Prognostic significance of Tumour infiltrating B-Lymphocytes in Breast Ductal Carcinoma *in Situ*

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ABSTRACT:

Background: Tumour infiltrating lymphocytes are an important component of the immune response to cancer and have a prognostic value in breast cancer. Although several studies have investigated the role of T-lymphocytes in breast cancer, the role of B lymphocytes (TIL-Bs) in Ductal carcinoma in situ (DCIS), remains uncertain. This study aimed to assess the role of TIL-Bs in DCIS. **Methods**: 80 DCIS cases (36 pure DCIS and 44 mixed with invasive cancer) were immunohistochemically stained for B lineage markers CD19, CD20 and the plasma cell marker CD138. TIL-Bs density and localisation were assessed including relation to the *in-situ* and invasive components. Association with clinico-pathological data and patient outcome was performed. **Results**: Pure DCIS showed a higher number of TIL-Bs and lymphoid aggregates than DCIS associated with invasion. In pure DCIS, a higher number of peri-tumoural and para-tumoural TIL-Bs was significantly associated with large tumour size (p=0.016), hormone receptor (ER/PR) negative (p=0.008) and HER2+ status (p=0.010). In tumours with mixed DCIS and invasive components, cases with high density B-lymphocytes, irrespective of their location or topographic distribution, were significantly associated with variables of poor prognosis including larger size, high grade, lymphovascular invasion, lymph node metastases, ER/PR negative and HER2+ status. Outcome analysis showed that pure DCIS associated with higher numbers of B-lymphocytes had shorter recurrence free interval (p=0.04), however the association was not significant with CD138+ plasma cell count (p=0.07). **Conclusion**: Assessment of TIL-B cells based on location and topographic distribution can provide prognostic information. Validation in a larger cohort is warranted.

INTRODUCTION

Although breast cancer is a heterogeneous disease, there is a great similarity between ductal carcinoma *in situ* (DCIS) and associated invasive carcinoma at the histological and molecular levels [1]. Such similarity not only supports DCIS as a pre-invasive stage before progression to invasive duct carcinoma (IDC) but also indicates that they share molecular and behavioural features [2]. To date, neither histopathological features nor conventional breast cancer biomarkers can accurately predict whether DCIS lesions will progress to invasive disease or recur [3].

Evasion of immune surveillance and host immune response is a hallmark of carcinogenesis and cancer progression [4]. Furthermore, the intensity of tumour immune response influences the effectiveness of cancer therapy, and positively affects clinical outcome in several solid tumours [4]. The association with patient outcome in majority of the studies have focussed on the role of tumour infiltrating T lymphocytes (TILs) [5-9]. However, there is also a suggestion of a critical role of tumour infiltrating B lymphocytes (TIL-B) with patient survival [10]. B-cells are commonly activated in cancer patients, supporting the possibility of a positive role in tumour immunity [11]. In breast tumours, TIL-Bs are present in approximately 25% and comprise up to 40% of the TIL population [12-14], appearing early during breast tumuorigenesis [15]. However, assessment of TILs in cancer tissues remains challenging [16]. Despite the reports implicating TIL-Bs in improving patient survival, the mechanisms, functional profiles and their allied antigens remain to be defined.

Of the common B-cell antigens, CD20 is expressed on all mature B cells except plasma cells [17]. T cell response is inhibited by resting B cells and facilitated by activated B cells [18]. CD19, another B-cell marker, is a member of the Ig superfamily that has a critical signal transduction function regulating the development, activation and differentiation of B lymphocytes [19]. The expression of CD19 is restricted only to B cells. Similar to CD20, it appears early during B cell maturation in the late pro-B cell

stage and remains throughout maturation but is lost when B cells differentiate into plasma cells [20, 21]. CD138, another B-lineage antigen, is a transmembrane heparin sulphate proteoglycan family member having several cellular functions including proliferation, programmed cell death as well as role as an extracellular matrix receptor [22]. Its expression is typically on the surface of mature epithelial cells and some stromal cells in developing tissues [23]. High levels of CD138 expression is detected in precursor B cells and plasma cell differentiation [24], with monoclonal antibodies of the CD138 cluster beings plasma cell specific among hematopoietic elements [24]. This study aimed to determine the density and pattern of distribution of CD20/CD19 positive lymphocytes and CD138 positive plasma cells in patients with DCIS including their prognostic significance.

