Prognostic Significance of Tumour Infiltrating Lymphocytes in Ductal Carcinoma in Situ of the Breast

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ABSTRACT

Tumour infiltrating lymphocytes (TILs) provide prognostic value in invasive breast cancer and guidelines for their assessment have been published. This study aims to evaluate; (a) methods of TILs assessment, and (b) their prognostic significance in breast ductal carcinoma *in situ* (DCIS). Haematoxylin and Eosin sections from two clinically annotated DCIS cohorts; a training set (*n*=150 pure DCIS) and a validation set (*n*=666 comprising 534 pure DCIS and 132 cases wherein DCIS and invasive breast carcinoma were coexistent) were assessed. Seven different scoring methods were applied to the training set to identify the most optimal reproducible method associated with strongest prognostic value. Among different methods, TILs touching ducts' basement membrane or away from it by one lymphocyte cell thickness provided the strongest significant association with outcome and highest concordance rate [inter-cluster correlation coefficient=0.95]. Assessment of periductal TILs at increasing distances from DCIS (0.2mm, 0.5mm and 1mm) as well as percent of stromal TILs were practically challenging and showed lower concordance rates than touching TILs. TILs hotspots and lymphoid follicles did not show prognostic significance. Within the pure DCIS validation set, dense TILs were associated with younger age, symptomatic presentation, larger size, higher nuclear grade, comedo necrosis and oestrogen receptor negativity as well as shorter recurrence free interval (*p*=0.002). In multivariate survival analysis, dense TILs were independent predictor of shorter recurrence free interval (*p*=0.002) in patients treated with breast conservation. DCIS associated with invasive carcinoma showed denser TILs than pure DCIS ($p=9.0 \times 10^{-13}$). Dense TILs is an independent prognostic variable in DCIS. Touching TILs provides a reproducible method for their assessment that can potentially be used to guide management.

Key words: Breast ductal carcinoma in situ, infiltrating lymphocytes, assessment, recurrence.

INTRODUCTION

The incidence of ductal carcinoma *in situ* (DCIS) of the breast has dramatically increased after introduction of mammographic screening programmes (1, 2). This, together with increasing use of breast conserving surgery for management of breast cancer, has heightened the need for robust and reliable predictors of disease recurrence and progression, for risk stratification of patients. Comparison of DCIS recurrence rates and progression (to invasive disease) with the rates of mastectomies, surgical re-excisions or intense course of radiotherapy suggests overtreatment, stemming from a lack of accurate risk stratification (3). Current application of molecular genetic signatures for DCIS categorisation to provide suitable individualised management remains challenging (4-9).

Tumour infiltrating lymphocytes (TILs) are indicators of the adaptive immune response against tumours and play a cornerstone role in cancer immunotherapy (10, 11). In previous studies on invasive breast cancer, dense TILs were shown to augment effect of chemotherapy providing better prognosis mainly in triple negative breast tumours (12, 13). Following demonstration of their prognostic significance in invasive breast carcinoma and their potential clinical application, guideline recommendations for TILs evaluation have been published (14, 15). However, the recommended method of TILs assessment in invasive breast carcinoma may not be applicable in DCIS as not all stromal TILs are directly in contact with malignant ducts and identification of stromal area surrounding DCIS can be confusing and ill-defined. Despite the reported role of TILs in DCIS (5, 16), studies utilising the invasive carcinoma guidelines for TILs assessment in the context of DCIS did not find any association with outcome (15, 17). This is likely related to the difference in nature and distribution of TILs within DCIS compared to invasive breast carcinoma and the sub-optimal method of their assessment. This study aims to identify the optimal method of TILs evaluation in DCIS in terms of reliability,

reproducibility and prognostic significance with recurrence through utilisation of a large well-annotated DCIS cohort with long term follow-up.

MATERIALS AND METHODS

Study cohort

This retrospective study included 982 cases diagnosed from 1990 to 2012 at Nottingham City Hospital, Nottingham, United Kingdom. Following histological review of tumour slides of all cases and retrieval of tissue blocks, representative Haematoxylin and Eosin stained sections from each case were prepared from 816 cases (684 pure DCIS and 132 DCIS mixed with invasive carcinoma). Representative sections were defined as those having the largest tumour burden. Clinicopathological data included patients' age, type of presentation (whether symptomatic or screen-detected), DCIS tumour size, nuclear grade, presence of comedo necrosis, associated Paget's disease, detailed information about management and outcome including surgery type, and number of operations, local radiotherapy treatment, occurrence and nature of local recurrence and recurrence free interval were collected from local data recording systems. Recurrence free interval is defined as time between first DCIS surgery and occurrence of ipsilateral tumour recurrence (either as DCIS or invasive carcinoma) in months. Cases with contralateral breast event or invasive breast carcinoma developed as a new primary tumour in a different quadrant were censored at the time of recurrences. For molecular characterisation of DCIS, oestrogen receptor immunohistochemical staining was performed in cases with available paraffin blocks. 4µm sections were stained on the Ventana BenchMark® ULTRA system (Tucson, Arizona, USA) using Ventana oestrogen receptor (SP1) Rabbit Monoclonal Primary Antibody as per recommended protocol.

The cohort was split into two groups; a training set and a validation set (clinicopathological parameters of both sets are shown in **Supplementary Table 1):**

Training set

The training set included 150 pure DCIS from patients older than 50 years of age with mixture of DCIS grades that was completely excised with free surgical margins (10mm or more) to avoid the confounding effect of age or margin of excision on the outcome analyses. Within a median follow-up period of 161 months; 41 cases (27%) developed ipsilateral recurrence.

Validation set

The validation set (*n*= 666) *was* further split into two subgroups; 1) Pure DCIS (*n*=534) which showed 63 ipsilateral local recurrence events (11.8%) within a median follow-up period of 109 months; 2) DCIS with co-existent invasive breast carcinoma (*n*=132) to compare the pattern of TILs density between pure DCIS cases and cases wherein DCIS is associated with invasion. **Supplementary Figure 1** shows the algorithm of the study cohorts.

Scoring of TILs

Freshly stained Haematoxylin and Eosin (4-5µm thick) full face sections were scanned using a high-resolution slide scanner (PANNORAMIC 250 FLASH III, 3D-HISTECH), followed by viewing the slides using ‘‘Pannoramic Viewer Software program, version 1.15.4’’. In this study, TILs were counted manually (eyeballing) using digital images. The International Working Group Recommendations for TILs assessment were modified and applied to our case series (15). All recognisable mononuclear inflammatory cells including lymphocytes and plasma cells were counted (polymorphonuclear cells were excluded). TILs were assessed around all DCIS duct profiles up to 20 ducts (the average number of ducts within the training set). For cases with more than 20 malignant ducts, we divided the section field into four identical quadrants using the Pannoramic Viewer software, and scored TILs around five ducts in each quadrant in order to keep the scoring more representative especially in cases with heterogeneously distributed TILs. During scoring, very large (i.e., mass forming papillary

carcinoma, branching or confluent DCIS ducts) or very small (terminal duct-lobular units involved by DCIS) ducts were not considered in TILs scoring to avoid skewing of the scores by the effect of extremes in duct sizes. Pure encapsulated papillary carcinomas, not associated with adjacent DCIS, were excluded from the scoring. Any type of circumferential TILs-infiltration was considered, including minimal, partial, subtotal and total circumferential TILs-infiltration around ducts. TILs beyond the lesion limits, surrounding the adjacent or intermingled fat, normal ducts, associated lobular carcinoma in situ, regressive hyalinosis or at crushing artefacts were excluded from the scoring.

