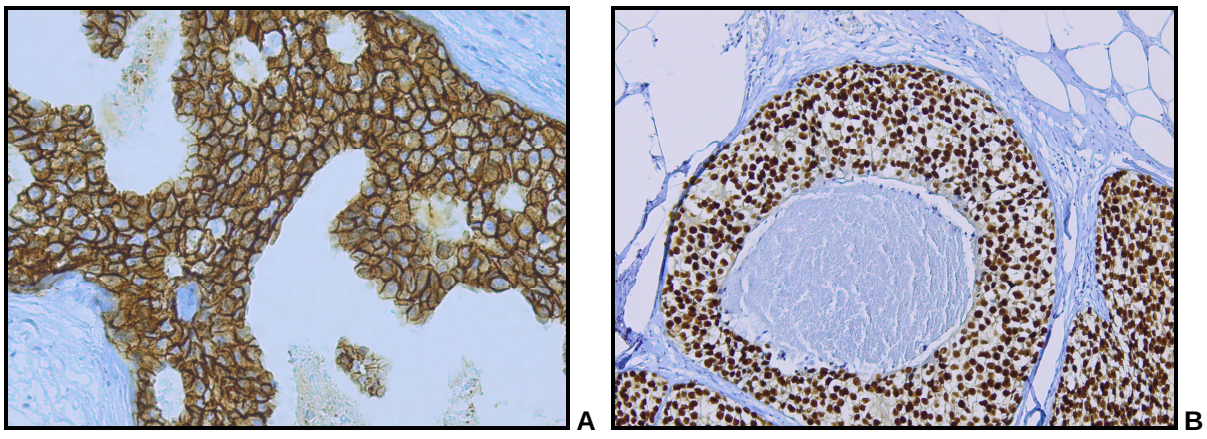


Table 9 Immunohistochemical features and *HER2* amplification in relation to the VNPI pathologic classification and the classification of Pinder et al. (3-tier). Values are n (%) or mean ± SD. The 'intermediate' group (intermed.) in both the 3-tier classification system of Pinder et al. and the 2-tier VNPI pathologic classification also contains a single case with grade I nuclear atypia. All p-values were calculated with Fisher's Exact test.

	VNPI pathologic classification			**Classification Pinder et al.**			
	Intermed.	**High**	**p value**	**Intermed.**	**High**	**Very high**	**p value**
ER expression			0.044				0.039
Negative	2 (5)	6 (26)		2 (5)	4 (24)	2 (33)	
Positive	36 (95)	17 (74)		36 (95)	13 (77)	4 (67)	
PR expression			0.043				0.051
Negative	4 (11)	8 (35)		4 (11)	6 (35)	2 (33)	
Positive	34 (90)	15 (65)		34 (90)	11 (65)	4 (67)	
HER2 expression			0.182				0.389
1+	12 (32)	3 (13)		12 (32)	3 (18)	0 (0)	
2+	7 (18)	3 (13)		7 (18)	2 (12)	1 (17)	
3+	19 (50)	17 (74)		19 (50)	12 (71)	5 (83)	

5.3.2. Immunohistochemical evaluation of stromal proteins.

Since 66% (40/61) of our patient cohort underwent mastectomy instead of BCS, and these patients were all recently treated (January 2007 till January 2011), no clinical follow-up data on DCIS recurrence and DCIS progression to invasive breast cancer were available. Therefore, we used the 2-tier VNPI pathological classification and the 3-tier Pinder classification as a surrogate prognostic marker, to correlate with stromal protein expression.

A. CD34 and SMA

CD34 immunostaining was positive in endothelial cells and also in stromal fibroblasts of some, but not all, DCIS cases. In this DCIS cohort, the incidence of absent, weak, moderate and strong stromal CD34 immunostaining is 10% (6/61), 31% (19/61), 56% (28/61) and 13% (8/61), respectively. SMA immunopositivity was present in stromal fibroblasts of some DCIS cases, and also in myoepithelial cells and the muscularized wall of blood vessels, which was already described by Barth et al. (*45*). Luminal epithelial cells of the DCIS lesions were negative for both CD34 and SMA. The incidence of absent, weak, moderate and strong stromal SMA immunostaining accounts for 16% (10/61), 57% (35/61), 11% (7/61) and 5% (3/61), respectively. Neither CD34 expression, nor SMA expression correlated with the VNPI pathologic classification or the Pinder classification (p-values, table 10).

Table 10 Stromal protein expression and VNPI pathologic classification or the Pinder classification.

	VNPI pathologic classification			Classification Pinder et al.			
	Inter-mediate	High	p value	Inter-mediate	High	Very high	p value
CD34 expression			0.796				0.573
Absent	3 (8)	3 (13)		3 (8)	1 (6)	2 (33)	
Weak	11 (29)	8 (35)		11 (29)	7 (41)	1 (17)	
Moderate	18 (47)	10 (44)		18 (47)	7 (41)	3 (50)	
Strong	6 (18)	2 (9)		6 (16)	2 (12)	0 (0)	
SMA expression			0.465				0.689
Absent	8 (21)	2 (9)		8 (21)	2 (12)	0 (0)	
Weak	21 (55)	14 (61)		21 (55)	10 (60)	4 (67)	
Moderate	8 (21)	5 (22)		8 (21)	4 (24)	1 (17)	
Strong	1 (3)	2 (9)		1 (3)	1 (6)	1 (17)	
CD10 expression			0.238				0.166
Absent	22 (58)	9 (39)		22 (58)	8 (47)	1 (17)	
Low	15 (40)	12 (52)		15 (40)	7 (41)	5 (83)	
High	1 (3)	2 (9)		1 (3)	2 (12)	0 (0)	
Caveolin-1 expression			0.390				0.538
Absent	8 (21)	3 (13)		8 (21)	3 (18)	0 (0)	
Weak	24 (63)	13 (57)		24 (63)	9 (53)	4 (67)	
Moderate	6 (16)	6 (26)		6 (16)	4 (24)	2 (33)	
Strong	0 (0)	1 (4)		0 (0)	1 (6)	0 (0)	
Caveolin-2 expression			0.046				0.042
Absent	14 (37)	5 (22)		14 (37)	5 (29)	0 (0)	
Low	23 (61)	13 (57)		23 (61)	8 (47)	5 (83)	
High	1 (3)	5 (22)		1 (3)	4 (24)	1 (17)	
Decorin expression			<0.001				<0.001
Weak	3 (8)	14 (61)		3 (8)	10 (59)	4 (67)	
Moderate	23 (61)	6 (26)		23 (61)	4 (24)	2 (33)	
Strong	12 (32)	3 (13)		12 (32)	3 (18)	0 (0)	
Laminin-β2 expression			0.043				0.039
Absent	4 (11)	9 (39)		4 (11)	8 (47)	1 (17)	
Low	28 (74)	12 (52)		28 (74)	8 (47)	4 (67)	
High	6 (16)	2 (9)		6 (16)	1 (6)	1 (17)	
Aquaporin-1 expression			0.233				0.636
Absent	3 (8)	0 (0)		3 (8)	0 (0)	0 (0)	
Weak	11 (29)	3 (13)		11 (29)	2 (12)	1 (17)	
Moderate	11 (29)	10 (44)		11 (29)	8 (47)	2 (33)	
Strong	13 (34)	10 (44)		13 (34)	7 (41)	3 (50)	
Necdin expression			0.774				0.306
Absent	1 (3)	1 (4)		1 (3)	0 (0)	1 (17)	
Weak	21 (55)	11 (48)		21 (55)	10 (59)	1 (17)	
Moderate	11 (29)	9 (39)		11 (29)	6 (35)	3 (50)	
Strong	5 (13)	2 (9)		5 (13)	1 (6)	1 (17)	

B. CD10

Myoepithelial cells underlying the DCIS lesions were CD10 positive, but luminal epithelial cells were negative, as has been described by Witkiewicz et al. (*46*). The incidence of absent, low and high stromal CD10 immunostaining is 51% (31/61), 44% (27/61) and 5% (3/61), respectively. No significant association with neither the VNPI pathologic classification nor the Pinder classification was noted (p-values, table 10).

C. Caveolin-1 and caveolin-2

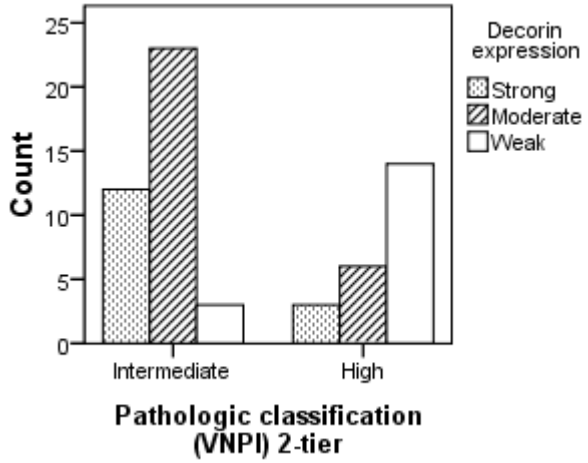
Caveolin-1 and caveolin-2 immunostaining was present in stromal fibroblasts, adipocytes, endothelial cells and myoepithelial cells, but luminal epithelial cells were negative, as was already described by Sloan et al., and Savage et al. (*42*, *49*). In this patient population, the incidence of absent, weak, moderate and strong stromal caveolin-1 immunostaining is 18% (11/61), 61% (37/61), 20% (12/61) and 2% (1/61), respectively. Stromal caveolin-1 expression was not associated with either VNPI pathologic classification or the Pinder classification (p-values, table 10).

Caveolin-2 expression in stromal fibroblasts was scored as absent, low or high in 31% (19/61), 59% (36/61) and 10% (6/61), respectively. Loss of stromal caveolin-2 expression was associated with lesions that were classified in the intermediate group, in both the VNPI pathologic classification and the Pinder classification (p=0.046 and p=0.042, respectively).

D. Decorin

Since decorin is an abundant small leucin-rich proteoglycan in the stroma of breast tissue, immunostaining was positive in the stroma of all DCIS cases, and in some cases, also nuclear staining of both the myoepithelial and luminal epithelial cells was noted.

Figure 8 Stromal decorin expression in relation to the VNPI pathologic classification.



Decorin expression was scored, based on fading of the staining in the periductal or 'specialized' stroma. Weak, moderate and strong decorin expression was present in 28% (17/61), 43% (29/61) and 25% (15/61) of DCIS cases. During scoring, it was noted that reduced decorin expression was often accompanied by the presence of myxoid stroma. Reduction of decorin expression in the periductal stroma correlated strongly with high grade lesions (Fig 8). When the VNPI pathologic classification was applied, 61 percent of the high grade DCIS presented weak decorin expression, while this was the case in only eight percent of the intermediate grade lesions ($p<0.001$). When using the Pinder classification, 93 percent of the intermediate grade DCIS displayed moderate or strong decorin expression, whereas 59 percent of high grade lesions and 67 percent of very high grade lesions presented with weak decorin expression ($p<0.001$). Strikingly, in the 'very high risk' group of the Pinder classification, none of the DCIS lesions exhibited strong decorin expression (table 10).

Because several in vitro studies provided sufficient evidence that decorin overexpression is associated with downregulation of members of the ErbB receptor tyrosine kinase family (*58, 59*), we investigated whether an association between stromal decorin expression and epithelial HER2 expression exists. We observed a strong correlation between diminished decorin expression and high HER2 IHC score ($p=0.001$). Stromal decorin was also obviously decreased in DCIS cases with *HER2* gene amplification ($p<0.001$), which is not surprising since HER2 protein expression and *HER2* amplification status strongly correlate ($p<0.001$, section 5.4.1.).

E. Laminin-beta-2

Nuclear laminin-beta-2 immunostaining was noted in periductal stromal fibroblasts, lymphocytes, endothelial cells and myoepithelial and luminal epithelial cells of DCIS lesions. The basement membrane of both affected and non-affected ducts stained positive as well. Laminin-beta-2 expression in stromal fibroblasts was scored as absent, low or high in 21% (13/61), 66% (40/61) and 13% (8/61), respectively. A reduction of stromal laminin-beta-2 expression was significantly associated with high grade DCIS lesions ($p=0.043$). Eleven percent of intermediate classified lesions presented with absent stromal laminin-beta-2 immunostaining, while this percentage amounted 39 in the high grade group. There was a significant correlation between stromal laminin-beta-2 expression and the Pinder classification as well ($p=0.039$).

F. Aquaporin-1

Membranous staining of aquaporin-1, a water channel protein, was seen in endothelial cells, in luminal epithelial and myoepithelial cells of DCIS lesions, and also in periductal stromal fibroblasts of several DCIS cases. Stromal aquaporin-1 immunostaining was scored as absent, weak, moderate and strong in 5% (3/61), 23% (14/61), 34% (21/61) and 38% (23/61), respectively. Neither VNPI pathologic classification, nor the Pinder classification correlated with aquaporin-1 expression in periductal stromal fibroblasts (p-values, table 10).