PATIENTS AND METHODS

Study patients

This retrospective study included cases of DCIS with or without an invasive component diagnosed from 1989 to 2000 at Nottingham City Hospital, Nottingham UK who underwent conservative breast surgery with standard adjuvant treatment that was based on risk stratification. High risk patients with pure DCIS received post-operative adjuvant radiotherapy. However low risk DCIS patients did not receive postoperative radiotherapy. Patients with IDC associated with DCIS were treated either with adjuvant hormonal, chemotherapy or radiotherapy or combination based on hormone receptor status and Nottingham Prognostic Index (NPI) of their invasive disease. Histological data for DCIS type including grade, presence or absence of necrosis was recorded together with patient outcome. Recurrence free survival (RFS) was calculated in months from the date of first operation until the first recurrence [25]. For invasive cases associated with DCIS, histological data lymph node status [26] and stage was recorded together with outcome data. Breast cancer specific survival (BCSS) was defined as the time (in months) from the date of the primary surgical treatment to the time of death from breast cancer [27].

Immunohistochemistry (IHC)

Haematoxylin and eosin stained full-face sections representative of each case were examined to confirm the diagnosis and assess the suitability of the tissue block for IHC. IHC staining was performed using the Novocastra Novolink Polymer Detection Kit (Leica Microsystems, Newcastle, UK) following the manufacturer's guidelines. Tissue sections were stained by optimised monoclonal anti-human CD20 antibody (clone L26, DAKO, Glostrup, Denmark, dilution 1:300), monoclonal anti-human CD19 antibody (clone LE-CD19, DAKO, Glostrup, Denmark, dilution 1:75) and monoclonal anti-human CD138 antibody (clone MI15, DAKO, Glostrup, Denmark, dilution 1:40). CD19, CD20 and CD138 were applied on sequential tissue sections from the same paraffin block and not on the same slide. All markers were included in the analysis. Paraffin sections of normal human tonsil were used as a positive control which showed positive cells distributed mainly in the germinal centres and mantle zone (B cell area) with some scattered inter-follicular positive cells. Negative controls were included with staining runs by omitting the primary antibody.

$CD20^+$/$CD19^+$/$CD138^+$ lymphocytes and plasma cells quantification

The number of B-lymphocytes, marked by CD20 and/or CD19 positive cells, and plasma cell count, evidenced by CD138 positivity, was counted in each tissue section using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). For the purpose of the analysis, cells showing membranous labelling with CD20 and/or CD19 were considered as TIL-Bs. For CD138, only cells with morphology consistent with plasma cells were considered during cell counting. If CD138 additionally showed membrane and cytoplasmic staining of DCIS and invasive tumour cells, this epithelial staining pattern was not considered in the analysis. Slide scanning was also done using 'Panoramic digital slide' scanner, followed by viewing the slide using 'Panoramic Viewer software'. Marking the zones of interest to be scored was done digitally (0.5mm, 1mm and 2mm).

Pure DCIS cases and the *in situ* component of the mixed cases were assessed, clearly defined and marked to delineate the tumour edge. TIL-Bs were identified within DCIS

(tumour infiltrating lymphoid cells) and at the periphery of DCIS (stromal TIL-Bs). All foci of DCIS were evaluated with average of 1-6 foci. The highest density focus (hot spot) was then selected as the final result for analysis. Presence and distribution of positively stained cells with morphologic features of lymphocytes and /or plasma cells were reported. Stromal TIL-Bs was divided into: a) cuffing (in direct contact with DCIS), b) peritumoural (less than 0.5mm distance from the DCIS profile margin), c) paratumoural TIL-Bs that was quantified by counting positive cells within the marked tumour area and not in direct/close contact with tumour cells (more than 0.5mm and up to 1mm distance away from the DCIS profile margin) and d) TIL-Bs present in up to 2mm away from the DCIS profile margin. Mean number of TIL-Bs was then calculated for each compartment. Within the marked area, each case was assigned a qualitative stromal TIL-Bs density score: A score of 1 referred to low density of TIL-Bs (less than or equal to 25% surrounding the duct circumference). A score of 2 (>25% and <50% TIL-Bs surrounding the duct circumference) and score of 3 referred to a diffuse/marked infiltration of more than, 50% (TIL-Bs surrounding most of the duct circumference).