In the training set, TILs were assessed using 7 different scoring methods (**Table 1**). These included: 1- Percentage of stromal TILs (assessed in a manner similar to the modified method for evaluation of TILs in invasive breast carcinoma. The stromal area was defined as the area surrounding the DCIS duct within two high-power microscopic fields and used for evaluation of stromal TILs percentage (15, 17, 18). In cases with numerous involved ducts, an evaluation of the area surrounding the whole lesion was performed, and percentage of stromal TILs in the total stromal area of all DCIS involved ducts was determined (5, 15, 19), 2-The mean number of touching TILs defined by TILs touching or within one lymphocyte cell thickness from the malignant ducts' basement membrane; 3-Mean number of TILs within 0.2mm distance from ducts' basement membranes; 4- Mean number of TILs located within 0.5mm distance from ducts' basement membranes; 5-Mean number of TILs located within 1mm from ducts' basement membranes; 6-TILs hotspots defined as the largest number of lymphoid cells aggregates directly surrounding or located between DCIS ducts within the boundaries of the lesion; and 7-Assessment of lymphoid follicles with reactive germinal centres. Counting of TILs at different topographic areas was carried out manually with aid of the Panoramic Viewer Software program scale. Overlapping TILs between adjacent ducts in each topographic area

were counted once. The detailed methods followed to assess TILs are summarised in **Table 1** and illustrated in **Figure 1.**

To check the reliability and reproducibility of the evaluation methods, training set was scored using all the previous parameters by 3 observers (MST, IMM and AK). Two observers discussed the detailed methodology before starting their scoring, while the third observer scored the cases based on a written protocol without any verbal discussion. The optimal method for TILs assessment was determined based on the association with outcome and reproducibility in terms of inter-observer concordance as well as practicality, which was then applied to the validation set. Validation set, including the pure DCIS and mixed cohorts, was scored for touching TILs by 2 observers (MST and AK) to confirm reproducibility and prognostic significance. In mixed cases, touching TILs were scored around the DCIS component only while TILs adjacent to invasive tumour were not considered in the scoring. TILs scoring carried out by the first observer (MST) were considered in the final statistical analysis (the main researcher for this study). Other observers' scores were used to check the reproducibility and concordance rate.

As assessment of stromal TILs was the method used to evaluate TILs in DCIS in previous studies (15, 17, 18), stromal TILs was also evaluated in the pure DCIS validation set using the same criteria applied in training set.

This study is ethically approved by the North West - Greater Manchester Central Research Ethics Committee (15/NW/0685).

Statistical Analysis

The optimal cut-off point for TILs density against recurrence free interval was defined using X-tile bioinformatics software (Yale University, version 3.6.1). TILs were classified into sparse infiltrates and dense infiltrates depending on these cut-off points (**Supplementary Table 2**).

For consistency, the same cut-off point used in dichotomisation touching TILs in the training set into sparse and dense groups was applied in the validation set. Furthermore, to mimic the three-tier prognostic classification system of TILs in melanoma (20) and based on the outcome analysis, TILs were further defined into three-groups; absent/very scanty (mean number of touching TILs/DCIS duct ≤5 cells), sparse (6-20 cells/DCIS duct) and dense (>20 cells/DCIS duct) TILs. This was based on counting TILs around DCIS ducts, in up to 20 ducts per case, and then the total number of TILs was divided by the number of DCIS ducts resulting in the mean TILs number. IBM-SPSS statistical software 22.0 (SPSS, Chicago, IL, USA) was used to analyse our findings. Inter-observer degree of agreement was assessed through inter-class correlation coefficient for continuous data and Kappa test for categorical groups. Linear correlation between TILs densities within different topographic areas and touching TILs was analysed using Spearman's test. Kaplan–Meier curves and log-rank test were used for univariate survival analyses while Cox regression model was used for the multivariate analysis for patients treated with breast conserving surgery. Two tailed *p*-value <0.05 was considered as statistically significant.

RESULTS

This study included two sets of cases; a training set comprising 150 pure DCIS cases scored by three observers using 7 different scoring methods and a validation set (*n*=666 cases comprising 534 pure DCIS and 132 DCIS cases with co-existing invasive carcinoma) which was scored for touching and stromal TILs by 2 observers.

Training set

The concordance rate of TILs assessment using different methods between the three observers is summarised in **Supplementary Table 3**. The highest degree of inter-observer agreement was observed in touching TILs (Inter-cluster correlation coefficient=0.96) whereas the least concordance rate was found with the percentage of stromal TILs evaluation (Inter-cluster correlation coefficient=0.79) which is the recommended method in invasive breast carcinoma. Mean TILs counts within different topographic areas around DCIS are summarised in **Supplementary Table 4**. The mean count of TILs increased from 37 cells/DCIS duct at touching area to 482 cells/DCIS duct within 1mm distance. Largest hotspot density ranged from 20 to 6,000 cells while percentage of stromal TILs ranged from 1-65%. Mean TILs density increased by 4-fold between touching and within 0.2mm distance, while it increased only by 2-fold and 1.5-fold from 0.2mm to 0.5mm and from 0.5mm to 1mm distances, respectively. No cases with absent TILs were observed. Touching TILs showed positive linear correlation with TILs within the other topographic areas (**Supplementary Table 5**). The highest correlation was observed between touching TILs and TILs counted at 0.2mm distance (Spearman's correlation =0.854, $p=1x10^{-13}$). Counting TILs in farer areas away from the ducts showed less correlation with touching TILs.

Percentage of the cases with dense TILs were 53%, 57%, 59% and 65% for touching TILs and within 0.2mm, 0.5mm and 1mm from DCIS ducts, respectively. Dense hotspots were observed in 31% of cases while dense stromal TILs were observed in 49% of cases. Lymphoid follicles

were observed in 31 cases only (21%). The percentages of cases with dense and sparse TILs in context of each topographic area as well as the percentage of cases that developed local recurrence within each category and their association with recurrence free interval are summarised in **Table 2**. Examples of dense and sparse TILs are shown in **Figure 2.**

More than 60% of DCIS cases that developed local recurrence showed dense TILs irrespective of the topographic area used for assessment (Touching, 0.2mm distance, 0.5mm, 1mm and % stromal TILs). Only five cases (12%) from those developed local recurrence showed lymphoid follicle formations. Dense TILs infiltrate at different compartments showed statistically significant association with shorter recurrence free interval (p=4.7x10^{-6}, p=0.001, p=0.002 and p=0.001 for touching TILs and TILs within 0.2mm, 0.5mm and 1mm distances, respectively). Stromal TILs density was also associated with shorter recurrence free interval but with less statistical significance (p=0.02) than dense TILs defined in context of circumferential distances from the malignant ducts. Neither hotspots nor lymphoid follicles formation showed significant association with recurrence (p=0.09 and p=0.15, respectively). Results of univariate survival analysis are detailed in **Table 2**. **Supplementary Figure 2** shows Kaplan Meier survival curves for TILs density in different compartments and recurrence free interval.

Validation set

The concordance rate between the two observers evaluated touching TILs in the validation set was comparable to the training set (Inter-cluster correlation coefficient=0.95). Dichotomisation of touching TILs using 20 cells/DCIS duct as a cut-off point, showed that 239 cases (45%) had dense TILs infiltrate while 55% of cases showed sparse infiltrate.

Association of TILs density with other clinicopathological parameters

Dense touching TILs were associated with parameters characteristic of aggressive tumour behaviour including younger age, symptomatic presentation and larger tumour size. Only 8

cases (14%) of low nuclear grade lesions showed dense TILs in comparison with 33% and 54% of intermediate and high nuclear grade lesions, respectively (p=1.1x10^{-9}). Half of the cases (50%) associated with comedo necrosis harboured dense TILs while this was observed only in 32% of cases without comedo necrosis (p=0.00009). Approximately two thirds (69%) of DCIS cases associated with Paget's disease showed dense TILs infiltrate compared with 46% of patients without Paget's disease (p=0.025). 72% of oestrogen receptor negative DCIS showed dense TILs infiltrate while only 36% of oestrogen receptor positive cases showed high TILs density (p=1.04x10^{-10}).