G. Necdin

Nuclear necdin immunostaining was positive in stromal fibroblasts of several DCIS cases, but also in lymphocytes and in luminal epithelial and myoepithelial cells of DCIS lesions. The incidence of absent, weak, moderate and strong stromal necdin expression is 3% (2/61), 52% (32/61), 33% (20/61) and 11% (7/61), respectively. Necdin immunostaining did not correlate with the VNPI pathologic classification and the Pinder classification (p-values, table 10).

5.4. Results of *HER2/Neu* FISH

5.4.1. HER2 amplification status in relation to histopathological and IHC features

The *HER2/Neu* amplification status was examined in sixty of sixty-one cases (98%), based on dual-probe FISH. According to the valid cut-off for *HER2/CEP17* ratio, 26 DCIS cases (43%) were non-amplified and 34 cases (57%) manifested amplification of the *HER2/Neu* gene (*57*). Of the 34 amplified DCIS lesions, thirty lesions (88%) displayed clusters.

Neither estrogen receptor expression nor progesterone receptor expression correlated with *HER2* amplification status ($p=0.446$ and $p=0.093$, respectively). The amplification status of the DCIS lesions correlated significantly with the HER2 immunohistochemical score ($p<0.001$, Fig 9A). In both the amplified and the non-amplified DCIS group, none of the lesions was assigned 0 as a HER2 IHC score. Most amplified lesions (88%) were assigned a 3+ HER2 IHC score, whereas the majority of non-amplified lesions (77%) had a negative or unequivocal IHC score (Fig 9A).

Table 11 shows the associations between amplification status and several histopathologic features or patient characteristics. Patient age and the size of the DCIS lesions did not correlate with amplification status. There was no significant correlation between amplification status and any of the two applied pathological classification systems, although there was a weak correlation with nuclear atypia ($p=0.045$). The two conditions for classifying a DCIS lesion in the 'very high risk' group of the Pinder classification, namely the presence of a predominantly solid (>50%) growth pattern, and the presence of extensive (>50%) comedo necrosis, were analysed separately. Amplified DCIS lesions pointed out to have significantly more extensive comedo necrosis than non-amplified lesions ($p=0.002$, Fig 9B). However, there was no significant correlation between the amplification status and the architectural pattern of the lesions.

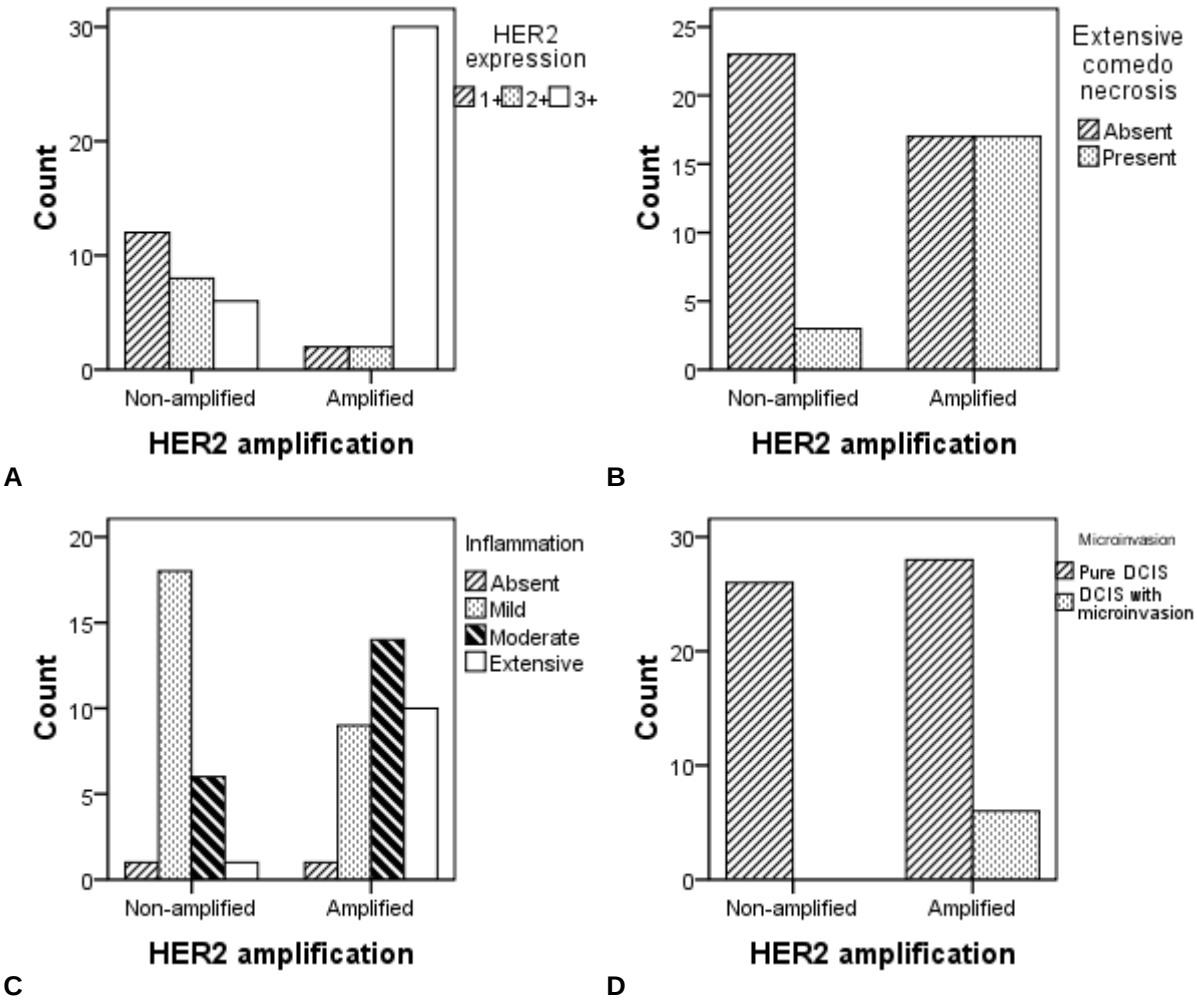
The presence of intraductal calcifications did not correlate with amplification status, nor did stromal morphology (p-values, table 11). Nevertheless, the degree of stromal inflammation was significantly higher in amplified than in non-amplified lesions ($p=0.003$, Fig 9C). Seventy percent of amplified DCIS displayed a moderate to extensive inflammatory infiltrate in the surrounding stroma, whereas 73 percent of the non-amplified cases manifested absent or mild chronic inflammation. Finally, the presence of microinvasion was correlated with the presence of *HER2* gene amplification, since all six cases with microinvasion were amplified ($p=0.031$), as is illustrated in Figure 9D.

Table 11 Histopathological features and patient age in relation to *HER2* amplification status for 60 (98%) of 61 DCIS cases. The 'intermediate' group in both the 3-tier classification system of Pinder et al. and the 2-tier VNPI pathologic classification also has a single case with grade I nuclear atypia.

	Non- amplified, n (%) or mean ± SD	**Amplified, n (%) or mean±SD**	**p-value[a]**
Age (years)	55.65±10.53	51.74±9.31	0.232
VNPI age groups			0.153
<40	0 (0)	4 (12)	
40-60	18 (69)	23 (68)	
>60	8 (31)	7 (21)	
Size (mm)	26.31±24.29	26.50±21.67	0.771
VNPI size groups (mm)			0.931
≤15	9 (35)	14 (41)	
16-40	14 (54)	16 (47)	
≥41	3 (12)	4 (12)	
Nuclear atypia			0.045
Grade 1	1 (3)	0 (0)	
Grade 2	19 (48)	17 (50)	
Grade 3	20 (50)	17 (50)	
Pathologic classification VNPI			0.060
Intermediate	20 (77)	17 (50)	
High	6 (23)	17 (50)	
Classification Pinder et al. 3-tier			0.115
Intermediate	20 (77)	17 (50)	
High	5 (19)	12 (35)	
Very high	1 (4)	5 (15)	
Extensive comedo necrosis			0.002
Absent	23 (89)	17 (50)	
Present	3 (12)	17 (50)	
Main growth pattern			0.113
Micropapillary	2 (8)	7 (21)	
Solid	14 (54)	15 (44)	
Cribriform	7 (27)	12 (35)	
Papillary	3 (12)	0 (0)	
Solid vs. non-solid architecture			0.603
Non-solid	12 (46)	19 (56)	
Solid	14 (54)	15 (44)	
Presence of calcifications			1.000
Absent	6 (23)	8 (24)	
Present	20 (77)	26 (77)	
Stromal inflammation			0.003
Absent	1 (4)	1 (3)	
Mild	18 (69)	9 (27)	
Moderate	6 (23)	14 (41)	
Extensive	1 (4)	10 (29)	
Stromal morphology			0.064
Sclerotic	25 (96)	26 (77)	
Myxoid	1 (4)	8 (24)	
Presence of microinvasion			0.031
Absent	26 (100)	28 (82)	
Present	0 (0)	6 (18)	

[a] Fisher's Exact test or Mann-Whitney U-test

Figure 9 Clustered bar charts showing the relation between *HER*2 amplification and HER2 expression (A), the absence or presence of extensive comedo necrosis (B), the amount of stromal inflammation (C) and the absence or presence of microinvasion (D); p-values are shown in table 11.



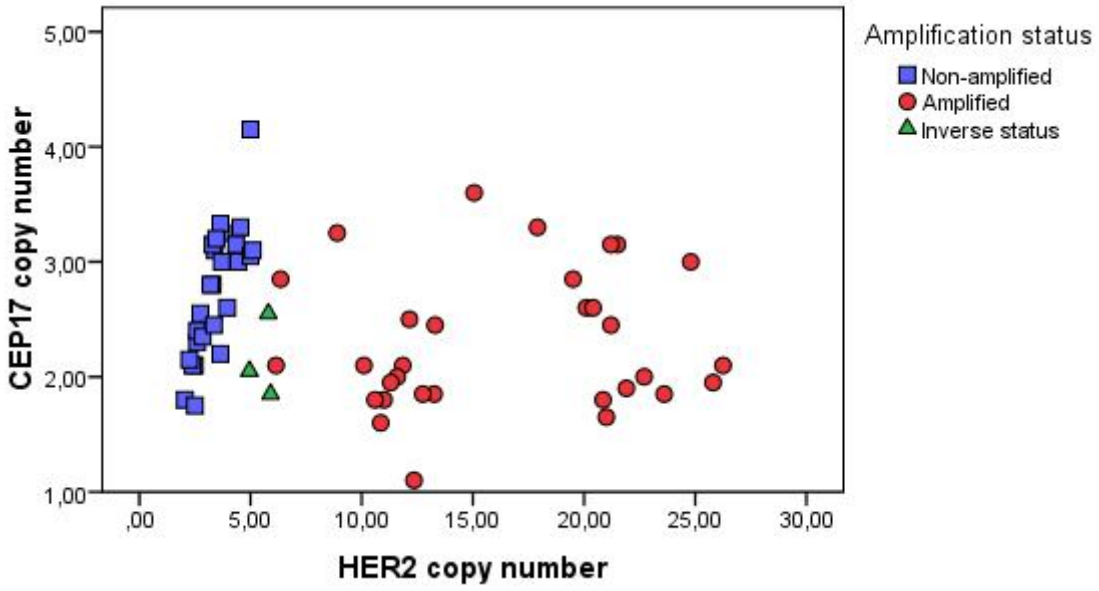
5.4.2. Mean HER2 copy number in association with mean CEP17 copy number

One DCIS case (2%) and 35 DCIS cases (58%) showed monosomy 17 and polysomy 17, respectively, when the validated cut-off of <1,4 and >2,2 mean *CEP17* copies per nucleus was used (*60*). Based on dual-probe FISH, 26 DCIS cases (43%) were non-amplified and 34 cases (57%) manifested amplification of the *HER2/Neu* gene.

We also simulated a single-probe FISH by determining the amplification status based merely on the *HER2* signals, setting the cut-off value at a mean *HER2* copy number of more than six per nucleus, analogous to the ASCO/CAP guidelines (*57*). This disclosed three cases with a discordant status: they were considered amplified because of their *HER2/CEP17* ratio, but their mean *HER2* copy number was lower than six (Fig 10). These findings imply that the amplification status based on dual-probe FISH is 95% concordant with the results of a simulated single-probe FISH for *HER2*.

There was no correlation between the mean *HER2* and *CEP17* copy number in the total cohort (p=0.254; r=1.000), but when this population was analysed separately according to amplification status, a strong correlation was revealed between mean *HER2* and *CEP17* copy number in the non-amplified DCIS lesions (p<0.001; r=0.778). In the group of amplified DCIS cases, a random distribution was observed (p=0.582; r=0.098), which is illustrated in Figure 10.