In the cases with mixed DCIS and invasive tumours, TIL-Bs were assessed separately in both components to indicate the difference in the density and pattern of distribution between components and between DCIS associated with invasion and pure DCIS. For TIL-Bs surrounding/infiltrating the DCIS components in these cases, the same approach as used as in pure DCIS cases. On the other hand, TIL-Bs were assessed in the invasive component according to the previously published guidelines [28, 29]; in three locations; (1) Intra-tumoural compartment (defined as TIL-Bs in tumour nests that had cell-to-cell contact with no intervening stroma and directly interacting with carcinoma cells), (2) Within stroma away from tumour (defined as TIL-Bs located dispersed in the stroma, more than one tumour cell diameter, and among the carcinoma cells but not directly interfacing carcinoma cells), and (3) Peri-tumoural stroma (defined as TIL-Bs within one tumour cell diameter of the tumour). In this study, the total number of TIL-Bs was determined by adding the counts for the three tumour compartments. TIL-Bs in areas

with crush artefacts, necrosis, inflammation around biopsy sites or extensive central regressive hyalinisation and adjacent normal lobules were not included [28, 29].

In addition to the presence, density, and location of TIL-Bs, the presence and distribution of lymphoid follicles or aggregates were assessed and their relation to DCIS and invasive disease, when present, were recorded. Lymphoid follicles (tertiary lymphoid structures, (TLSs) were considered as aggregates of lymphocytes with a germinal centre while lymphoid aggregates were considered as a collection of lymphocytes without germinal centre formation.

This study was approved by Nottingham Research Ethics Committee 2 (REC C202313) under the title of "Development of a molecular genetic classification of breast cancer".

Statistical Analyses

IBM-SPSS statistical software 22.0 (SPSS, Chicago, IL, USA) was used to analyse the correlation between the number of $CD20^+$, $CD19^+$ lymphocytes and $CD138^+$ plasma cells and the various clinico-pathological parameters. The optimal cut-off point for $CD20^+$, $CD19^+$ lymphocytes and $CD138^+$ plasma cells against patient survival was defined using X-tile bioinformatics software (Yale University, version 3.6.1). Kaplan–Meier curves and log-rank test were used for survival analyses. A p-value less than 0.05 (two-tailed) was considered as statistically significant.

RESULTS

Patient characteristics

Patient characteristics for the 80 patients on the study are shown in Table 1. Patients were aged 70 years or less (median = 55 years) with long-term follow-up (median follow-up = 266 months). No patients received neoadjuvant therapy. Thirteen patients received chemotherapy and 17 cases were hormonally treated for the invasive disease. Of the two categories, 36 cases were of DCIS alone (none of the pure DCIS cases contained microinvasion), while in 44 cases, DCIS was associated with an invasive

component (all cases included invasive carcinoma measuring more than 1mm). Most pure cases (28/36: 77.8%) were from postmenopausal women. Histological assessment for DCIS is summarised in Figure 1A. During the follow-up period, Ipsilateral local recurrence in pure DCIS occurred in 7/36 (19.4%) patients, of which 2 (5.5%) cases recurred as invasive disease.

Forty-four female patients with DCIS had associated invasive disease, 27/44 of which were postmenopausal. Figure 1B & C summarise the histological data for the invasive disease including tumour size, type grade, presence or absence of comedo necrosis, lympho-vascular invasion [30], lymph node status [26]. Median follow-up was 143 months (19-307 months), during which period, recurrence of invasive disease occurred in 12 (27.3%) patients, 7 (15.9%) patients developed ipsilateral local recurrence and 8 (18.2%) cases progressed into distant metastasis.

To summarise the pathology parameters in this series, solid DCIS with necrosis (comedo type) was the predominant type. Most cases showed high nuclear grade (55%). The mean DCIS size was 24 mm. Positive hormone receptor status (ER/PR) and negative HER2 was the predominant pattern in both groups.

Assessment of ER, PR and HER2 assays were based on the American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update (ASCO). [31, 32]. For ER and PR, considered positivity if there are at least 1% positive tumour nuclei. For HER2 status, intense complete/circumferential IHC membrane staining within more than 10% of tumour cells was considered positive (3+).

DCIS TIL-Bs; frequency, and localisation

Intra-tumoural TIL-Bs were very rare within DCIS compared with the peri-ductal stromal TIL-Bs. Both diffuse and aggregate patterns of TIL-Bs were observed in the stroma. The density of CD19 was lower than that of CD20 and the number of follicles stained with CD19 was fewer than those identified by the CD20. Plasma cells did not show any specific pattern or distribution. Pure DCIS cases showed a significantly higher number of TLSs (maximum 20 lymphoid aggregates) than those cases associated with invasion

(maximum 12 lymphoid aggregates) (p=0.04). Tertiary Lymphoid Structures (TLSs) were mainly localised around the DCIS component of the mixed group.