Moreover, dense TILs were observed in 80% of DCIS mixed with invasion compared with 54% of pure grade-matched DCIS (χ^2=51.29, p=8.96x10^{-13}). This association was observed also when touching TILs was assessed as a continuous variable (p=3.14x10^{-12}) (**Supplementary Figure 3)**. The association between TILs density and various clinicopathological parameters are summarised in **Table 3**.

Outcome analysis

Ipsilateral local recurrence rate in the pure DCIS validation cohort was 11.8% (n=63). Recurrence occurred in 39/239 cases with dense TILs (62% from total recurrences), in comparison with 24/295 cases with sparse TILs (38% from total recurrences). Within patients treated with breast conserving surgery, local recurrence was reported in 24/80 cases with dense TILs (56%) compared to 19/139 cases with sparse infiltrate (44%). When assessed based on oestrogen receptor status, there were 22 recurrent events among oestrogen receptor positive DCIS with dense TILs (n=114) compared with 40 events among cases with sparse TILs (n=198). Among oestrogen receptor negative DCIS cases, 14/83 cases with dense TILs had local recurrence while only 1/33 cases with sparse TILs experienced a recurrence.

When TILs were classified into three categories defined as; absent/very scanty TILs (mean number of touching TILs/DCIS duct ≤5 cells), sparse TILs (from 6-20 cells/DCIS duct) and dense TILs (>20 cells/DCIS duct), recurrence occurred in 1/37 patients (3% of the group, 1.5% of the total recurrences), 23/258 patients (9% of the group, 36.5% of the total recurrences) and 39/239 (16% of the group, 62% of the total recurrences); respectively.

In univariate survival analysis using two-tier classification system of TILs, dense TILs showed statistically significant association with shorter recurrence free interval. This was observed when the analysis was conducted on the whole cohort (p=0.002), or confined to patients treated with breast conserving surgery (p=0.001). In context of oestrogen receptor status, dense TILs infiltrate was associated with shorter recurrence free interval in both oestrogen receptor positive (p=0.011) and oestrogen receptor negative (p=0.025) DCIS. Supporting the reproducibility of touching TILs evaluation in outcome prediction, univariate analysis using the second observer's scoring showed significant association with recurrence in the whole cohort and in patients treated with breast conserving surgery (p=0.0001 and p=0.004, respectively). Moreover, when the analysis was conducted using the average score between the two observers, it also showed significant association (p=0.002, p=0.01 for the whole cohort and for patients treated with breast conserving surgery, respectively).

Similar findings were observed when the three-tier classification system was used in the univariate analysis where dense TILs infiltrate was associated with shorter recurrence free interval in the whole cohort and breast conserving surgery treated patients (p=0.005 and p=0.004; respectively). The detailed results of univariate analyses between different clinicopathological parameters as well as TILs against recurrence free interval, and Kaplan-Meier curves are provided in **Table 4 and Figure 3,** respectively. Interestingly, there was no statistically significant association between recurrence free interval and TILs density when

assessed based on the percentage of overall stromal TILs (p=0.117 for the whole cohort and p=0.138 for patients treated with breast conserving surgery).

Cox regression multivariate model with other clinicopathological parameters including patient's age, tumour size, nuclear grade, presence of comedo necrosis, adjuvant radiotherapy and oestrogen receptor status showed that dense touching TILs is the only independent predictor of shorter recurrence free interval in patients treated with breast conserving surgery (*Hazard ratio* =2.6, 95% *Confident interval* = 1.41-4.7, p=0.002) (**Table 5**).

DISCUSSION

Currently, clinicopathological parameters used for DCIS risk stratification such as age, nuclear grade, tumour size and associated comedo necrosis are still insufficient to accurately estimate recurrence risk associated with DCIS (21, 22). TILs in DCIS are thought to have a role in tumour behaviour and progression; however, there are currently no consensus guidelines to evaluate TILs in clinical practice. Previous studies of TILs evaluation in DCIS neither used clear or uniform definition of the stromal area surrounding DCIS for TILs assessment nor identified cut-off points that can prognostically stratify DCIS. Some studies used the International Working TILs Group guidelines (14) with modification to DCIS and assessed percentage of stromal TILs at the stroma within the boundaries of the whole lesion (5, 19). *Pruneri* et.al (15) defined stromal TILs surrounding DCIS as those located at the area within two high power microscopic fields form the DCIS ducts and this method was adopted by other authors (17, 18, 23). These studies have reported lack of association between TILs density and DCIS recurrence. In the current study, TILs were assessed in a large cohort of DCIS with long-term follow-up data using various scoring methods to determine the most reproducible method that can additionally provide prognostic value. Prognostic stratification system has also been proposed to facilitate TILs application in DCIS in routine practice.

Assessment of TILs in the training set revealed that touching TILs is the optimal method for TILs scoring in DCIS. This conclusion was based on several criteria. Firstly, touching TILs had the highest concordance rate between the observers with or without prior methodology discussion. Secondly, they were positively correlated with TILs in the other topographic areas, hence representing TILs density within the whole lesion without requirement to assess TILs in wider areas around ducts which is time consuming. Thirdly, touching TILs assessment was the easiest and fastest method which can be performed without the need for accurate measurement of distance around ducts or adjustment of the scale and area's dimensions. Moreover,

assessment of touching TILs avoids confusion/variability in scoring cells within overlapping areas between adjacent ducts. Lastly, touching TILs showed the strongest significant association with other prognostic clinicopathological parameters and DCIS outcome (recurrence free interval).

In this study, touching TILs showed significant association with recurrence free interval in both training and validation cohorts as well as when the analysis was conducted using different observers' scores. Notably, dense touching TILs were associated with shorter recurrence free interval when the analysis was performed either in the whole cohort or when confined only to patients treated with breast conserving surgery. Importantly, touching TILs was an independent prognostic factor for DCIS recurrence in patients treated with breast conserving surgery regardless of other known determinants of tumour behaviour (24-27).

Interestingly, the percentage of overall stromal TILs assessed using the same methods for TILs evaluation described in the previous studies (5, 15, 17-19) showed not only the least concordance rates but also variability in association with the outcome. Unlike stromal TILs evaluation in invasive breast carcinoma, the low inter-observer and intra-observers' agreement may reflect the subjectivity of assessment of stromal TILs in DCIS, even with the use of predefined criteria of stromal area surrounding DCIS.

Studies of TILs in invasive breast carcinoma reported associations between TILs and better prognosis especially in triple negative breast cancer and supported their synergistic effect with chemotherapy (12, 13). By contrast, the current study demonstrates that dense TILs in DCIS are associated not only with other potential risk factors for aggressive DCIS behaviour but also with increased risk of tumour recurrence and progression. Supporting our results, *Pruneri et al*. reported that dense TILs are correlated with more aggressive DCIS (15). *Hendry et al*. have also reported dense TILs are associated with high grade, comedo type, oestrogen receptor

negative and Her2 positive DCIS lesions (17). Although both studies failed to find a significant association between TILs density and tumour outcome, this might be due to different assessment methodology. A recent molecular study showed that dense TILs were associated with aggressive DCIS. Copy number variation in DCIS with dense TILs was shown to be more profound than lesions with low TILs density which might indicate the higher immunoediting capability of DCIS with dense TILs making them more likely to progress and recur (17). In addition, dense TILs were associated with high DCIS Oncotype DX score (28), providing evidence to support our results that dense TILs are associated with poor outcomes in DCIS. Association between dense TILs infiltrate and poor prognosis has also been reported in oral, colonic, prostatic and pancreatic preinvasive neoplasia (29, 30).