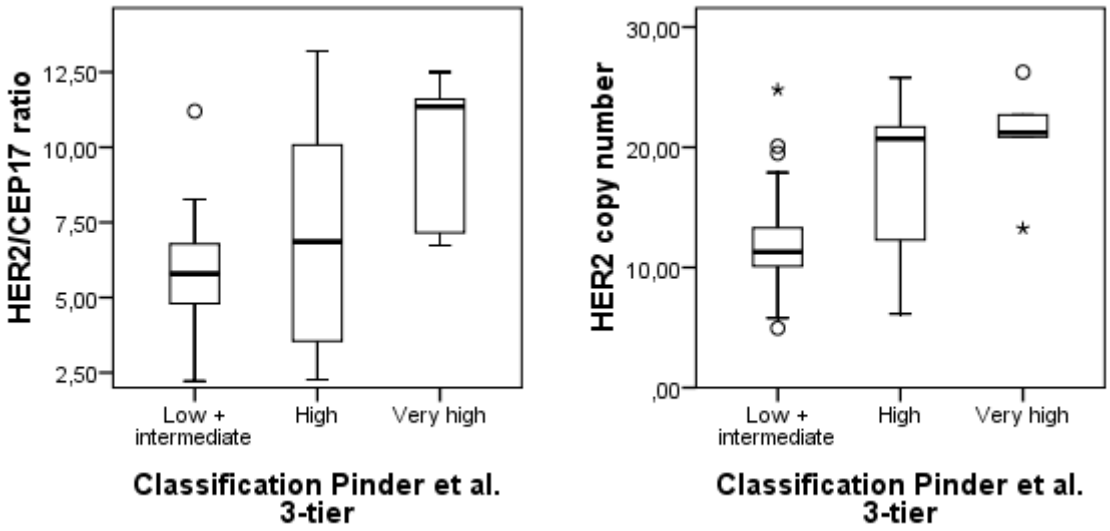
Figure 10 This scatter plot shows the distribution of the mean *HER*2 and *CEP17* copy number. The blue squares and red circles indicate non-amplified and amplified DCIS lesions, respectively. The green triangles indicate DCIS cases with an inverse status, which means that they are amplified according to dual-probe FISH (*HER2/CEP17* ratio > 2.2) but non-amplified based on single-probe FISH (mean *HER2* copy number < 6.00).



5.4.3. HER2/CEP17 ratio and mean HER2 and CEP17 copy number in amplified DCIS: relation with pathological and IHC features

In the amplified DCIS lesions of this cohort, age did not correlate with *HER2/CEP17* ratio, mean *HER*2 copy number and mean *CEP17* copy number (p=0.385; p=0.326 and p=0.389, respectively). No significant correlation was found between the mean *CEP17* copy number and any of the studied variables, i.e. the 2-tier VNPI pathological classification system, the 3-tier pathological classification system of Pinder et al., and expression of ER, PR and HER2 (p-values are shown in table 12).

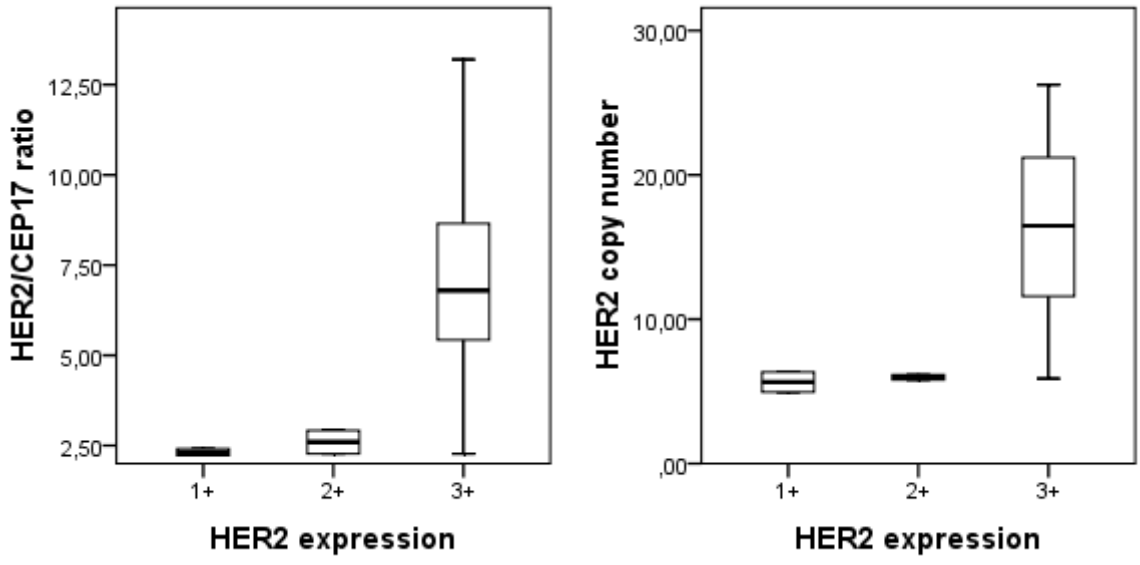
Figure 11 *HER2/CEP17* ratio (A,C) and mean *HER*2 copy number (B,D) in relation to the classification of Pinder et al. (A,B) and the VNPI pathologic classification (C,D) in amplified DCIS lesions. The 'intermediate' group in both the 3-tier classification system of Pinder et al. and the 2-tier VNPI pathologic classification also contains a single case with grade I nuclear atypia.



Both *HER2/CEP17* ratio (p=0.037) and mean *HER2* copy number (p=0.007) correlate with the classification system of Pinder et al., as is illustrated in Figure 11. *HER2/CEP17* ratio, as well as mean *HER2* copy number, increase with higher grading, and significance is due to the obvious difference between the 'intermediate' and 'very high' groups (p=0.026 and p=0.018, respectively). No significant correlation was noted between the 'intermediate' and 'high' groups, or between the 'high' and 'very high' groups, for both *HER2/CEP17* ratio (p=0.554 and p=0.283, respectively) and mean *HER2* copy number (p=0.064 and p=0.884, respectively). *HER2/CEP17* ratio (p=0.036) and mean *HER2* copy number (p=0.003) also correlate with the VNPI pathological classification system (Fig 11).

Neither *HER2/CEP17* ratio nor mean *HER2* copy number correlate with estrogen and progesterone receptor expression. (p-values are shown in table 12). DCIS lesions with a higher *HER2/CEP17* ratio displayed a higher HER2 IHC score (p=0.010), and the same significant correlation was seen between mean *HER2* copy number and HER2 protein expression (p=0.008; Fig 12). Thirty of all 34 amplified DCIS cases were assigned a 3+ HER2 IHC score, and all these cases showed clusters. Strikingly, the two cases with a 2+ HER2 IHC score and the two cases with a 1+ HER2 IHC score were the only four cases without clusters.

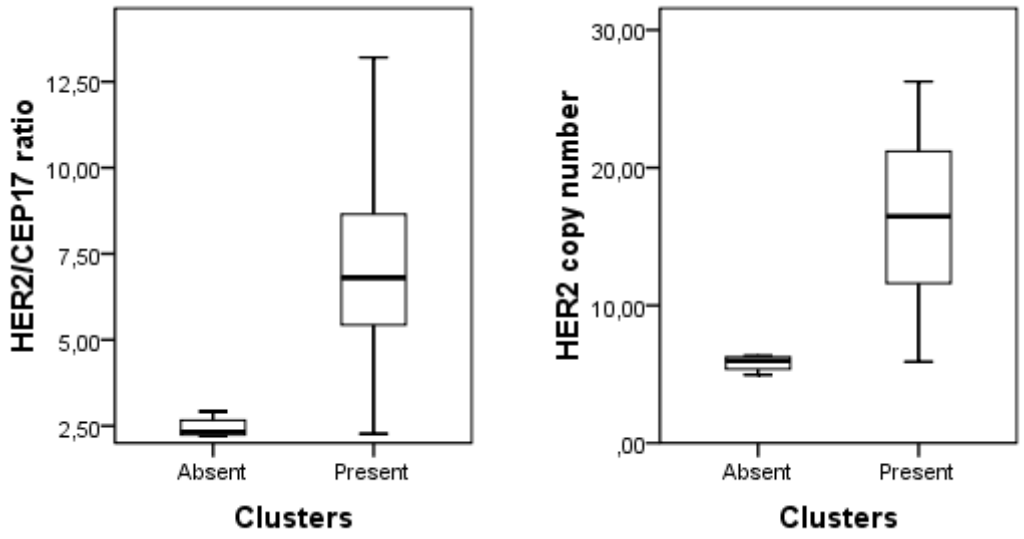
Figure 12 *HER2/CEP17* ratio (A) and mean *HER2* copy number (B) in relation to HER2 expression in amplified DCIS lesions. Corresponding p-values are shown in table 12.



A **B**

The presence of clusters correlated significantly with a positive HER2 IHC score ($p<0.001$). In accordance with these findings, the presence of clusters also strongly correlated with a higher *HER2/CEP17* ratio ($p=0.003$) and a higher mean *HER2* copy number ($p=0.002$, Fig 13). Moreover, all four cases with absent clusters have a mean *HER2* copy number close to the valid cut-off value of more than six copy numbers per nucleus. To be precise, two of these cases have a mean *HER2* copy number of 6.15 and 6.35, and the remaining two cases display a discordant amplification status, with a mean *HER2* copy number of 4.95 and 5.80, and a *HER2/CEP17* ratio of > 2.2 (Fig 10).

Figure 13 *HER2/CEP17* ratio (A) and mean *HER2* copy number (B) in relation to the presence of clusters in amplified DCIS cases.



A B

	***HER2/CEP17* ratio**	**p value**	**Mean *HER2* copy number**	**p value**	**Mean *CEP17* copy number**	**p value**
Classification Pinder et al. 3-tier		0.037		0.007		0.820
Intermediate	5.59±2.32		12.27±5.52		2.26±0.57	
High	7.12±3.84		17.51±6.32		2.37±0.64	
Very high	9.87±2.71		20.85±4.76		2.18±0.56	
VNPI pathologic classification		0.036		0.003		0.904
Intermediate	5.59±2.32		12.27±5.52		2.26±0.57	
High	7.93±3.69		18.49±5.96		2.31±0.61	
ER expression		0.198		0.804		0.928
Negative	5.06±2.06		14.63±5.20		2.33±0.69	
Positive	7.12±3.38		15.54±6.79		2.28±0.57	
PR expression		0.585		0.711		0.075
Negative	7.34±3.79		15.99±5.64		2.06±0.70	
Positive	6.55±3.11		15.16±6.85		2.37±0.53	
HER2 expression		0.010		0.008		0.692
1+	2.32±0.13		5.65±0.99		2.45±0.57	
2+	2.60±0.46		5.98±0.25		2.32±0.32	
3+	7.33±3.03		16.66±5.78		2.27±0.61	

Table 12 Histopathological and immunohistochemical features in relation to *HER2/CEP17* ratio and mean *HER2* and *CEP17* copy number for amplified DCIS lesions.

The 'intermediate' group in both the 3-tier classification system of Pinder et al. and the 2-tier VNPI pathologic classification also has a single case with grade I nuclear atypia. Values are mean ± SD (standard deviation). All p-values are calculated using Mann-Whitney U test or Kruskal-Wallis test.

	***HER2/CEP17* ratio**	**p value**	**Mean *HER2* copy number**	**p value**	**Mean *CEP17* copy number**	**p value**
Classification Pinder et al. 3-tier		0.447		0.749		0.218
Intermediate	1.26±0.20		3.53±0.89		2.79±0.58	
High	1.27±0.21		3.13±1.02		2.42±0.44	
Very high	1.08±0.00*		3.45±0.00*		3.20±0.00*	
VNPI pathologic classification		0.626		0.542		0.411
Intermediate	1.26±0.20		3.53±0.89		2.79±0.58	
High	1.24±0.20		3.18±0.92		2.55±0.50	
ER expression		0.630		0.386		0.531
Negative	1.15±0.04		2.95±0.49		2.55±0.35	
Positive	1.26±0.21		3.49±0.91		2.75±0.58	
PR expression		0.630		0.386		0.531
Negative	1.15±0.04		2.95±0.49		2.55±0.35	
Positive	1.26±0.21		3.49±0.91		2.75±0.58	
HER2 expression		0.525		0.159		0.230
1+	1.20±0.15		3.10±0.84		2.59±0.67	
2+	1.30±0.24		3.59±0.90		2.75±0.48	
3+	1.31±0.23		3.93±0.80		3.01±0.34	

Table 13 Histopathological and immunohistochemical features in relation to *HER2/CEP17* ratio and mean *HER2* and *CEP17* copy number for non-amplified DCIS lesions.

The 'intermediate' group in both the 3-tier classification system of Pinder et al. and the 2-tier VNPI pathologic classification also has a single case with grade I nuclear atypia. Values are mean ± SD (standard deviation). All p-values are calculated using Mann-Whitney U test or Kruskal-Wallis test.