Intra-tumoural, peri-tumoural, para-tumoural and stromal lymphocytes' results in pure and mixed groups are summarised in Table 2. Mean count of peri-tumoural lymphocytes was 80.4 in pure DCIS cases, compared to 37.7 in cases associated with invasive component (p= 0.002). In para-tumoural TIL-Bs, the mean number was 108.1 in pure DCIs cases, compared to 56.7 in DCIS cases mixed with an invasive component (p= 0.006). High level of B-cells as defined by CD20 and or CD19 positivity was observed around the DCIS component of the tumour in 65.9% of cases, and was observed around the invasive component in 27.3% (p=0.01) (Figure 1D-F). It was observed that the number of B-lymphocytes around the DCIS foci was higher in the pure group when compared to the DCIS foci in the mixed cases (p<0.001). Pure DCIS cases showed a higher number of plasma cell count (mean=91) than mixed cases (mean=44), though this does not achieve statistical significance (p=0.4). Stromal distribution was the detected pattern of plasma cell distribution. No intratumoural plasma cells were found.

Association with clinicopathological variables

Overall associations of TIL distribution with clinic-pathological variables are summarised in Table 3. In pure DCIS cases, increased number of TLSs and dense peri-tumoural and para-tumoural TIL-Bs were significantly associated with larger tumour size (p=0.016), hormone receptor (Oestrogen/Progesterone receptor; ER/PR) negative tumours (p=0.008) and HER2 positive status (p=0.01). No association between plasma cells and the clinicopathological parameters was identified in the pure cases.

In the mixed cohort, higher number of B-lymphocytes, irrespective of their location and topographic distribution, were significantly associated with larger (invasive and *in situ*) tumour size (p=0.019), higher invasive tumour grade (p=0.005), presence of DCIS necrosis (p=0.042), lympho-vascular invasion (p=0.022), lymph node metastases (p=0.033), negative ER/PR status (p=0.04) and positive HER2 status (p=0.008). A higher number of plasma cells were significantly associated with ER/PR negative tumours (p=0.01) and HER2 positivity (p=0.019).

Outcome analysis (Figure 2) revealed that pure DCIS cases, associated with low number of peri-tumoural and para-tumoural B-lymphocytes had a longer recurrence free survival (RFS) ($p=0.008$ and $p=0.04$ respectively). Less dense peri-tumoural and low count of stromal B-lymphocytes was associated with longer recurrence free survival (RFS) ($p=0.04$ and $p=0.01$ respectively). There was a non-significant association between low plasma cell count around the invasive component and a longer survival ($p=0.07$). Intratumoural TIL-Bs did not show significant association with patient outcome.

DISCUSSION

The role of immune cells in breast carcinogenesis remains questionable [33]. It was initially thought that tumour infiltrating immune cells play a protective role in tumorigenesis [34]. However, there is increasing evidence supporting the fact that the infiltrating immune cells play a role in carcinogenesis [35] and there is a plethora of data to propose powerful links between infiltrating immune cells and carcinogenesis [33]. Tumour infiltrating lymphocytes (TILs) are an important immune component of the response to cancer [36]. It is now well accepted that the immune system has a dual role in cancer development and progression. It can eradicate emerging malignant cells by an orchestrated action of innate and adaptive branches thus preventing tumour growth. On the other hand, it can paradoxically promote growth of malignant cells, their invasive capacity and their ability to metastasise. The presence of immune cells with tumour-suppressive and tumour-promoting activity in the cancer microenvironment and in peripheral blood is usually associated with good clinical outcome and poor clinical outcome, respectively [36]. Furthermore, the intensity of tumour immune response influences the effectiveness of cancer therapy, and positively affects the clinical outcome in several solid tumours [4]

TILs have previously been identified as prognostic and predictive biomarkers in several cancers, including breast cancer. However, the exact role of the different components of

TILs remains unclear [16]. Although most the TILs in breast cancer have focused on T-lymphocytes [5-7], the role tumour-infiltrating B lymphocytes remains poorly defined [37] with few studies having assessed the role of tumour infiltrating B lymphocytes (TIL-Bs) in the breast [38-40]. In this study, we aimed to determine the potential role of TIL-Bs in DCIS and its role in DCIS behaviour and progression to invasive disease.