Interestingly, dense TILs were associated with shorter recurrence free interval irrespective of oestrogen receptor expression in DCIS. This might indicate that the crosstalk between the immune microenvironment and tumour cells contributing to DCIS recurrence and/or progression is unrelated to oestrogen receptor pathway. Comparing TILs density in DCIS associated with invasion with pure DCIS indicates more TILs density in the former. This finding supports the hypothesised role of inflammatory cells in DCIS progression and aggressiveness (5, 16, 31, 32). TILs density assessment and reporting in the routine practice for DCIS diagnosed by core biopsy may provide a predictive factor for presence of invasion in these lesions as previously observed (33, 34).

Involvement of different immune cell subpopulations in DCIS behaviour has been speculated. Regulatory T cells (T-regs) play key roles in tumour evasion from the immune system (35). Homeostasis of the immune response in the body is regulated by T-regs, however a paradoxical action may occur through over-suppression of the immune cells attacking the tumour cells leading to tumour progression (36). It was shown that high grade DCIS lesions harbour higher percentage of FOXP3+ cells (19). Moreover, tumour cells surrounded by dense TILs may

produce some protective proteins to evade the host immune system. *Thompson et al*. have reported that DCIS with dense TILs show higher level of programmed cell death ligand 1 (PDL-1) positive tumour cells (5). This was supported with two similar studies that characterised immune microenvironment in DCIS (17, 19). Although the role of B-lymphocytes in tumour immunity and behaviour is unclear, a study on a small cohort of DCIS by *Miligy et al*., showed increased B lymphocytes infiltrate was associated with increased recurrence liability and with other poor outcome parameters (37).

In conclusion, using touching TILs as an assessment method for TILs in Haematoxylin and Eosin stained full-face DCIS sections is a reproducible and practical method to predict tumour behaviour and progression. Application of this method in routine practice would aid in risk-stratification of DCIS for improved, individualised management.

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Figures and Figures' legends:

Figure 1: Parameters of TILs assessment; A) Touching lymphocytes (x40) defined by lymphocytes that touch the basement membrane (BM) or are located within one lymphocyte cell thickness distance from basement membrane (yellow arrows); inset closer view for touching TILs, B) TILs assessment within 0.2mm, 0.5mm and 1.0mm distance from the involved ducts, and C) evaluation of hotspots (largest number of lymphoid cells aggregates within the lesion as shown in area surrounded by black dashed circle).

Figure 2: Touching TILs density around DCIS; A) Dense infiltrate where mean number of touching TILs is more than 20 cells/DCIS duct, B) High power view for dense touching TILs, and C) Sparse infiltrate where the mean number of touching TILs within the lesion is 20 cells or less/DCIS duct.

Figure 3: Kaplan-Meier Curves showing association of touching TILs density (Two-groups) with Recurrence free interval (in months); A) all cases irrespective of surgical treatment, B) cases treated with breast conserving surgery, (C and D) according to oestrogen Receptor status, as well as when TILs density defined as three-groups in; E) the whole cohort, and F) breast conserving surgery treated patients.

Table 1. Methods and parameters of Tumour Infiltrating Lymphocytes (TILs) assessment in DCIS

Methods for evaluation of TILs in DCIS
1- TILs were assessed in Haematoxylin and Eosin-stained sections. Only full-face sections from surgically excised specimen were assessed. Lesions diagnosed on core biopsy were not included. 2- One representative section (4μm), per patient, which has the largest tumour burden, was selected for TILs assessment. 3- All mononuclear inflammatory cells apart from polymorphonuclear leukocytes were counted. 4- TILs within the boundaries of the DCIS were assessed. TILs beyond the tumour limits, surrounding normal ducts/lobules, adjacent fatty tissue, lobular carcinoma in situ, regressive hyalinosis, crushed artefacts or sites of previous biopsy were excluded. 5- TILs within tumour cells (intra-tumour TILs) were not assessed. 6- TILs were assessed around all malignant ducts up to 20 ducts. For lesions with more than 20 malignant ducts, we assessed TILs surrounding 20 ducts (5 ducts from each quadrant of the lesion). 7- TILs were assessed around average sized ducts only (case specific). TILs around very large DCIS ducts such as mass forming papillary carcinoma, branching or confluent DCIS ducts or very small ducts such as terminal duct-lobular system involved by DCIS were excluded. 8- Any TILs infiltrating the ducts' circumference were considered. Overlapping TILs between adjacent ducts were counted once.
Parameters used for TILs assessment*
A- Estimation of stromal TILs (as previously published): • The stromal area was defined as the area surrounding the DCIS duct within two high power microscopic fields and used for evaluation of stromal TILs percentage (15, 17, 18). In cases with numerous involved ducts, an evaluation of the area surrounding the whole lesion was performed, and percentage of stromal TILs in the total stromal area of all DCIS involved ducts was determined (5, 15, 19). B-Estimation of periductal TILs (based on counting TILs around all DCIS duct profiles up to 20 ducts) 1- Evaluation of the mean number of TILs touching DCIS involved ducts (defined as TILs touching or within one lymphocyte cell thickness from ducts' basement membrane). 2- Evaluation of the mean number of TILs within 0.2mm distance from the ducts 3- Evaluation of mean number of TILs within 0.5mm distance from the ducts 4- Evaluation of mean number of TILs with 1.0mm distance from the ducts 5- Evaluation of the TILs hotspot defined by largest number of lymphoid aggregates directly surrounding or located between DCIS ducts within the boundaries of the lesion 6- Evaluation of lymphoid follicles formations with reactive germinal centres in the stroma directly surrounding or located between DCIS ducts within the boundaries of the lesion.

***All parameters were assessed in the training set while touching and stromal TILs assessment were conducted to validation set.**

Table 2: Frequency of TILs density in different topographic areas and their association with outcome in terms of recurrence free interval in the training set

Parameter	TILs density (mean number of TILs/DCIS duct as cut-off)	Number of cases (%)	Recurrence (%)	*p*-value (Log rank test)
Touching TILs (Two-tier system)	**Sparse** (≤ 20) Dense (>20)	70 (47) 80 (53)	6 (15) 35 (85)	**$4.7x10^{-6}$**
Touching TILs (Three-tier system)	Absent/very scanty (≤5) Sparse (6-20) Dense (>20)	7 (5) 63 (42) 80 (53)	0 (0) 6 (15) 35 (85)	**$2.0x10^{-5}$**
TILs at 0.2mm distance	Sparse (≤ 60) Dense (> 60)	64 (43) 86 (57)	8 (20) 33 (80)	**0.001**
TILs at 0.5mm distance	Sparse (≤ 100) Dense (> 100)	61 (41) 89 (59)	8 (20) 33 (80)	**0.002**
TILs at 1.0mm distance	Sparse (≤ 120) Dense (> 120)	53 (35) 97 (65)	6 (15) 35 (85)	**0.001**
Hotspot	Sparse (≤ 1200) Dense (>1200)	103 (69) 47 (31)	33 (80) 8 (20)	0.089
Lymphoid follicles	No Yes	119 (79) 31 (21)	36 (88) 5 (12)	0.150
Percentage of Stromal TILs	Sparse (≤ 5%) Dense (>5%)	77 (51) 73 (49)	15 (37) 26 (63)	**0.020**

Table 3: Correlation between TILs density (based on mean number of touching TILs with cut-off 20 cells/DCIS duct) and clinicopathological parameters in the validation set