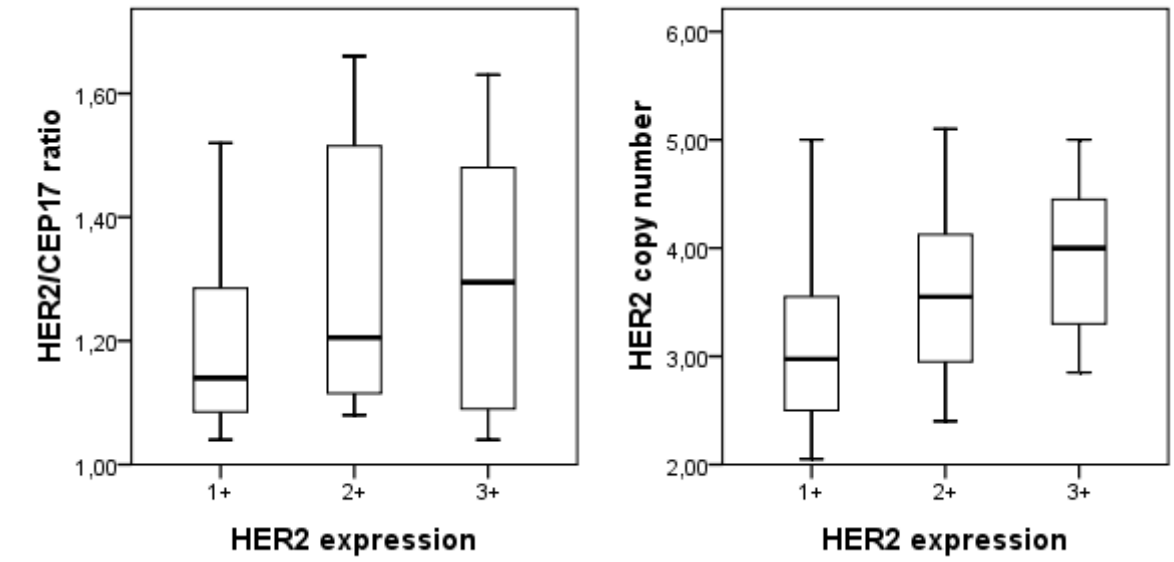
* Single case in this group.

5.4.4. HER2/CEP17 ratio and mean HER2 and CEP17 copy number in non-amplified DCIS: relation with pathological and IHC features

In contrast with the amplified DCIS cases, the non-amplified DCIS lesions did not show any relation between *HER2/CEP17* ratio and mean *HER2* copy number and the classification system of Pinder et al. or the VNPI pathologic classification (p-values, table 13). No association was observed between *HER2/CEP17* ratio or mean *HER2* copy number and hormone receptor status. Although no significant correlation was found, the HER2 IHC score tended to be higher with increasing *HER2/CEP17* ratio and increasing mean *HER2* copy number (p=0.525 and p=0.159, respectively), as is illustrated in Figure 14.

No significant correlation was found between the mean *CEP17* copy number and any of the studied variables, i.e. the 2-tier VNPI pathological classification system, the 3-tier pathological classification system of Pinder et al., and expression of ER, PR and HER2 (p-values are shown in table 13).

Figure 14 *HER2/CEP17* ratio (A) and mean *HER*2 copy number (B) in association with HER2 expression at the protein level.



A **B**

6. DISCUSSION

6.1. Patient features and histopathologic characteristics

6.1.1. The impact of surgical intervention: lumpectomy (BCS) versus mastectomy

Although the VNPI was originally developed as a tool to guide treatment decisions and to predict disease recurrence in patients with pure DCIS treated with BCS (*34*), we calculated the VNPI for every patient in this cohort, even if the patient was treated with mastectomy. This permitted us to use the VNPI and its pathologic classification as a surrogate prognostic marker, because of lack of follow-up data. Next, we correlated the total VNPI score and its four predictive components with the performed surgical procedure (BCS versus mastectomy).
The VNPI age groups did not correlate with the type of surgical intervention, but age as a continuous variable was significantly lower in the mastectomy group than in the lumpectomy group. Size of the DCIS lesions was significantly higher in patients who underwent mastectomy. Although this observation is rather logic, it confirms that patients are not treated in an arbitrary manner, but in accordance with a straightforward policy. BCS is reserved for patients with small DCIS lesions at imaging, and more extensive DCIS lesions require mastectomy, which is evident, since the treatment of DCIS is mainly based on (complete) resection of the lesion.

Strikingly, mean margin width was not significantly different between the two treatment groups, and VNPI margin width groups did not correlate with the type of intervention, which indicates mastectomy specimens do not necessarily have a wide resection margin. The total VNPI score was significantly higher in the mastectomy group. This difference was probably caused by the extent of the lesions, since the other VNPI predictors did not correlate with the type of surgery.
Because younger age was related to undergoing mastectomy and mastectomy was associated with more extensive lesions, we analysed whether age correlated with DCIS size. Younger age was not significantly associated with more extensive DCIS lesions, which is in contrast with the observations of Collins et al. (*61*). However, the authors determined DCIS extent as the number of low power fields (*61*), and we preferred following the ASCO/CAP guidelines (*55*). On the other hand, Collins et al. investigated 657 DCIS patients (*61*). Our cohort of 61 patients might be too small to demonstrate a significant correlation between patient age and size, since we did notice a tendency of smaller lesion in older patients, and vice versa.

In conclusion, we were not able to demonstrate a significant correlation between patient age and extent of the DCIS lesions, probably due to the small size of this patient population. We observed that mastectomy was generally reserved for extensive DCIS lesions, confirming that DCIS treatment in this patient population was mainly guided by the extent of the lesion. Noteworthy, mastectomy was associated with younger patient age and this intervention does not always result in wide resection margins.

6.1.2. Histopathologic features and their heterogeneity

In the present study, we analysed the relations between various histopathologic characteristics and two different pathologic classification systems (*34*, *35*). In addition, we assessed the occurrence of heterogeneity in nuclear atypia, architectural pattern and stromal morphology. We observed a marked architectural heterogeneity: only 19% of DCIS lesions presented a single growth pattern, whereas 34% of DCIS cases displayed 2 types, 39% presented 3 types and 7% presented all four architectural patterns.

Although architectural heterogeneity in DCIS has been described to be more common than cytonuclear heterogeneity (*32, 33*), we noted a remarkable cytonuclear heterogeneity in the majority (54%) of the DCIS cases in our cohort. Only 46% of lesions presented with a single grade of nuclear atypia. It has been reported that visual inspection by light microscopy might miss differences in nuclei that are classified as having the same grade (*62*). Miller et al. performed image cytometry of 81 DCIS cases and reported interductal heterogeneity in nuclear grade in 42% of their patient population (*62*). Since therapy is also partially guided by nuclear grade (for instance, as a part of the VNPI), this nuclear heterogeneity might have implications for patient outcome.

Stromal morphology appeared to be the least heterogeneous feature, with 56% of cases presenting a single morphologic type, and 44% of lesions displaying both sclerotic and myxoid stroma. Stromal morphology and stromal inflammation were the only two histopathologic features that correlated strongly with both pathologic classification systems. High grade lesions presented more often a moderate or extensive inflammatory infiltrate in the periductal stroma, and in addition, myxoid stroma was more common in these high grade lesions as well. This observation about stromal inflammation was already reported by Lee et al., who noted an association between the extent of the inflammatory infiltrate and poor differentiation of DCIS in a cohort of 41 patients (*63*).

Since we described the least heterogeneity in stromal morphology, one may wonder whether we need to progress to a classification system taking into account this feature. As DCIS constitutes a heterogeneous group of lesions and heterogeneity is also common within one individual lesion, a good classification system should employ features that are the least heterogeneous as possible throughout these proliferations. In this way, it is possible to avoid creating large mixed categories (*32*). Moreover, the more homogeneous a certain feature is, the more inter-observer variability is prevented. In addition, if a histopathologic characteristic is homogeneous throughout a lesion, the number of tissue blocks analysed is less decisive for the final score of this characteristic.

However, before new features such as stromal morphology and stromal inflammation are applied in a new classification system, these features need to be investigated in larger patient populations, and the possible associations with patient outcome need to be evaluated. Perhaps stromal morphology and stromal inflammation might be used to indentify a 'very high risk' group, just like the new classification of Pinder et al. (*35*). These authors described a 'very high risk' group, based on the presence of >50% solid architecture and >50% ducts bearing comedo necrosis (which we defined as extensive comedo necrosis) (*35*). In the present study, we noted a correlation between this 'very high risk' group and both stromal morphology and inflammation, so it is not unthinkable that these two features might be prognostic markers.

The presence of extensive comedo necrosis did not correlate with the VNPI pathologic classification, but it was significantly more frequent in the high and very high risk groups of the Pinder classification. This finding is not surprising, since extensive comedo necrosis is one of the conditions for a DCIS lesion to be classified as very high risk (*35*). The presence of necrosis in general is also a part of the VNPI pathologic classification (*34*), but since almost all (97%) of the DCIS cases in our cohort presented with some form of necrosis, this feature was probably too common and thus classification was mainly based on nuclear grade.

In summary, we question the prognostic value of classification systems that are mainly based on nuclear grades, since we and others (*62*) observed marked heterogeneity in nuclear atypia. To our opinion, nuclear grade should not be abandoned, but other histopathologic features that are more homogeneous throughout a DCIS lesion should be added, resulting in a new classification system. A correlation of these histopathologic features to both disease recurrence and breast cancer-specific mortality is required, preferably in large patient cohorts. Among such candidate prognostic markers are stromal morphology and stromal inflammation. The purpose of such a new classification system is achieving a higher prognostic value, contributing to a more individualized treatment.

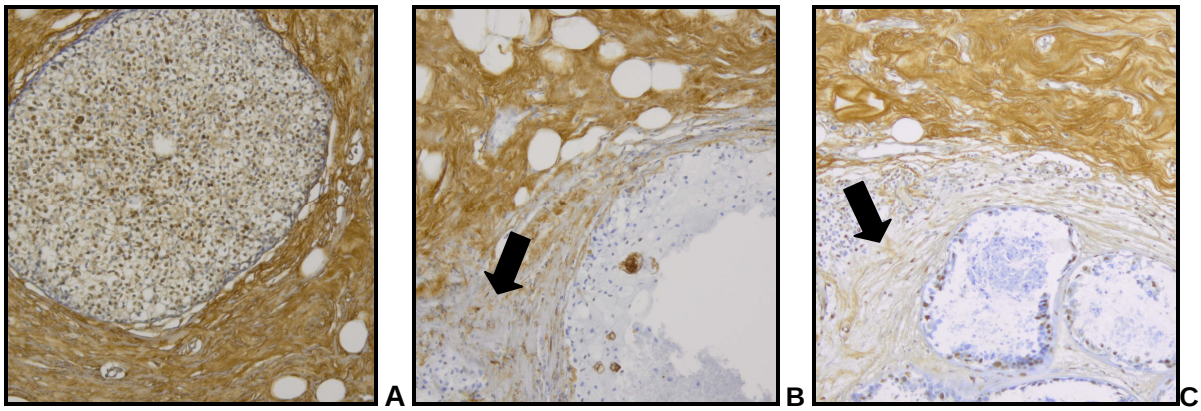
6.2. Stromal protein expression scrutinized

6.2.1. Decorin expression in the stroma surrounding DCIS

In this study, we have shown that reduced decorin expression at the protein level was strongly associated with high grade DCIS lesions. During scoring of the decorin immunostaining, we remarked that staining was clearly positive in the stroma adjacent to normal TDLU. In addition, we noticed that reduced periductal decorin expression in DCIS was often accompanied by the presence of myxoid stroma (Fig 15C). This seems logical, because periductal myxoid stroma was strongly associated with high grade DCIS as well, in both the VNPI pathologic classification and the Pinder classification.

Our findings at the protein level contrast with the results of Brown et al. about decorin expression at the mRNA level (*64*). Decorin appeared to be a strongly expressed stromal cell mRNA in normal breast tissue. Remarkably, increased mRNA expression was also noted adjacent to DCIS and invasive ductal carcinoma, and the patterns of mRNA expression in the stroma surrounding DCIS and IDC were very similar (*64*). This apparent discrepancy could be explained by an increased turnover of decorin protein, which leads to a positive feedback mechanism in order to maintain the stromal integrity, resulting in augmented decorin mRNA expression. However, other studies have shown that both decorin mRNA and protein expression are reduced in the stroma adjacent to IDC, in comparison with normal breast tissue (*65*). Thus, our results could also be explained by a reduced mRNA expression, which in turn causes a low protein level.

Figure 15 Decorin immunostaining, 10x objective. (A) Solid DCIS lesion, with strong decorin expression and no fading of the staining in the periductal stroma. (B) Moderate decorin expression with focal fading of the periductal stroma (black arrow). (C) Micropapillary DCIS lesion with weak decorin expression. Extensive fading of the periductal stroma is observed (black arrow).



Decorin is an abundantly secreted protein in the stromal compartment of many organs, belonging to a small leucin-rich proteoglycan (SLRP) gene family. Data from knock-out mouse experiments provided evidence that decorin plays an important role in regulating the formation of collagen fibres and maintaining the structural integrity of the skin (*66*). Interestingly, mice harbouring a targeted disruption of the decorin gene not only display skin fragility, but they also spontaneously develop intestinal adenomas and adenocarcinomas, providing further evidence that decorin probably acts as a tumour suppressor gene (*67*).