In current study, the density of CD19 was much lower than that of CD20 and the number of follicles identified by the CD19 antibody was a subset of those identified by the CD20 antibody. This finding goes in line with findings in other tumours such as chronic lymphocytic leukaemia where Ginaldi et al in 1998 showed that CD19 had low density and lower number than CD20 positive cells [41]. This can be explained as a reflection of an early stage of maturation of tumour infiltrating B lymphocytes in comparison to their counterparts in normal blood [41]. This may suggest that tumour immunity develops as a response to early stage of tumour development such as DCIS.

Some authors have reported that TILs are more commonly observed in DCIS than in invasive carcinoma [42-44] and this was confirmed in the current study. One study reported that the proportion of luminal-like subtypes decreased, while HER2+ and basal-like subtypes increased, with the development of invasion [45]. On the other hand, other studies have reported that microinvasive carcinoma might be a distinct entity.

Martinet et al [43] showed that tumour associated lymphocytes and mature dendritic cell densities were significantly higher in DCIS than in invasive carcinoma. However, the relationship between the presence or abundance of TLSs and specific DCIS subtypes has been unclear [46] though the presence of TLSs around HR-/HER2 positive tumours are also corroborated in the current study. It might be explained that once the carcinogenic events have settled (i.e. invasive cancers), a generalised increase in lymphocyte infiltration is observed, that does not differ among various tumour sub-types [33]. Moreover, DCIS tumour microenvironment over-expresses variety of inflammatory mediators (probably released from infiltrating leukocytes), including interleukin signalling [47]. Taken together, these findings suggest a role of leukocytes in early stages of breast

cancer development. In support of this, there is evidence that lymphocytes play a key role in creating a tumour promoting microenvironment [34]. However, this needs to be further investigated in breast carcinogenesis.

Our study showed that higher numbers of B-lymphocytes and TLSs were associated with higher tumour grade, presence of necrosis, vascular invasion, negative hormone status and HER2 positivity. These findings are supported by Schalper et al [48] who suggested that the tumour biology itself may play a possible role in lymphocytes induction. Higher CD20 positive TILs have also been similarly observed in high grade DCIS by Campbell et al. and as indicated, an orchestrated increase of FoxP3+, CD68+, HLA-DR+ and CD4+cells are observed in higher grades of DCIS [49]. The relationship with vascular invasion is interesting as in studies as early as 1997 by Lee et al, it was apparent from morphology correlates that clusters of B and T cells may be recruited in DCIS by high endothelial venules and the authors speculate that cytokines released by the DCIS along-with its immune cells may stimulate new vessel formation and create a pro-metastatic milieu [49]. DCIS cells should adapt to the hypoxic and nutrient-deprived ductal microenvironment. We assume that the presence of necrosis in DCIS might be associated with increased release of damage associated molecular substances such as ATP that could result in the subsequent recruitment of immune cells into the tumour microenvironment [50]. Ma et al [51] reported a strong immune response signature resulting in activation of other leukocytes and interferon signalling present particularly around high grade DCIS. They speculate that the presence of an immune response signature around high-grade DCIS may represent a phase where the cancer cells resist immune attack and instead are able to utilise the abundant cytokines produced by immune cells to facilitate invasion [51].

Unlike invasive cancer, where presence of abundant tumour infiltrating lymphocytes has been linked to better prognosis, this does not appear to be the case for DCIS [29]. In the current study, we found decreased RFS in cases associated with more TIL-Bs. This

goes with Knopfelmacher et al [52] who found that the presence of dense chronic inflammation surrounding DCIS was significantly associated with a high Oncotype DX DCIS Score and hence high recurrence risk. Although there are no genes directly related to the immune system in the DCIS Score, research has elucidated that there are genetic changes in the microenvironment, including stromal fibroblasts, myoepithelium, and inflammatory cells, which are associated with progression from *in situ* to invasive disease [53, 54]. In cases where DCIS is associated with early invasion, a dense chronic inflammatory infiltrate often surrounds these microinvasive foci. We could speculate that dense periductal chronic inflammation around DCIS suggests a role for the immune response in DCIS progression and deserves further investigation. Despite the prognostic role of TIL-Bs, it was not independent prognostic factor in Cox regression model and this might be explained by the small number of cases included in the study. In this study, it is noteworthy that, assessment of TILs in DCIS was different from that of invasive cancer. Evaluation of stromal associated lymphocytes, within the confinement of invasive tumour, was challenging because of presence of scattered ducts in DCIS. However the scoring was done as objectively as possible. For better reproducibility, standardisation of TILs scoring method in DCIS is needed.