Parameter	TILs density		Chi square (χ^2)	*p* value
	Dense N (%)	Sparse N (%)		
Patient age				
≤50 years	71 (52)	65 (48)	4.09	**0.043**
>50 years	168 (42)	230 (58)		
Presentation				
Screening	114 (41)	166 (59)	3.89	**0.049**
Symptomatic	125 (49)	129 (51)		
DCIS Size				
≤20mm	85 (37)	147 (63)	10.66	**0.001**
>20mm	152 (51)	147 (49)		
DCIS Grade				
Low	8 (14)	49 (86)	41.18	**$1.1x10^{-9}$**
Moderate	44 (33)	88 (67)		
High	187 (54)	158 (46)		
Comedo type necrosis				
Yes	185 (50)	182 (50)	15.16	**0.00009**
NO	54 (32)	113 (68)		
Associated Paget's disease				
Yes	18 (69)	8 (31)	5.03	**0.025**
No	156 (46)	180 (54)		
Oestrogen receptor status				
Negative	83 (72)	33 (28)	41.73	**$1.04x10^{-10}$**
Positive	114 (36)	198 (64)		
Type of DCIS				
Mixed with invasion	105 (80)	27 (20)	51.29	**$8.96x10^{-13}$**
Pure DCIS	239 (45)	295 (55)		

Table 4: Univariate association of TILs and other clinicopathological parameters with recurrence free interval

Parameter	Recurrence (%)	*p*-value
Patient age		
≤50 years	21 (33)	**0.042**
>50 years	42 (67)	
Presentation		
Symptomatic	36 (57)	0.110
Screening	27 (43)	
DCIS Size		
≤20mm	40 (65)	**0.002**
>20mm	22 (35)	
DCIS Grade		
Low	3 (5)	0.409
Intermediate	15 (24)	
High	45 (71)	
Comedo type necrosis		
No	25 (40)	0.191
Yes	38 (60)	
Associated Paget's disease		
No	32 (89)	0.552
Yes	4 (11)	
Final Operation type		
Mastectomy	20 (32)	**$1.1x10^{-6}$**
Breast conserving surgery	43 (68)	
Radiotherapy		
No	55 (87)	0.714
Yes	8 (13)	
Oestrogen receptor status		
Negative	15 (27)	0.992
Positive	40 (73)	
TILs density (Touching TILs) Two-tier system*		
Sparse	24 (38)	**0.002**
Dense	39 (62)	
TILs density (Touching TILs) Three-tier system*	1 (1.5)	
Absent/Very scanty	23 (36.5)	**0.005**
Sparse	39 (62)	
Dense		
TILs density (stromal TILs)		
Sparse	47 (75)	0.117
Dense	16 (25)	

***Classifications (Definitions) of various touching TILs densities**

- Two-tier (Two-groups) classified as **Sparse** where the mean number of TILs within the lesion is 20 cells/DCIS duct or less and **Dense** where the mean number of TILs within the lesion is more than 20 cells/DCIS duct.
- Three-tier (Three-groups) classified as **Absent/very scanty** where the mean number of TILs within the lesion in 0-5 cells/DCIS duct, **Sparse** where the mean number of TILs within the lesion is 6-20 cells/DCIS duct and **Dense** where the mean number of TILs within the lesion is more than 20 cells/DCIS duct.

Table 5: Multivariate analysis results (Cox regression model) *

Parameters	Hazard ratio (*HR*)	95.0% confident interval (*CI*)		Significance *p*-value
		Lower	Upper	
Patient Age	0.599	0.318	1.127	0.112
DCIS Size	0.755	0.403	1.415	0.381
DCIS Grade	1.531	0.899	2.608	0.117
Comedo Type necrosis	0.639	0.341	1.197	0.162
Radiotherapy	0.423	0.172	1.037	0.060
Oestrogen receptor status	0.934	0.477	1.829	0.841
Dense TILs	2.573	1.412	4.690	**0.002**

***Performed for patients treated with Breast conservative surgery only.**

Figure 1

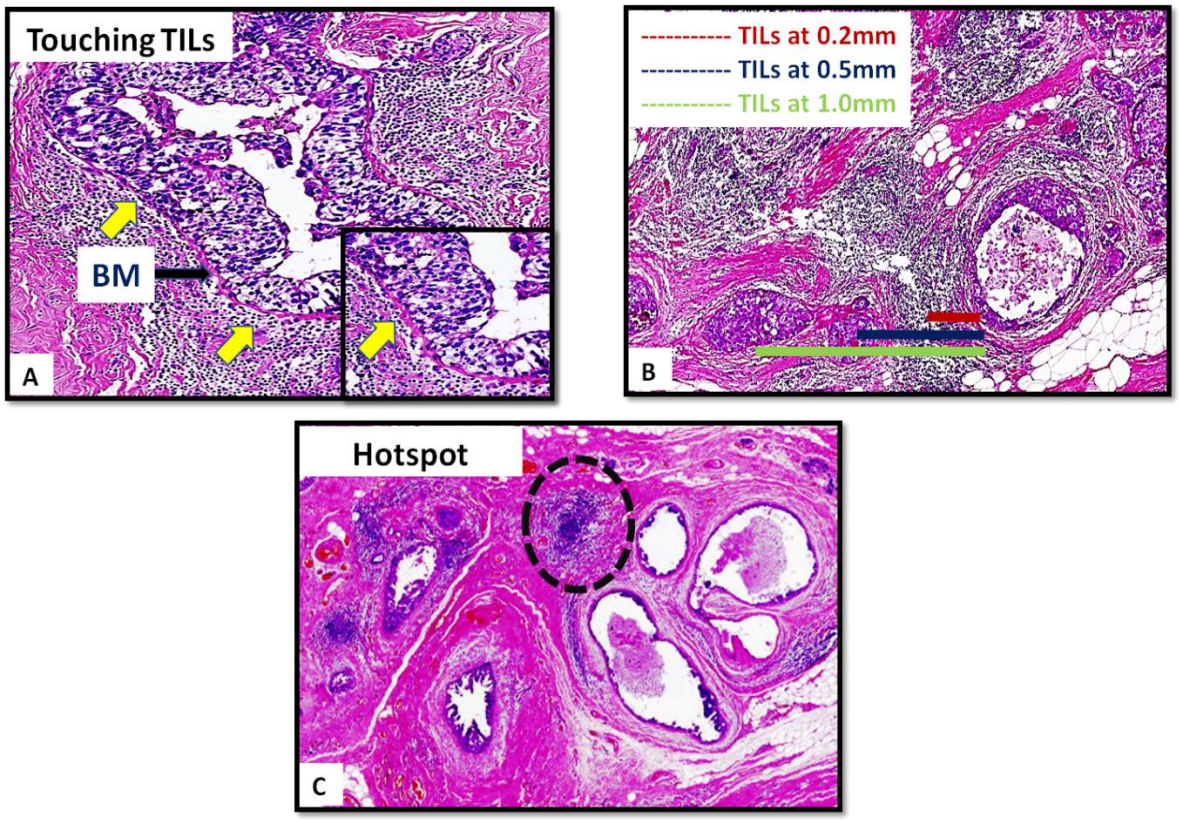

Figure 1: Parameters of TILs assessment; A) Touching lymphocytes (x40) defined by lymphocytes that touch the basement membrane (BM) or are located within one lymphocyte cell thickness distance from basement membrane (yellow arrows); inset closer view for touching TILs, B) TILs assessment within 0.2mm, 0.5mm and 1.0mm distance from the involved ducts, and C) evaluation of hotspots (largest number of lymphoid cells aggregates within the lesion as shown in area surrounded by black dashed circle).