Many in vitro and in vivo studies have shown an anti-oncogenic effect of this abundantly expressed protein (*58, 59, 68, 69*). Marked growth suppression was induced in established cancer cell lines of various histogenetic origins, by inducing ectopic expression of decorin, and also by adding recombinant decorin (*68, 69*). Notably, de novo expression of a mutated protein, a decorin protein core without any glycosaminoglycan side chains, caused the same effect. This growth suppression appeared to pass through an upregulation of p21, an inhibitor of cyclin-dependent kinase activity, and was retinoblastoma gene and p53 independent (*68*). Since p21 controls the G_1-S transition, this explains why a large proportion of cancer cells was arrested in the G_1 phase of the cell cycle (*68*). In vivo studies using animal models of human breast tumour xenografts, provided evidence for decorin-induced suppression of tumourigenesis and inhibition of metastatic spreading to the lungs (*58, 69*).

Evidence for the anti-oncogenic effect of decorin transcends the in vitro and animal studies, since decorin expression has also been examined in human breast cancer tissue. Both decorin mRNA and protein expression were reduced in peritumoural stroma, compared to adjacent normal stroma of the breast (*65*). A reduced decorin expression at the protein level was associated with larger tumour size, more aggressive disease and a worse prognosis in patients with lymph-node negative invasive breast cancer (*40*). These observations highlight the aforementioned anti-tumorigenic effects of decorin.

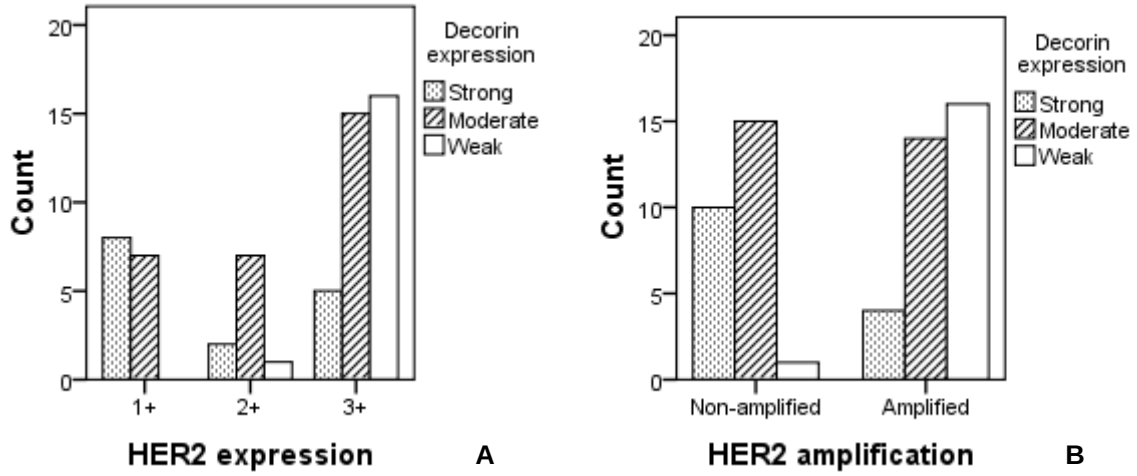
However, these results are mainly derived from in vitro and animal studies, and from investigations of human IDC. We report the first immunohistochemical analysis of stromal decorin expression in DCIS, and our data on reduced decorin expression in association with high grade DCIS lesions support the tumour suppressor function of decorin as well. Although Troup et al. did not observe a significant association between decorin expression and stromal inflammation or histological grade in their population of invasive breast cancers (*40*), we noticed a clear correlation between decorin expression and pathological classification.

Moreover, since stromal inflammation in our cohort also strongly correlated with the histological grade of DCIS, we presume there might exist a connection between extensive stromal inflammation, decreased decorin expression and high grade lesions. Unfortunately, our patient population is too small to perform multivariate analysis. Therefore it is not possible to determine which factor correlates most with histological grade.

One may question through which receptors decorin exerts its growth suppressing and anti-metastatic effects. Decorin suppressed cell growth in a squamous carcinoma cell line through activation of EGFR, with subsequent MAPK pathway activation and p21 induction, resulting in cell cycle arrest (*70*). In a cervical cancer cell line, decorin proved to be activate Met, the Hepatocyte Growth Factor Receptor (HGFR), resulting in its downregulation and inhibition of cell growth and cell migration (*71*).
Remarkably, decorin appears to inhibit ErbB2 phosphorylation and cause downregulation of this receptor in an ErbB2-overexpressing human breast cancer cell line, by activating ErbB4 and subsequent heterodimerization of ErbB4 with ErbB2 (*59*). This was confirmed by other studies in a metastatic rat mammary adenocarcinoma cell line (*58, 69*) and in HeLa cells (*71*). We assume decorin is not a ligand for the HER receptor family, but influences the activity of these receptors by interactions with other receptors, like integrins. Laminin-beta-2 is a protein that is also produced by stromal fibroblasts, just like decorin is, and laminins have been shown to serve as a ligand for multiple integrins (*72*). Thus, we hypothesize decorin might be a ligand for integrins as well. Further studies will have to elucidate the mechanisms of interactions that are responsible for the growth-suppressing and migration-inhibiting effects of decorin.

Since the aforementioned studies provided sufficient evidence to highlight that decorin induces downregulation of ErbB2 (also known as HER2 receptor), we analysed whether there exists a correlation between stromal decorin expression and epithelial HER2 expression. We observed a strong association between diminished decorin expression and high HER2 IHC score (Fig 16A). Periductal decorin was also remarkably decreased in DCIS cases with *HER2* gene amplification (Fig16B), which is not surprising since HER2 protein expression and *HER2* amplification status strongly correlated. Although our patient cohort is to small to permit multivariate analysis, we can probably exclude histological grade as a confounding factor, because HER2 expression and *HER2* amplification status did not correlate with histological grade (both the VNPI pathologic classification and the Pinder classification).

Figure 16 Reduced periductal stromal decorin expression in DCIS is significantly associated with a high HER2 IHC score (p=0.001) (A) and with amplification of the *HER2* gene (p<0.001) (B).



The key question is: how to explain this noteworthy association? If decorin is naturally present in the mammary stroma as a constitutive inhibitor of ErbB2/HER2 and other RTKs, either in a direct or indirect way, one might hypothesize that *HER2* gene amplification and increased HER2 expression are a kind of 'escape mechanism' of the tumour cells to overcome this growth suppressing agent, by presenting more membranous HER2 receptors.

Furthermore, tumour cells could produce substances that destruct the periductal decorin, or they could activate other (stromal) cells to produce such substances, like matrix metalloproteinases. The latter would also explain the presence of myxoid periductal stroma in high grade DCIS cases with reduced decorin protein.

The gene profile study of Ma et al. showed that MMP are upregulated in the stroma adjacent to IDC (*17*). We presume that DCIS lesions, prior to invasion, also influence their neighbouring stromal fibroblasts and instigate them to produce MMP. By modulating the composition of the host connective tissue, the tumour could influence its microenvironment and prepare its way for invasion into the mammary stroma.

Hence, the presence of decorin protein may serve as a protective barrier against tumour invasion, not only by modulating the integrity of the peritumoural connective tissue, but also because of its capability to directly influence tumour cells and suppress their growth. Perhaps this mechanism could explain why some patients with DCIS develop invasive ductal carcinomas, and some patients don't.

One limitation of this study, is its lack of follow-up data. Future studies should certainly assess the recurrence risk of DCIS in larger patient cohorts, either as DCIS or IDC, in correlation with stromal decorin expression, both at the protein and mRNA level. Moreover, it would be interesting to explore decorin expression in patients with coexistent IDC and DCIS. It could be valuable not to limit this research to decorin, but to expand it to other members of the SLRP family, like lumican and fibromodulin. Until now, the role of decorin in breast cancer progression seems to be a relatively neglected domain. Hence, future research should elucidate its role in mammary tumour biology.

In conclusion, our study is the first to report an immunohistochemical evaluation of stromal decorin expression in a cohort of DCIS patients. Although follow-up data were not availale, we observed that reduced periductal decorin expression is strongly correlated with high grade DCIS lesions. Moreover, in this DCIS cohort, reduced decorin protein expression was clearly associated with epithelial HER2 protein expression and *HER2* gene amplification status. These findings offer perspectives for the future, as decorin protein expression might be used as a prognostic marker in predicting disease progression. Furthermore, decorin might serve as a possible therapeutic target in breast cancer patients, considering the capability of decorin to (indirectly) antagonize several receptors by inhibition of RTKs and to function as a natural anticancer substance (*71*).

6.2.2. Laminin-beta-2

Laminins are a family of heterotrimeric glycoproteins, and together with type IV collagens, nidogens and proteoglycans, they are the major components of the basement membrane (*72*). They affect cell differentiation, migration and adhesion, and their biological function is exerted through integrins (*73*). Each laminin glycoprotein consists of one alpha, one beta and one gamma chain, assembling through a coiled coil-domain at the C-terminus of each chain and making up a cross-shaped glycoprotein (*72, 73*).

Laminin-beta-2 is an example of such a beta chain, and is part of laminin-3 (α1β2γ1), laminin-4 (α2β2γ1), laminin-7 (α3β2γ1), laminin-9 (α4β2γ1) and laminin-11 (α5β2γ1) (*73*). It is not surprising that truncating mutations in the *LAMB2* gene, coding for this widely expressed protein, give rise to several symptoms, constituting the Pierson syndrome (*74*). Neither in patients suffering of the Pierson syndrome, nor in *LAMB2* knock-out mice experiments, development of tumours has been reported (*74*).

To date, the expression of laminin-beta-2 in DCIS has not been thoroughly investigated yet, nor in breast tissue in general. Fujita et al. analysed the expression of several laminin isoforms in normal breast tissue, DCIS and IDC (*75*). They reported a shift from beta2-containing isoforms to beta1-containing isoforms during breast cancer progression: laminin-9 expression was reduced in favour of laminin-8, and laminin-11 expression was diminished in favour of laminin-10. This isoform switch starts in DCIS and becomes more obvious in IDC, in which laminin-9 expression appeared to be completely absent (*75*). Laminin-beta-2 immunostaining was present in vascular basal membranes and around epithelial structures of normal breast tissue. Protein expression was conserved in four out of five DCIS cases, but revealed to be almost completely absent in IDC (*75*). Notably, the same laminin-9 to laminin-8 shift observed in breast cancer progression was also present in glial tumours, and was associated with glial tumour grade, recurrence and patient survival (*76*).

In the present study, we performed an immunohistochemical analysis of the stromal laminin-beta-2 expression in a cohort of 61 DCIS cases. To date, no other immunohistochemical analyses of stromal laminin-beta-2 expression in large DCIS or IDC cohorts have been reported. A significant association was noted between reduced periductal expression of laminin-beta-2 and high grade DCIS lesions. This correlation is in line with the observations of Fujita et al. (*75*).

The basement membrane separates epithelia from connective tissue, and also surrounds vascular structures. A switch in the constituents of the basal lamina again emphasizes the probability that tumour cells influence the host microenvironment. The proliferating cells of a pre-invasive lesion like DCIS might interact with normal stroma cells, in order to provoke changes in the neighbouring basal lamina and connective tissue as a means to penetrate the basement membrane, and to subsequently invade the surrounding connective tissue. An altered laminin production can be caused by downregulation of the mRNA expression in the stromal cells, but changes in the extracellular matrix can also be provoked by production of proteases by stromal cells. The stromal gene expression profiling study of Ma et al. shows that the DCIS-IDC transition is associated with an increased expression of various matrix metalloproteases (*17*).
This remodelling process of the host microenvironment is probably an important step in the progression of mammary tumours, and further research will prove whether laminin-beta-2 can play a major role in predicting the behaviour of DCIS lesions.

6.2.3. Stromal CD10 expression

CD10 is a cell surface zinc-dependent metalloproteinase and its increased stromal expression was found to be associated with disease recurrence in DCIS patients, but not with nuclear grade in DCIS (*46*). Makretsov et al. reported that an increased stromal CD10 expression correlated with poor prognosis and high grade in invasive breast cancer (*47*). Iwaya et al. observed the same association between increased stromal CD10 positivity and worse prognosis in patients with IDC, but they did not find a significant correlation with tumour grade (*77*).

In the present study, we used two pathological classification systems for DCIS as a surrogate prognostic marker, because of lack of follow-up data. Considering the aforementioned previous reports, we expected to find a reduced CD10 expression in intermediate grade DCIS, and an elevated CD10 level in high (or very high, according to the Pinder classification) grade DCIS.

Nevertheless, we could not demonstrate a significant association between increased stromal CD10 immunostaining and both pathological classification systems. This observation does not exclude CD10 as a potential prognostic marker, because DCIS classification and disease recurrence remain two different entities. More research is required to assess the potential of stromal CD10 as a prognostic marker in DCIS, and in breast cancer in general.

6.2.4. Caveolin-1 and caveolin-2

Caveolins are the major structural components of caveolae, small plasma membrane-associated vesicles that participate in vesicular and cholesterol trafficking (*78*). Furthermore, multiple studies suggest that caveolins also play a role in transmembrane signaling, and caveolin-1 has been shown to inhibit cellular proliferation, suggesting a role as a tumour suppressor (*78*). Several studies about stromal caveolin-1 expression in breast cancer have already been published, and according to these formerly reported results, we expected to find an inverse correlation between stromal protein level and pathologic classification for both caveolin-1 and caveolin-2, reasoning that high grade is associated with worse prognosis (*41-43, 49*).