The use of CD138 as a clinical marker remains controversial. In our study, CD138 was expressed in the entire epithelium as well as plasma cells, but it did not stain lymphocytes and this finding is consistent with the finding of Barbareschi, et al [55]. We also found that more plasma cells were observed in pure DCIS cases when compared to DCIS cases associated with invasion. High plasma cell count represented by CD138 positivity is correlated with ER/PR negativity. In fact, it could be hypothesised that in ER-negative tumours that have lost the ability to respond to the oestrogen-dependent proliferative pathway, high CD138 expression may confer a particularly important growth advantage by enhancing the response to other growth factors [55]. This correlates with poor prognosis; findings consistent with those reported by Barbareschi et al [55].

CD138 is in fact implicated in several essential physiologic cell functions, such as control of cell proliferation, differentiation, adhesion, and migration [56]. One of the best known biologic functions of CD138 is related to its interaction with fibroblast growth factors (FGFs), which are known angiogenic and mitogenic growth factors for breast carcinoma cells [57], binding to FGFs and to their receptors in a ternary signalling complex [58]. CD138 also can function as a potent FGF-2 activator through physiologic shedding and degradation of its extracellular domain by enzymes, such as heparanase [59].

The limitations of the study are that a limited subset of cases were studied, as well as the limited availability of data for hormone receptor status and HER2 expression especially for the DCIS component. This might have affected the statistical associations with some parameters. Expansion of this study to include a larger patient cohort is therefore warranted.

In conclusion, this study suggests that B cells, perhaps as part of the adaptive humoral immune response, may have a role in breast cancer. Expansion of this work on a larger series of patients as well as the development of a standardised scoring approach is warranted. Studies of a holistic nature exploring the cross-talk of both B and T cell pathways may reveal the immune switch enabling tumour progression from the *in-situ* stage to invasive disease. As B-cell activation may be both T cell dependent and independent, studying them side by side alongside released mediators, will help better understanding of the correlations between biology and morphology. As the role of B cells in the pre-invasive to invasive stages becomes clearer, novel options for immune modulation to prevent breast cancer progression may become evident.

Figure Legends:

Figure 1: A) High grade comedo DCIS with central necrosis and adjacent cuffing lymphocytic aggregates (H&E x10). B) DCIS with micro-invasion and surrounding cuffing lymphocytes and some intra-tumoural lymphocytes (H&E x10). C) Solid DCIS mixed with invasive component and scattered stromal lymphocytes (H&E x10). D) A case of DCIS with peri-tumoural and intra-tumoural CD20 positive lymphocytes forming aggregates (IHC x10). E) Peri-tumoural and stromal CD19 positive lymphocytes (IHC x10). F) CD19 positive lymphocytic aggregate in a case mixed with invasive component (IHC x 4).

Figure 2: A and B: For combined pure DCIS cases and DCIS cases mixed with invasion, decreased density of peri-tumoural and para-tumoural B lymphocytes tend to be associated with better RFS ($p=0.008$ and $p=0.04$).

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Authors' disclosures of potential conflicts of interest

The author(s) indicated no potential conflicts of interest.

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Table 1: Clinico-pathological variables of the study cohort (80 cases)

Clinico-pathological Variables	**Total Number of cases**	**DCIS (pure cases) Number (%)**	**DCIS (mixed cases) Number (%)**	***X*²/(P value)**
Age <50 ≥50	 25 55	 6 (16.7) 30 (83.3)	 19 (43.2) 25 (56.8)	6.48 (**0.01**)
Menopausal Status Pre-menopausal Post-menopausal	 25 55	 8 (22.2) 28(77.8)	 17 (38.6) 27 (61.4)	2.48 (0.11)
Tumour size <2.0cm ≥2.0cm	 44 36	 16 (44.4) 20 (55.6)	 28 (63.6) 16 (36.4)	2.95 (0.08)
Histologic type Comedo DCIS Cribriform DCIS Micropapillary DCIS Solid DCIS	 54 9 4 13	 22 (61.1) 6 (16.7) 4 (11.1) 4 (11.1)	 32 (72.7) 3 (6.8) 0 (0) 9 (20.5)	80 (<**0.0001**)
Comedo Necrosis No Yes	 37 43	 10 (27.8) 26 (72.2)	 27 (61.4) 17 (38.6)	8.98 (**0.008**)
Grade 1 2 3	 18 18 44	 5 (13.9) 6 (16.7) 25 (69.4)	 13 (29.5) 12 (27.3) 19 (43.2)	5.63 (0.06)
Lymph node status Negative Positive	 56 11	 23 (100) 0 (0)	 33 (75) 11 (25)	7.67 (**0.006**)
Oestrogen receptor Negative Positive	 22 32	 3 (30) 7 (70)	 19 (43.2) 25 (56.8)	0.58 (0.44)
Progesterone receptor Negative Positive	 22 22	 3 (30) 7 (70)	 19 (43.2) 15 (56.8)	0.58 (0.44)
HER2 Status Negative Positive	 33 18	 4 (57.1) 3 (42.9)	 29 (65.9) 15 (34.1)	0.2 (0.56)