Figure 2

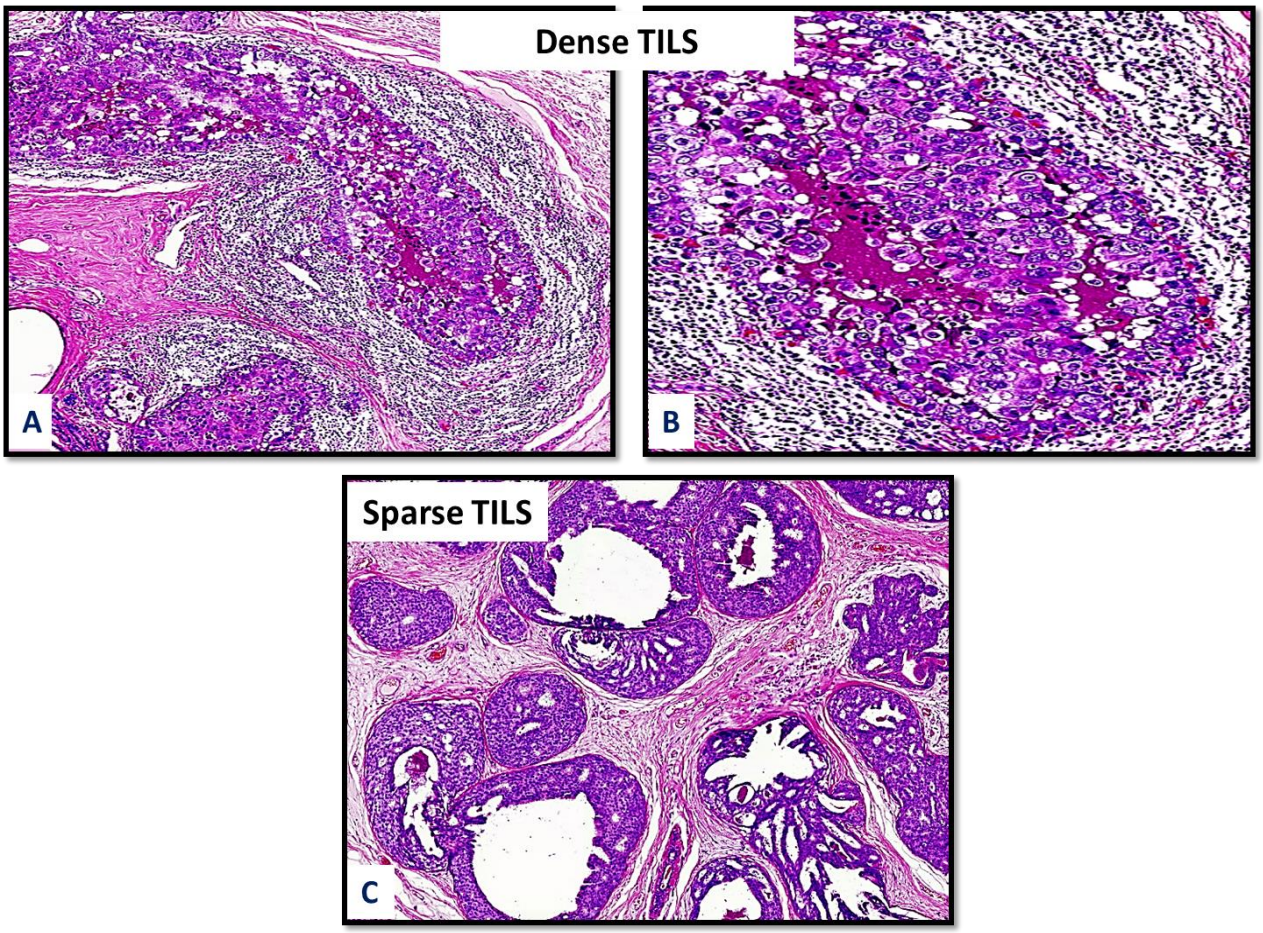

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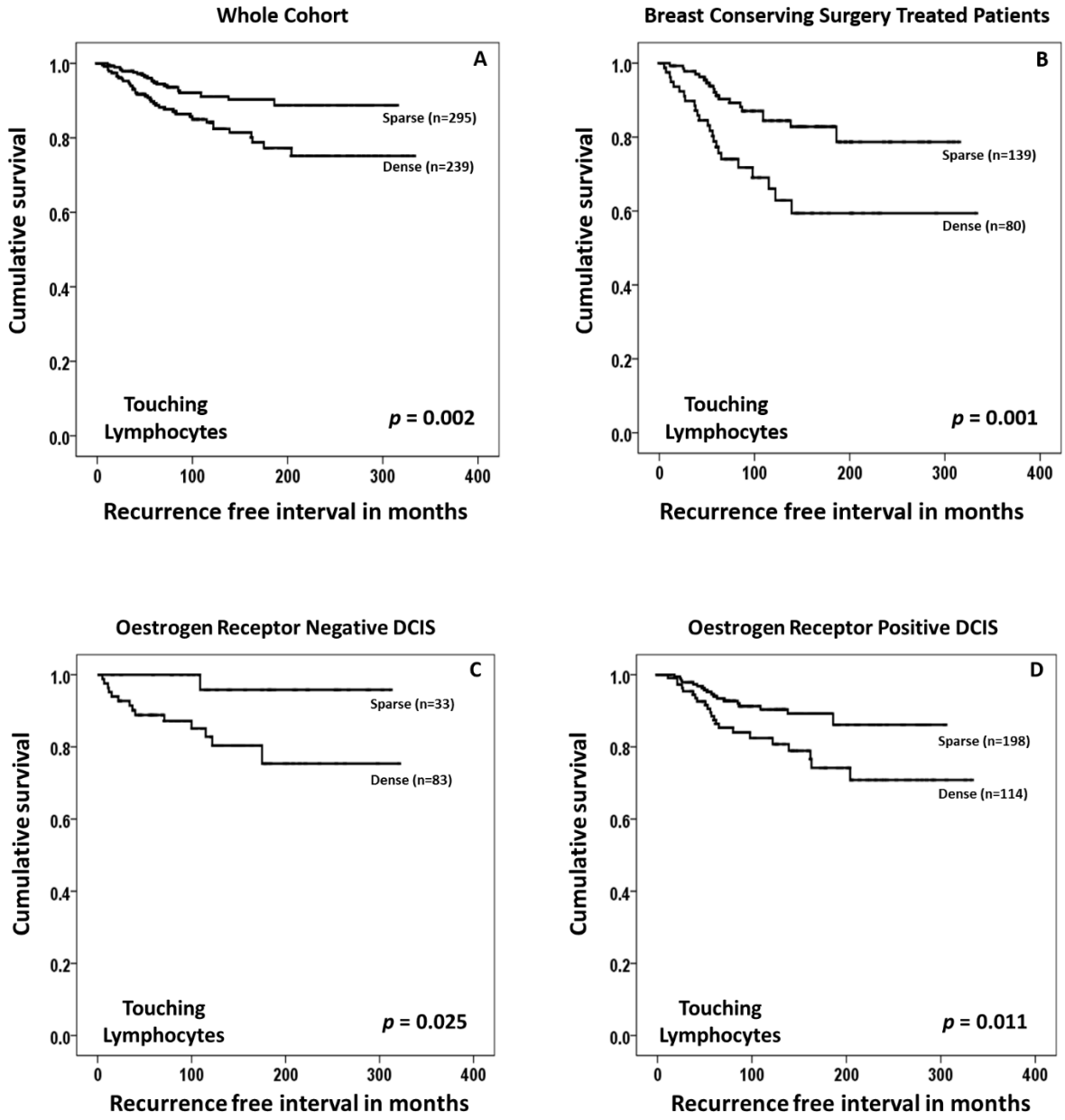
Figure 3