Nevertheless, in the present study, stromal caveolin-1 expression (Fig 17A) did not significantly correlate with histological grade of the DCIS lesions. This finding was in line with the reported absence of an association between stromal caveolin-1 and nuclear grade, although stromal caveolin-1 predicted early DCIS progression to IDC (*43*).

Stromal caveolin-2 expression (Fig 17B) did correlate with histological grade of DCIS, albeit in a different manner than expected. We selected both caveolin-1 and caveolin-2 out of the additional file of the aforementioned taxonomy study of Gevaert et al., because the expression of these two proteins was associated with the 'better outcome group', embodied by the lobular carcinoma cluster in this study (*48*).
Since we used two pathological classification systems as surrogate prognostic marker, we expected to find a lower stromal expression of both caveolins in the presence of high-grade DCIS lesions. Surprisingly, we observed the reverse association, i.e. an elevated stromal caveolin-2 expression correlated with higher histological grade. These results are confirmed by a recent publication in which mammary tumour progression is associated with upregulation of the stromal caveolin-2 expression (*79*).

This discrepancy between our findings and the observations in the gene expression profile study of Gevaert et al. can be explained in many ways. First of all, we selected this molecular marker out of this gene expression profile study, which was based on whole tumour tissue and not solely on peritumoural stroma (*48*). This implies that the association between elevated caveolin-2 expression and better prognosis might be due to an increased epithelial caveolin-2 expression instead of increased stromal expression. Indeed, reduced epithelial caveolin-2 mRNA expression has been suggested to play a role in breast cancer progression (*80*).
However, this contrasts with the observation that epithelial expression of caveolins is a marker for breast carcinomas with basal-like and triple negative phenotypes, and is associated with a more aggressive clinical behaviour (*81*). Somehow, the link between caveolin expression and basal-like phenotype is logic, since caveolin expression in normal breast has been described in myoepithelial cells, and not in luminal epithelial cells (*49, 80*).
Another possible explanation of our findings is that gene expression was biased in the taxonomy study (*48*), since caveolin expression is not confined solely to stromal fibroblasts or myoepithelial cells of normal TDLU. Adipocytes and endothelial cells all consistently express caveolin-1 and -2, which can influence gene expression. Since the amount of stroma in their tumour samples was not defined, this possibility cannot be confirmed, nor excluded.

In conclusion, we were not able to show a correlation between stromal caveolin-1 expression and high-grade DCIS. Increased stromal caveolin-2 expression was associated with high-grade DCIS, and this observation was confirmed by Koo et al., who demonstrated that mammary tumour progression correlates with augmented stromal caveolin-2 expression (*79*).

Figure 17 (A) Caveolin-1 immunostaining in a predominantly solid DCIS lesion, strong stromal expression (score 3 on a four-point scale), 10x objective. (B) Caveolin-2 immunostaining in a predominantly solid DCIS lesion with extensive comedo necrosis, high stromal expression (score 2 on a three-point scale), 10x objective.

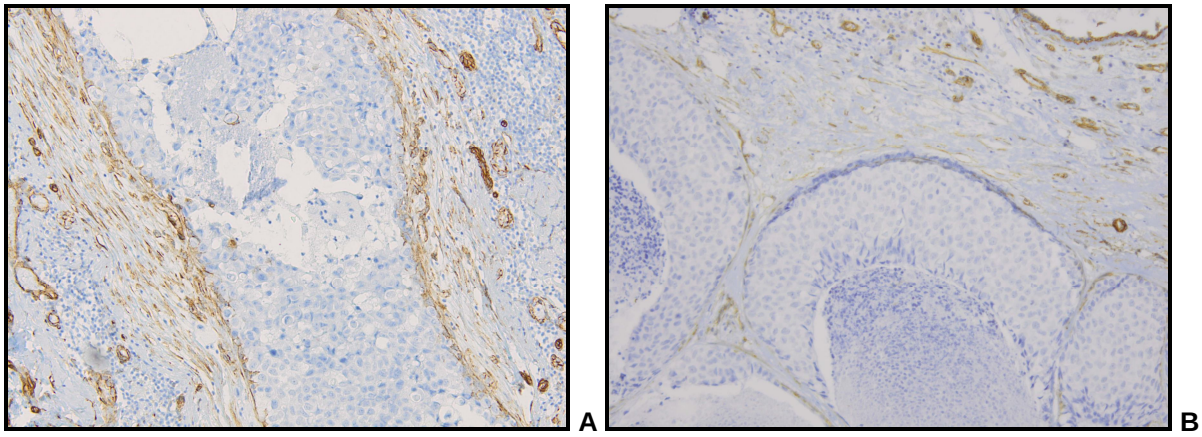


Figure 18 (A) CD34 immunostaining, strong stromal expression (score 3 on a four-point scale), 10x objective. (B) SMA immunostaining in a predominantly micropapillary DCIS lesion, strong stromal expression (score 3 on a four-point scale), 10x objective.

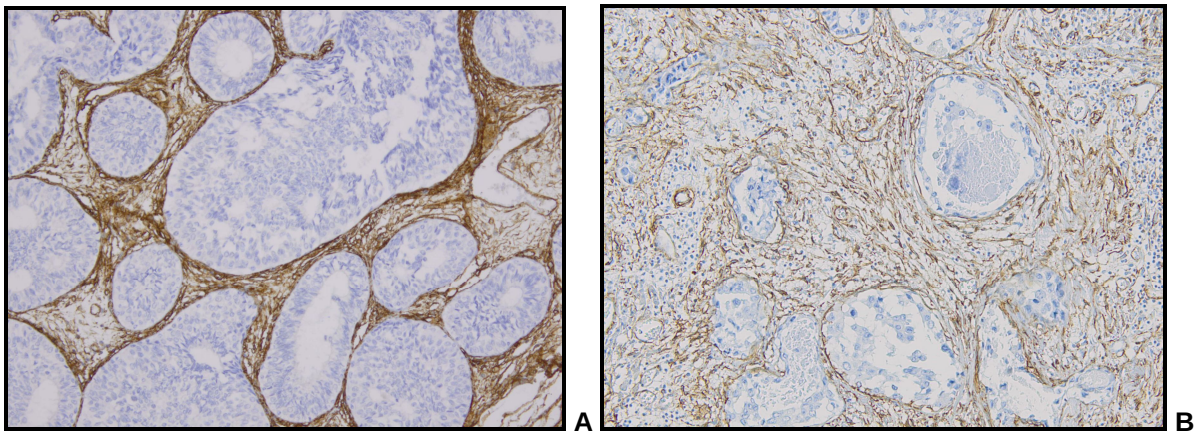
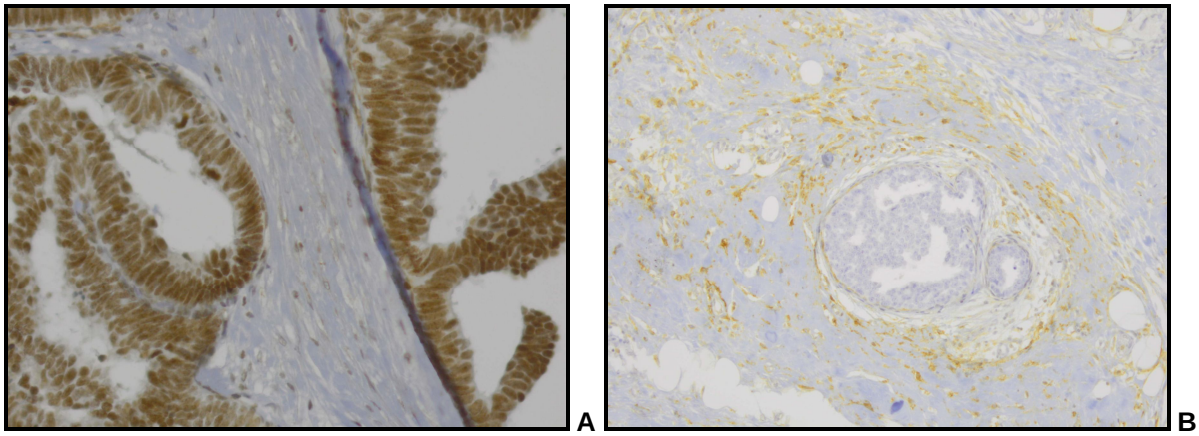


Figure 19 (A) Laminin-beta-2 immunostaining, in a papillary DCIS lesion which also exhibited nuclear staining of the malignant cells, high stromal expression (score 2 on a three-point scale), 20x objective. (B) CD10 immunostaining, high stromal expression (score 2 on a three-point scale), 10x objective.



6.2.5. Evaluation of stromal expression of CD34 and SMA

In the past decade, the stromal compartment in DCIS has been increasingly focused on. Pavlakis et al. investigated stromal expression of CD34 and SMA in 78 DCIS cases and compared with the expression in normal breast tissue (*44*). They observed no difference in immunostaining between the low grade DCIS and normal breast tissue, but a significant difference in protein expression was present between all three DCIS groups (*44*). High-grade DCIS was significantly associated with reduced CD34 and increased SMA expression, and these observations were also reported by others (*44, 45, 82*). In addition, Yamashita et al. reported that the presence of SMA-positive myofibroblasts in invasive breast cancer was associated with a worse prognosis (*83*).

Considering the observations reported in literature, we expected to find an association between reduced stromal CD34 expression, augmented SMA expression and higher grade in DCIS. However, we were not able to demonstrate a significant increase in stromal CD34 expression, or a reduction in stromal SMA expression, between intermediate and high-grade DCIS cases. This might be caused by the small size of our patient population, or by the lack of low-grade DCIS cases in our cohort.

6.2.6. Stromal expression of necdin

Necdin is a member of the melanoma-associated antigen (MAGE) family (*84*). This small nuclear protein functions as a growth suppressor and as an anti-apoptotic protein in early neurons (*85*). Furthermore, this protein is suspected to act as a tumour suppressor (*84*). To our knowledge, necdin protein expression has not been investigated yet in patient populations with breast cancer.

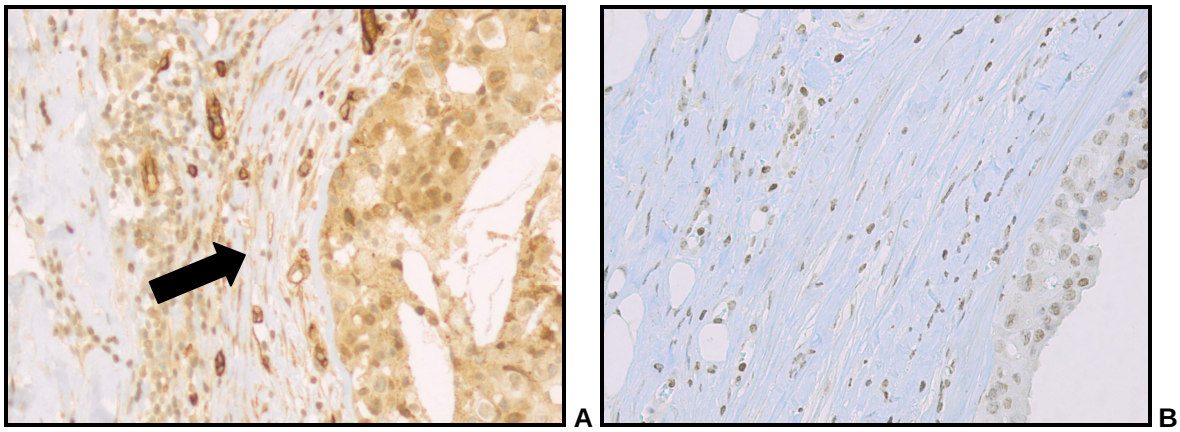
We present the first immunohistochemical analysis of stromal necdin expression in a patient cohort with DCIS. We selected necdin out of a gene list, of which was known that upregulation was associated with a good prognosis breast carcinoma subgroup (*48*). In this DCIS cohort, we could not demonstrate a significant relation between stromal necdin expression and pathological classification.

6.2.7. Stromal aquaporin-1 expression

Aquaporin-1 is a water channel protein that plays a role in regulating water flux across cell membranes (*86*). This protein was proved to be an independent prognostic marker in a subgroup of basal-like breast tumours, which were associated with a poor prognosis (*86*). Aquaporin-1 was one of the molecular markers we selected out of the additional file 4 of the gene expression profile study of Gevaert et al., because its expression was associated with a good prognosis subgroup of breast carcinomas (*48*). Furthermore, this protein was also present in the additional file 1 of the stromal gene expression profile study of Ma et al., in which it was observed to be downregulated in the stroma adjacent to IDC compared to the stroma in DCIS (*17*). The role of aquaporin-1 in breast cancer or in the stroma surrounding mammary tumoural lesions has not been thoroughly investigated yet.