DCIS: Ductal carcinoma in situ ***X*²:** Chi square

Table 2: Distribution of TIL-Bs in different compartments of DCIS in both pure and mixed groups)

Compartment	DCIS (pure group) Mean Number of TIL-Bs	DCIS (mixed group) Mean Number of TIL-Bs	X^2 (P value)
Intra-tumoural	15.6	4.8	6.5 **(0.04)**
Cuffing (direct contact)	45.3	30.6	4.8 (0.1)
Peri-tumoural (<0.5mm)	80.4	37.7	10.2 **(0.002)**
Para-tumoural (0.5-1mm)	108.1	56.7	14.9 **(0.006)**
Stromal (1-2mm)	134.2	80.8	3.1 (0.3)

TIL-Bs: Tumour infiltrating lymphocytes, B cells.

DCIS: Ductal carcinoma in situ

***X²*:** Chi square test

Table 3: Distribution of TIL Bs and association with clinico-pathological parameters.

Clinico-pathological parameters	Intra-tumoural TIL-Bs P value (test value)	Cuffing TIL Bs P (test)	Peri-tumoural TIL-Bs P (test)	Para-tumoural TIL-Bs P (test)	Stromal 2mm TIL-Bs P (test)
Age*	0.075 (1.58)	0.12 (1.6)	0.375 (1.1)	**0.001** (2.76)	0.7 (1.8)
Menopausal status*	0.66 (1.35)	0.089 (2.96)	**0.011** (6.76)	**0.005** (8.387)	0.078 (1.9)
Tumour size*	0.275 (1.21)	0.06 (1.55)	**0.005** (2.31)	**0.000** (7.81)	0.07 (2.01)
Histologic tumour type**	0.24 (1.69)	0.07 (2.12)	0.078 (2.5)	0.65 (1.21)	0.58 (1.66)
Necrosis*	0.45 (1.67)	0.216 (1.55)	**0.051** (3.93)	**0.026** (5.13)	0.68 (2.11)
Grade**	0.13 (2.06)	0.21 (1.9)	**0.028** (3.71)	**0.02** (4.13)	0.08 (2.21)
ER/PR status*	0.06 (1.67)	**0.032** (2.62)	**0.008** (2.91)	**0.001** (3.29)	**0.001** (4.5)
Her2neu status*	0.1 (1.83)	**0.02** (1.42)	**0.01** (2.53)	**0.002** (3.34)	0.07 (1.42)

TIL Bs: Tumour infiltrating lymphocytes, B cells

Test value using Mann-Whitney U test (*) or Kruskal-Wallis test (**) and the figured data used as continuous variables for TLI-Bs.

Figures and tables:

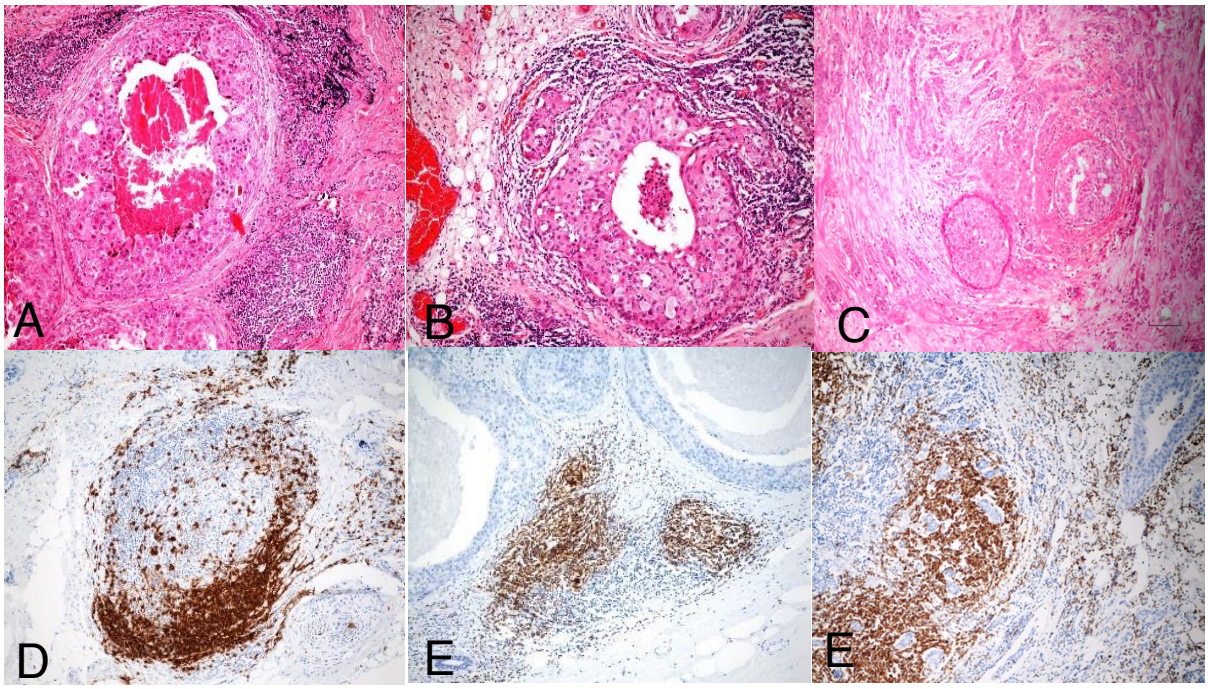

Figure 1: A) High grade comedo DCIS with central necrosis and adjacent cuffing lymphocytic aggregates (H&E x10). B) DCIS with micro-invasion and surrounding cuffing lymphocytes and some intra-tumoural lymphocytes (H&E x10). C) Solid DCIS mixed with invasive component and scattered stromal lymphocytes (H&E x10). D) A case of DCIS with peri-tumoural and intra-tumoural CD20 positive lymphocytes forming aggregates (IHC x10). E) Peri-tumoural and stromal CD19 positive lymphocytes (IHC x10). G) CD19 positive lymphocytic aggregate in a case mixed with invasive component (IHC x 4).

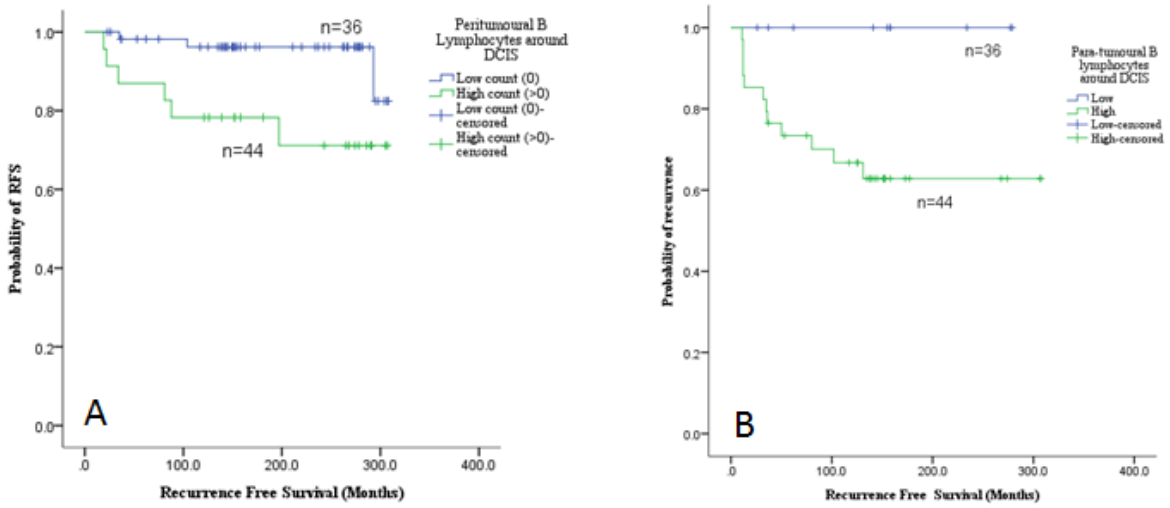

Figure 2: A and B: For combined pure DCIS cases and DCIS cases mixed with invasion, decreased density of peri-tumoural and para-tumoural B lymphocytes tend to be associated with better RFS (p=0.008 and p=0.04).