Whole Cohort
A
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
Sparse (n=295)
Dense (n=239)
Touching Lymphocytes
p = 0.002
0
100
200
300
400
Recurrence free interval in months
Breast Conserving Surgery Treated Patients
B
Cumulative survival
Sparse (n=139)
Dense (n=80)
Touching Lymphocytes
p = 0.001
Recurrence free interval in months
Oestrogen Receptor Negative DCIS
C
Cumulative survival
Sparse (n=33)
Dense (n=83)
Touching Lymphocytes
p = 0.025
Recurrence free interval in months
Oestrogen Receptor Positive DCIS
D
Cumulative survival
Sparse (n=198)
Dense (n=114)
Touching Lymphocytes
p = 0.011
Recurrence free interval in months

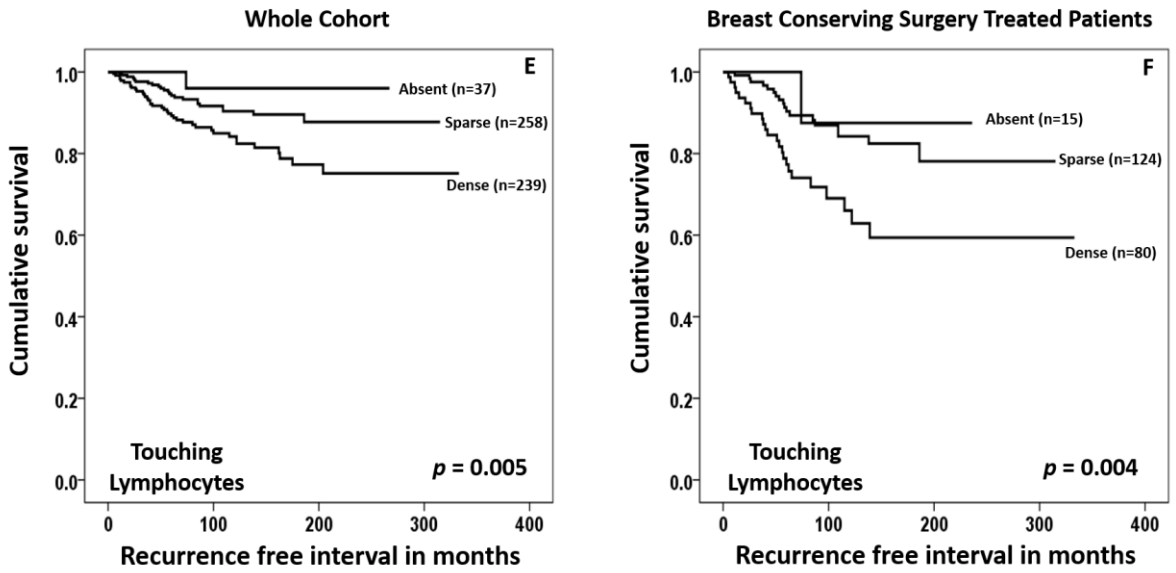
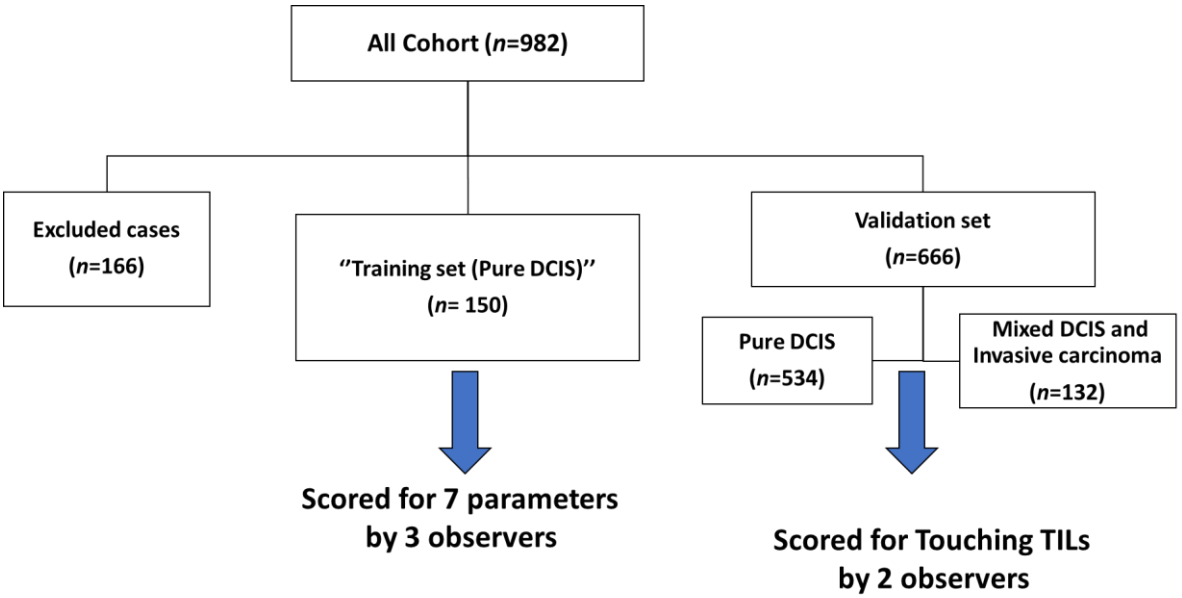
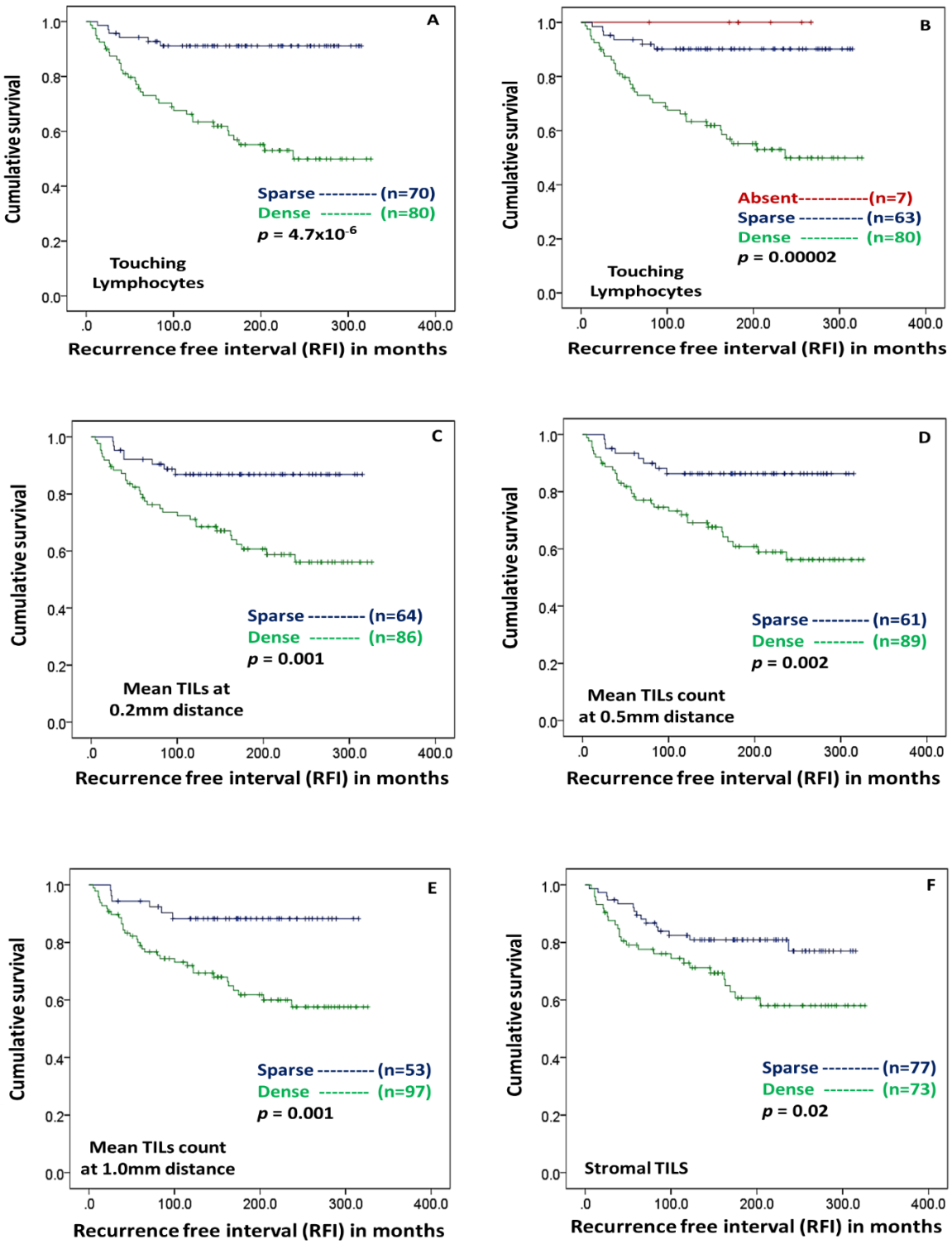