In the present study, we investigated the stromal aquaporin-1 expression in DCIS, but we were not able to demonstrate a significant correlation with pathological classification, which we used as a surrogate prognostic marker. However, since we did not possess clinical follow-up data, and since our patient cohort is rather small, we cannot exclude stromal aquaporin-1 yet as a future prognostic marker. More research in larger patient cohorts is warranted.

Figure 20 (A) Stromal aquaporin-1 immunostaining (black arrow), strong stromal expression (score 3 on a four-point scale), 10x objective. (B) Stromal necdin immunostaining, strong stromal expression (score 3 on a four-point scale), 20x objective.



6.3. Hormone receptor status

Since tamoxifen was found to be associated with decreased disease recurrence rates in patients with DCIS undergoing breast preserving surgery, it is recommended as an adjuvant systemic therapy in case of estrogen receptor positive DCIS (*30*). Therefore, we determined the hormone receptor status in our DCIS cohort. An Allred score of two or more was considered positive, for both estrogen and progesterone receptor expression.

In this cohort, 87% DCIS lesions were ER positive, and 80% were PR positive, which implies the majority of patients is eligible for treatment with tamoxifen. ER expression was significantly more common in intermediate grade lesions than in high grade lesions, in both the VNPI pathologic classification and the Pinder classification. The lower frequency of ER positivity in high grade lesions has been reported in various studies (*18, 21, 36, 52*) and we can confirm these observations. PR expression correlated with the VNPI pathologic classification, but not with the Pinder classification. Other studies also have demonstrated a higher frequency of PR positivity in non-high-grade DCIS than in high grade lesions (*18, 21, 36*).

It is difficult to compare our rate of ER and PR positive DCIS lesions with that of other studies, since the distribution of histological grade varies from study to study. Moreover, the manner of assessing histological grade and scoring hormone receptor expression differs between studies. However, the 87% ER positivity we observed in our cohort is in agreement with the findings of the 72% ER positivity reported by Altintas et al. (*36*) and the 82% positivity rate in DCIS described by Horimoto et al. (*87*).

Baquai and Shousha reported a 40% ER positivity and a 55% PR positivity in their pure DCIS cohort (*88*). These expression rates are remarkably lower than our observations, but this is probably due to their relatively higher proportion of high grade lesions (52%, versus 38% in our cohort) and their method for scoring the immunostaining (>10% of cells positive) (*88*).
Meijnen et al. observed an ER positivity of 68% and a PR positivity of 46% (*18*). These lower receptor expression rates might be due to the fact that the authors used a TMA (tissue microarray), which can be subject to bias because of heterogeneous receptor expression. In fact, during scoring of the ER and PR immunostaining, we noticed a heterogeneity in nuclear expression for both receptors in several cases. Meijnen et al. used three 0.6mm-tissue cores per case, but it has been reported that for most purposes, at least four tissue cores are recommended, to take tumour heterogeneity into account (*89*).

Concerning immunohistochemical analysis of DCIS, Lin et al. demonstrated that the use of a TMA can significantly underestimate the expression of progesterone receptor and HER2 because of a heterogeneous staining pattern in the whole tissue section (*90*). The lower the core number, the higher the non-concordance between the biopsy cores and the whole tissue section.

In summary, we report that 87% of the DCIS lesions in our cohort was ER positive, suggesting that the majority of patients is eligible for treatment with adjuvant tamoxifen, following breast preserving surgery. In addition, we noted that ER and PR positivity was significantly less common in high grade lesions, which confirms the observations of others (*18, 21, 36, 52*). We recommend the use of whole tissue sections instead of TMA, since tumour marker heterogeneity leads to significant underestimations when TMA analysis is used (*90*).

6.4. The HER2 story

6.4.1. HER2 protein overexpression in DCIS

Patients with IDC exhibiting HER2 overexpression are treated with trastuzumab or lapatinib (*50*), unlike patients with HER2 positive DCIS (*87*). In IDC, HER2 does not only forecasts prognosis, but it also predicts the response to these targeted therapies. Several studies have assessed HER2 expression in pure DCIS or in coexisting DCIS/IDC, but despite their observations, the significance of HER2 overexpression in DCIS and its role in mammary tumour progression is not completely understood (*51, 87*). Learning more about the significance of HER2 in DCIS and mammary tumour biology should lead to a better management of DCIS.

Taking into account the entire cohort, we did not observe a significant correlation between HER2 protein expression and histological grade. This finding contrasts with the observations of other studies, in which HER2 positivity was manifestly more common in high-grade lesions (*21, 36, 51, 52, 87*). The fact that these other studies were performed in considerably larger patient populations might be an explanation for this discrepancy. Perhaps our patient cohort, containing 61 DCIS cases, was too small to demonstrate this association. Moreover, all these studies contained a certain percentage of low-grade DCIS cases, whereas our DCIS population contained only one low-grade case, which was merged with the intermediate-grade group.

In the present study, the majority of DCIS lesions (59%) was assigned a 3+ positive IHC score. If we regard a 2+ score positive instead of equivocal, 75% of the DCIS lesions is considered positive at IHC analysis. In line with our remarks about comparing hormone receptor expression between different studies, we have to call attention to the different definitions of HER2 IHC positivity, and also to the dissimilar guidelines or classification systems that are used to determine histological grade. Moreover, the percentage of high-grade and non-high-grade DCIS lesions varies among studies. This might explain why reported HER2 expression rates in other studies were clearly lower than in our study, ranging from 28 to 40% (*18, 51, 52, 88*). Moreover, the quality of HER2 IHC staining needs to be questioned. We scored HER2 protein expression in the same way as Altintas et al. did, but their combined 2+/3+ positive rate of 40% is remarkably lower than our 75% HER2 positivity (*36*). Horimoto et al. used a scoring system which was similar to ours, and they reported a 2+/3+ positive rate of 61% (*87*), which is more in line with our findings.

One constant observation can be found in literature: HER2 protein overexpression is definitely less common in IDC than in DCIS, with reported rates ranging 13-29% (*51, 53, 54, 91*). This finding is rather peculiar, since HER2 overexpression and *HER2* gene amplification is associated with a worse prognosis in IDC (*92*). Provided that DCIS is a precursor lesion of IDC, one would expect that HER2 positive DCIS lesions are more likely to progress to IDC, resulting in a higher rate of HER2 positive IDC cases than the current clinical observations.

Park et al. demonstrated a 93% concordance in HER2 protein expression between primary IDC and their lymph node metastases, which proves that dedifferentiation and subsequent loss or gain of HER2 expression does not occur often during the process of invasion and tumour spread throughout the body (*53*). Santiago et al. also reported that HER2 status is stable during axillary metastatic progression (*93*). In our opinion, the fact that HER2 expression is maintained in metastases makes it highly unlikely that HER2 expression is often lost during the transition of DCIS to IDC. Some authors proposed this to explain the different expression frequencies between DCIS and IDC (*18*), while others suggested HER2 probably does not have a critical role in the progression from DCIS to IDC (*19, 53*).

It is questioned whether DCIS is an obligate precursor lesion of invasive ductal carcinoma, and if so, what proportion of DCIS progresses to invasive cancer (*8*). The higher rate of HER2 positivity in DCIS compared to IDC might be another argument for the existence of the so-called parallel pathway, as described by Sontag and Axelrod (*12*) (see section 2.1.3.). HER2 overexpression (and the possible but not obligate underlying gene amplification) has been described as being an early event in the development of breast cancer (*19*), and this might be as well an early genetic change in a common progenitor cell, which can progress to either DCIS or IDC lesions (*12*). The parallel pathway theory of Sontag and Axelrod is rather controversial and defying, because this model questions the current treatment strategies, which are based on prevention of further progression to IDC (*19*).
However, this HER2 discrepancy may match the commitment theory too (see section 2.1.3.). Although we did not observe a significantly higher HER2 positivity rate in our cohort, others have reported this association in larger patient populations. According to this commitment model, HER2 positive high-grade DCIS will progress to HER2 positive high-grade IDC. The high concordance in HER2 expression between the invasive and non-invasive components of coexistent DCIS/IDC also supports this theory and suggests that DCIS frequently is a precursor lesion of IDC (*20, 21*). A major objection against this commitment model is that, even if histological grade is taken into account, HER2 expression is still higher in DCIS (*51*).

Supposing DCIS can be a precursor lesion of IDC, another possible explanation might be that HER2 positive DCIS lesions simply less frequently evolve to invasive breast cancer, as proposed by Meijnen et al. (*18*), although this contrasts with the observed increased aggressiveness in HER2 positive IDC (*92*). An indirect argument for less frequent progression of HER2 positive DCIS lesions is the observation of Altintas et al., that disease recurrence after BCS or mastectomy was not associated with HER2 protein expression (*36*).

All these observations nourish several models of breast cancer progression. To date, we are still in uncertainty as to which model describes mammary tumour progression best. Moreover, some studies provide evidence that suggest multiple theories might be correct. Lin et al. already reported that a mixture of breast cancer progression pathways is most consistent in approaching the 'real' clinical observations (*15*). In addition, Tang et al. demonstrated that DCIS lesions can be subdivided into three subtypes, according to their expression of five CK markers (*94*). The distribution of these CK markers appeared to correlate with the nuclear grade. Their findings suggests that DCIS lesions can originate from different progenitor cell types, which would explain the morphological and genetic heterogeneity of DCIS lesions (*20, 94*).

These findings suggest some types of DCIS lesions will progress and become invasive, and other sorts of DCIS will remain quiescent and will keep their DCIS identity. Or, in other words, both the Sontag-Axelrod parallel pathway and the commitment model can coexist. It is now up to us to identify these subsets of patients with DCIS lesions that will keep their DCIS identity and do not require adjuvant therapies, or perhaps do not need surgery at all.

6.4.2. **HER2** *gene amplification status and the importance of copy numbers*

In the present study, 43% of DCIS lesions were non-amplified, and 57% were amplified, based on results of dual-probe FISH. Strikingly, 30 of 34 amplified DCIS (88%) displayed clusters, whereas in invasive breast cancer, this is only 56% (*54*). All cases with clusters were assigned a 3+ IHC score, whereas the cases without clusters received a 1+ or 2+ IHC score.

We wonder whether we should consider the four DCIS cases without clusters as non-amplified, because their mean *HER2* copy number was very close to the cut-off value of six per nucleus (4.95; 5.80; 6.15 and 6.35), despite the fact that their *HER2/CEP17* ratio was >2.2. Two of these cases would be regarded as non-amplified DCIS if merely *HER2* signals were considered. Since there are no previous reports about the presence of clusters in DCIS, comparison with data of other publications was impossible.

Three cases presented an inverse status (two of them without clusters): their mean *HER2* copy number was lower than six per nucleus, but their *HER2/CEP17* ratio was >2.2. This implies a 95% concordance rate between the results of dual-probe and simulated single-probe FISH. A similar concordance rate of 98% was demonstrated in an IDC patient cohort (*54*). The results of Lambein et al. suggested that the actual *HER2/CEP17* ratio is less important in IDC than de mean *HER2* copy number (*54*). This might be the case in DCIS as well.

A few studies have been published about *HER*2 gene amplification status in DCIS (*19, 53*). Park et al. reported an amplification rate of 50% in their patient population with pure DCIS (*53*), which is in line with our own findings. In contrast, Burkhardt et al. reported a remarkable lower amplification rate of 23% (*19*). This lower rate might be caused by their use of a TMA instead of whole tissue sections. In their study, one tissue core was used per DCIS case, and one additional core was used in case of coexistent IDC (*19*).
Goethals et al. already described that the exact number of tissue cores, required for accurately assessing tumour marker positivity, is influenced by the degree of heterogeneity in the protein expression pattern, and should at least account four tissue cores for most purposes (*89*). Lin et al. confirmed this tumour marker heterogeneity by assessing ER, PR and HER2 expression in whole tissue sections and a TMA of DCIS cases (*90*). The authors warn for significant non-concordance and underestimation in the interpretation of results when TMA analysis is used for breast cancer (*90*).
Although we agree that TMA has its advantages (it is cheaper than using whole tissue sections and less time-consuming), we support the opinion that results of a TMA analysis should be interpreted with caution (*90*), and we believe there is enough evidence to recommend the use of at least four tissue cores, as demonstrated by Goethals et al. (*89*). Beside the heterogeneity in protein expression, the use of more tissue biopsies will also abrogate the bias caused by sampling errors during core extraction, or loss of tissue cores during the staining procedure (*90*).

Amplification status was not associated with hormone receptor expression. This contrasts with IDC, in which *HER2* amplification is associated with ER and PR negativity (*54*). *HER2* amplification status was independent of patient age or DCIS extent. Although amplification status did not correlate with any of the applied pathological classification systems, amplified lesions presented significantly more high-grade nuclear atypia and extensive comedo necrosis. There was no correlation between amplification status and growth pattern, the presence of calcifications or stromal morphology.

Amplified cases appeared to be significantly associated with a more extensive inflammatory infiltrate. Notably, all six DCIS cases with microinvasion displayed *HER2* gene amplification. Although the number of DCIS with microinvasion in our cohort is too small to conclude anything, this suggests HER2 might play a role in the progression of DCIS to IDC. However, if HER2 gene amplification were necessary for the transition of the non-invasive to the invasive stage, one would expect more *HER2* amplified IDC cases than is currently observed in clinic.