Figure 3: Kaplan-Meier Curves showing association of touching TILs density (Two-groups) with recurrence free interval (in months); A) all cases irrespective of surgical treatment, B) cases treated with breast conserving surgery, (C and D) according to oestrogen Receptor status, as well as when TILs density defined as three-groups in; E) the whole cohort, and F) breast conserving surgery treated patients.

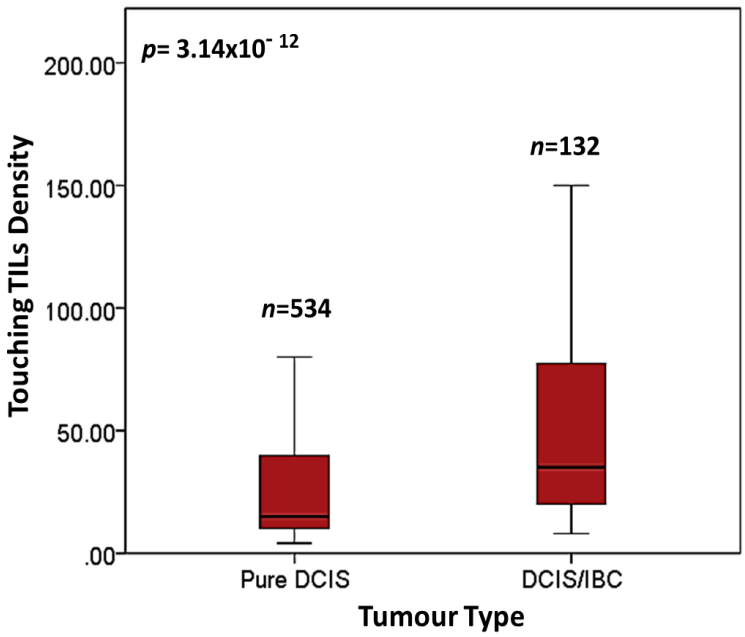
Supplementary Figures



Supplementary Figure 1: Algorithm for Study Cohort



Supplementary Figure 2: Kaplan-Meier curves showing association between TILs density within different topographic areas and recurrence free interval (RFI) (in months) for training set; a) Touching TILs (two-tier), b) Touching TILs (three-tier), c) TILs within 0.2mm distance, d) TILs within 0.5mm distance, e) TILs within 1mm distance, and f) Stromal TILs



Supplementary Figure 3: Box and Plot shows difference in TILs density between pure DCIS and DCIS associated with invasive breast cancer (IBC) (*p*-value conducted from Mann-Whitney test).

Supplementary Tables

Supplementary Table 1: Clinicopathological characteristics of the cases in the training and validation sets

Parameter	Training set (*n*=150) Number of cases (%)	Validation set (*n*=534) Number of cases (%)
Patient Age		
≤ 50 years	0 (0)	136 (25)
> 50 years	150 (100)	398 (75)
Presentation		
Symptomatic	78 (52)	254 (48)
Screening	72 (48)	280 (52)
DCIS Size		
≤ 20mm	78 (52)	232 (43)
>20mm	67 (45)	299 (56)
DCIS Grade		
Low	11 (8)	57 (11)
Intermediate	20 (13)	132 (25)
High	119 (79)	345 (64)
Comedo type necrosis		
No	45 (30)	167 (31)
Yes	105 (70)	367 (69)
Associated Paget's disease		
No	106 (71)	336 (63)
Yes	6 (4)	26 (5)
N/A	38 (25)	172 (32)
Estrogen receptor status		
Positive	80 (64)	312 (73)
Negative	45 (36)	116 (27)
Final operation type*		
Mastectomy	86 (57)	314 (59)
Breast conserving surgery	64 (43)	219 (41)
Radiotherapy**		
No	127 (85)	139 (64)
Yes	23 (15)	80 (36)
Recurrence		
No	109 (73)	471 (88)
Yes	41 (27)	63 (12)

N/A: Data not available

***One case in the validation set had no surgical data.**

****For patients treated with breast conserving surgery.**

Supplementary Table 2: Cut-off points of mean TILs count/DCIS duct as generated by the X-tile software based on association with patient's outcome (recurrence free interval).

Parameter	Definitions of TILs density	
	Sparse	**Dense**
Mean count of Touching TILs *	≤20 cells	>20 cells
Mean count of TILs within 0.2mm distance *	≤60 cells	>60 cells
Mean count of TILs within 0.5mm distance *	≤100 cells	>100 cells
Mean count of TILs within 1mm distance *	≤120 cells	>120 cells
Hotspots	Largest hotspot within the lesion's boundaries compromising 1200 cells or less	Largest hotspot within the lesion's boundaries compromising more than 1200 cells
Stromal TILs	TILs represent 5% or less from the total surrounding stromal area	TILs represent more than 5% of total surrounding stromal area

***Represent mean number of TILs count within a specified area/DCIS duct.**

Supplementary Table 3: TILs Inter-rater concordance results in the training set

Parameter	Intra-cluster correlation coefficient between all observers
Mean TILs count Touching ducts	0.96
Mean TILs count within 0.2mm distance	0.89
Mean TILs count within 0.5mm distance	0.92
Mean TILs count within 1mm distance	0.90
Hotspots	0.89
Lymphoid follicles*	0.86
Stromal TILs percentage	0.79

***Performed using Kappa test between first and second observers.**

Supplementary Table 4: Results of TILs assessment in the training set

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

****Defined as largest number of lymphoid cell aggregates within the boundaries of the DCIS.**

Supplementary Table 5: Correlation between Touching TILs scores and TILs scores at other topographic areas (training set)

Parameter	**Spearman's correlation**	***p* value**
Mean TILs count within 0.2mm distance	0.85	**1.0×10^{-13}**
Mean TILs count within 0.5mm distance	0.75	**1.0×10^{-13}**
Mean TILs count within 1mm distance	0.69	**1.0×10^{-13}**
Hotspot	0.57	**1.1×10^{-13}**
Percentage of stromal TILs	0.69	**1.0×10^{-13}**