In the group of non-amplified cases, there was a significant association between mean *HER2* and *CEP17* copy number, which was also described in non-amplified IDC (*54*). We hypothesized this finding may be related to cell cycling. Duplicated DNA in interphase nuclei probably accounts for additional signals for *CEP17* and *HER2*, which are equal in number. This results in a correlation between the mean copy number of these genes.

In invasive breast cancer, mean *HER2* copy number and *HER2/CEP17* ratio significantly correlated with HER2 IHC score in both amplified and non-amplified cases (*54*). This gene dosage effect at the protein level was also present in the amplified cases of our DCIS cohort: the higher the HER2 IHC score, the higher the mean *HER2* copy number (or the *HER2/CEP17* ratio). In the non-amplified cases of our cohort, we observed a tendency towards this association, but it was not significant. We hypothesize this might be due to the small size of our cohort. We plan further investigations in a larger patient population and we assume that this gene dosage effect is also present in non-amplified DCIS, just as in non-amplified IDC (*54*).
The degree of HER2 immunostaining depends on the amount of receptor molecules on the surface of a cell. Our results show that amplified lesions need more *HER2* gene copies than non-amplified lesions in order to acquire a similar protein expression, and thus a similar HER2 IHC score (*54*). Moreover, it suggests HER2 plays a different biologic role in amplified and non-amplified DCIS.

In the group of amplified DCIS lesions, no association was observed between mean *HER2* copy number or *HER2/CEP17* ratio and hormone receptor status, although this correlation was present in amplified IDC (*54*). Amplified DCIS lesions that were high-grade displayed a significantly higher mean *HER2* copy number and *HER2/CEP17* ratio. This association was absent in the group of non-amplified DCIS cases.

In summary, our study demonstrates the presence of a *HER2* gene dosage effect at the protein level in amplified DCIS. Although we did not find a significant association between *HER2* copy number and protein expression in non-amplified DCIS, we presume a larger patient population is required to demonstrate this *HER2* gene dosage effect in non-amplified DCIS. We assume HER2 might have a different biological role in DCIS than in IDC, because of three reasons: 1) the *HER2* gene amplification frequency is clearly higher in DCIS than in IDC; 2) unlike IDC, amplification status in DCIS appears not associated with hormone receptor status; and 3) amplified DCIS lesions present remarkably more often clusters of *HER2* signals than amplified IDC.

Since 13% of non-amplified DCIS cases had a genuine 3+ IHC score as the majority of the amplified DCIS lesions, we recommend FISH instead of IHC analysis as the primary HER2 testing modality.

7. PERSPECTIVES FOR FURTHER RESEARCH

Our strategy of selecting molecular markers out of a prognostic signature of the aforementioned gene expression profile study of Gevaert et al. (*48*), has enabled us to identify decorin and laminin-beta-2 as promising prognostic markers for DCIS. Regarding our findings about stromal decorin expression, and its association with higher grade, we will investigate the stromal expression of this SLRP in a larger patient population (including follow-up data), in cooperation with the Antwerp University Hospital. In our opinion, the possible connection between decorin and HER2, and the relation between stromal decorin and patient outcome, warrant exploration. This multicentre study will allow us to perform a multivariate analysis, to determine whether stromal decorin expression correlates stronger than histological grade with HER2 protein expression and *HER2* amplification status.

This immunohistochemical analysis of decorin expression can also be extended to DCIS cases with coexistent IDC. Moreover, like decorin, other molecular markers can be selected out of additional file 4 of the taxonomy study of Gevaert et al. (*48*), in order to perform an immunohistochemical analysis of their stromal expression. Fibromodulin, also a member of the SLRP family, was associated with a better prognosis as well, and its stromal expression in DCIS warrants further investigation.
In addition, the stromal expression of other SLRP proteins (lumican, biglycan) should be explored in DCIS, since it has been reported that lumican and decorin are differentially expressed in neoplastic breast tissue (*65*). Moreover, Troup et al. reported that increased lumican mRNA expression is associated with poor outcome in lymph node-negative breast cancer, which implies lumican and decorin are inversely regulated (*40*). Unfortunately, to date no lumican and fibromodulin antibodies are available for IHC analysis on paraffin embedded tissue sections.

Since we noticed a significant association between stromal morphology or inflammation and DCIS histological grade, it would be interesting to explore whether these two histological features are related to patient outcome, and whether they might be used as a prognostic marker, as a part of a new pathological classification system for DCIS. These histopathological features appear to be less heterogeneous than growth pattern and nuclear grade, which would improve inter-observer variability.

Regarding the observed presence of heterogeneity in architectural pattern, nuclear atypia and stromal morphology, it would be interesting to explore in a retrospective way to which extent the cylindrical biopsy specimens, obtained after needle biopsy, are representative for the final entire resection specimen, either after lumpectomy or mastectomy. Nuclear grade, presence of necrosis, architectural pattern, stromal inflammation and stromal morphology are among the features that need to be assessed in such a retrospective study. Indirectly, this assessment would also help us to determine which histopathologic features are the least heterogeneous, and which of these features would be suitable to include in a new pathological classification system for DCIS.

Considering our observations about the *HER2* gene dosage effect in amplified DCIS, and our previous report about this gene dosage effect in both amplified and non-amplified invasive breast cancer (*54*), we will investigate HER2 protein expression and amplification status in a larger patient population with DCIS, with available clinical follow-up data. This cohort will be extended with cases of coexistent IDC/DCIS, which would permit us to compare copy numbers, *HER2/CEP17* ratio and the presence of clusters between the invasive and non-invasive component.

We are currently investigating the expression of Rab27B in this DCIS patient population, and we aim to perform this immunohistochemical analysis of Rab27B in an extended DCIS population, established by cooperation with the Antwerp University Hospital. Rab27B is a secretory GTPase that plays a role in vesicle exocytosis. It was shown that Rab27B regulates invasive growth and metastasis in ER-positive breast cancer cell lines (*95*). Moreover, increased expression of Rab27B in human invasive breast cancer is associated with a worse prognosis (*95*).
Interestingly, Rab27B activity is associated with MMP-2 activation (*95*) and since we hypothesize MMP activity plays a role in the destruction of stromal decorin, it is worth investigating Rab27B expression in DCIS and, if possible, correlating it with stromal decorin expression.

Many questions, few answers and... a lot of research ahead.

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9. ABBREVIATIONS

ADH: atypical ductal hyperplasia

ASCO/CAP: American Society of Clinical Oncology / College of American Pathologists

BCS: breast conserving surgery

BCT: breast conserving treatment

CALLA: Common Acute Lymphocytic Leukemia Antigen

CAV1: caveolin-1

CAV2: caveolin-2

CD10: Cluster of Differentiation 10

CEP17: chromosome enumeration probe 17

CK: cytokeratin

DAB: 3,3' diaminobenzidine

DCIS: ductal carcinoma in situ

DCIS-Mi: ductal carcinoma in situ with microinvasion

DCN: decorin

DAPI: 4', 6-diamidino-2-phenylindole

EBCTCG: Early Breast Cancer Trialists' Collaborative Group

ER: estrogen receptor

ErbB2: Erythroblastic Leukemia Viral Oncogene (synonym for HER2)

FEA: flat epithelial atypia

FISH: fluorescence-in-situ-hybridisation

GTP: guanosine triphosphate

H&E: hematoxylin and eosin

HeLa cells: cervical cancer cells taken from Henrietta Lacks

HER2: human epidermal growth factor receptor type 2

HGFR: hepatocyte growth factor receptor

IHC: immunohistochemistry

LAMB2: laminin-beta-2

LCIS: lobular carcinoma in situ

MAGE: melanoma-associated antigen

MAPK: mitogen activated protein kinase

MMP: matrix metalloproteinase

PR: progesterone receptor

RTK : receptor tyrosine kinase

SD: standard deviation

SLRP: small leucin-rich proteoglycan

SMA: Smooth Muscle Actin

TDLU: terminal ductal lobular unit

TMA: tissue microarray

UDH: usual duct hyperplasia

UK: United Kingdom

UKCCCR/ANZ: United Kingdom Coordinating Committee on Cancer Research / Australia and New Zealand

VNPI: Van Nuys Prognostic Index

DANKWOORD

Eén van de doelstellingen van deze onderzoeksstage was „een smaakmaker te zijn voor wetenschappelijk onderzoek", alsook een "een bijzondere en nuttige leerervaring" te zijn. Op dat vlak is deze stage dubbel en dik geslaagd! Het waren vijf leerrijke en boeiende maanden, waarin ik heb kunnen proeven van research. En het smaakt eigenlijk naar meer…

Het verwezenlijken van dit onderzoeksrapport zou echter niet mogelijk geweest zijn zonder de hulp van vele helpende handen, zowel letterlijk als figuurlijk. Verscheidene mensen hebben in meerdere of mindere mate bijgedragen aan het tot stand komen van dit werk, en daarom is een dankwoord hier zeker op zijn plaats.

Aan mijn promotor, prof. dr. Louis Libbrecht, en aan mijn co-promotor, dr. Kathleen Lambein, ben ik een woord van dank verschuldigd omwille van de uitstekende begeleiding bij dit onderzoek, maar in de eerste plaats voor de introductie in "The Sea of Uncertainty Surrounding DCIS" (dixit Welch et al.).
Door het uitschrijven van dit onderzoeksproject boden ze me de kans gedurende vijf maanden een onderzoeksstage te lopen op de dienst pathologische anatomie, waarbij ik werd ingewijd in de wereld van de DCIS (en toch ook een beetje in de wereld van de LCIS, de UDH, de ADH en de apocriene metaplasie). Ik wil hen van harte bedanken voor het geven van talrijke nuttige tips en praktische aanwijzigen, voor de immer terugkerende "coupe pingpong", voor de leerrijke en boeiende DCIS discussies die bij tijd en wijle het e-mailverkeer deden pieken, voor het nalezen van deze tekst, en voor hun aanstekelijke enthousiasme.

Eveneens ben ik een uitgebreid dankwoord verschuldigd aan alle laboranten van het routine labo van de dienst pathologische anatomie, wiens namen ik hier niet zal opsommen, uit schrik iemand te vergeten. Dank aan allen die direct of indirect hebben meegeholpen aan het tot stand komen van dit onderzoek, in de eerste plaats voor het snijden van ontelbaar veel coupes (van niet te evenaren eerste klasse kwaliteit!), maar ook voor de vele aanwijzingen bij het gebruik van de geautomatiseerde Ventana immunostainer en de automatische coverslipper (en eveneens dank voor hun oneindige geduld, want Caroline en Els hebben met eigen ogen mogen aanschouwen dat scherven geluk brengen). Voor alle vragen, groot en klein, kon ik steeds bij hen terecht. Dit was misschien wel de belangrijkste les van deze onderzoeksstage: zonder enthousiaste, gedreven en nauwgezet werkende laboranten, geen goeddraaiend lab pathologische anatomie.

Een bijzonder woord van dank dient ook uit te gaan naar mevr. Ann Nuyts, voor het uitvoeren van de FISH analyse.

Julie Ghyselinck, mijn ‘partner in crime’, en weldra afgestudeerd industrieel ingenieur, wil ik danken voor de aangename samenwerking aan dit gezamenlijk project. Ik ben ervan overtuigd dat jouw masterproef/scriptie een pareltje is, en ik wens je veel succes bij je toekomstige loopbaan als… (misschien moet je toch het laboleven overwegen, in plaats van het lesgeven?)

Alle assistenten van de dienst pathologische anatomie wil ik bedanken voor de aangename samenwerking tijdens zowel de klinische als onderzoeksstage gedurende het academiejaar 2010-2011, en in het bijzonder Alexandra en Marilyn, omdat ze me geleerd hebben hoe tumorectomie- en mastectomiespecimina verkleind dienen te worden. Ik kijk er al naar uit om bij jullie te starten op 1 september!

Mevr. Kristel Mareels van de biomedische bibliotheek dank ik voor de leerrijke introductie over werken met EndNote X4.

Ook dank aan… Charlotte, voor de wekelijkse nieuwtjes en het enthousiaste supporteren vanaf het Cambodjaanse front. Cleo, voor alle aanmoedigingen tijdens het schrijven en een stevige portie optimisme. Gezim, voor alle succeswensen en het zorgen voor pdf Creator. Sara, voor het voorraadje Oegandese thee en omdat je het levende bewijs bent dat carpoolen nooit saai hoeft te zijn! Sven, voor het schenken van een exemplaar van Owen Meany en het uitwisselen van vele leestips gedurende al die jaren.

Mijn ouders wil ik bedanken, voor alle mogelijkheden die ze me geboden hebben, en omdat ze me steeds hebben laten studeren wat ik wilde, zolang ik wilde.

Tot slot, eveneens dank aan prof. dr. Claude Cuvelier, mijn toekomstige stagemeester, en prof. dr. Marleen Praet, mijn toekomstig diensthoofd, omdat ik gedurende de afgelopen vijf jaar steeds welkom was op hun dienst.

Aan iedereen, heel erg bedankt!
Mieke