Optimized tumour infiltrating lymphocyte assessment for triple negative breast cancer prognostics

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A B S T R A C T
The tumour microenvironment has been shown to be a valuable source of prognostic information for different cancer types. This holds in particular for triple negative breast cancer (TNBC), a breast cancer subtype for which currently no prognostic biomarkers are established. Although different methods to assess tumour infiltrating lymphocytes (TILs) have been published, it remains unclear which method (marker, region) yields the most optimal prognostic information. In addition, to date, no objective TILs assessment methods are available.

For this proof of concept study, a subset of our previously described TNBC cohort (n = 94) was stained for CD3, CD8 and FOXP3 using multiplex immunohistochemistry and subsequently imaged by a multispectral imaging system. Advanced whole-slide image analysis algorithms, including convolutional neural networks (CNN) were used to register unmixed multispectral images and corresponding H&E sections, to segment the different tissue compartments (tumour, stroma) and to detect all individual positive lymphocytes. Densities of positive lymphocytes were analysed in different regions within the tumour and its neighbouring environment and correlated to relapse free survival (RFS) and overall survival (OS).

We found that for all TILs markers the presence of a high density of positive cells correlated with an improved survival. None of the TILs markers was superior to the others. The results of TILs assessment in the various regions did not show marked differences between each other.

The negative correlation between TILs and survival in our cohort are in line with previous studies. Our results provide directions for optimizing TILs assessment methodology.

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1. Introduction

Breast cancer is the most common type of cancer in women in the world, showing a still increasing incidence. In the Netherlands, an increase in breast cancer incidence from about 8000 women in 1990 to nearly 15,000 in 2015 was observed [1]. To support treatment planning and obtain prognostic information, all invasive breast tumours are routinely classified into histological subtypes according to the WHO classification [2] and assigned a histological grade using the Nottingham grading system [3]. In addition, for prognostic and predictive purposes, all invasive breast tumours are tested for expression of the oestrogen receptor (ER) and progesterone receptor (PR) as well as for overexpression of the human epidermal growth factor receptor 2 (HER2).

Approximately 15% of all breast cancers test negative for these three receptors [4], hence referred to as triple negative breast cancers (TNBC). TNBC has a high incidence among young women [5] and the course of the disease is characterized by a high risk of recurrence in the first three years after initial diagnosis [6]. About one fourth of TNBC patients will develop a recurrence after which the median survival is only 9–13 months [7,8]. Because of the high...
CD8+ phocytes is associated with improved survival, particularly for prognosis in TNBC [14]. The presence of highly diverse assessment methods, the general conclusion of these studies is that increased TIL density is associated with a better prognosis in TNBC [14]. The presence of specific subsets of lymphocytes is associated with improved survival, particularly for CD8+ [15,16] and FOXP3+ [14] lymphocytes. However, an increase of the total amount of mononuclear cells was found beneficial for survival [18]. Attempts to standardize individual evaluation methods for TILs in TNBC are ongoing [19] but do not consider, in a structured manner, which TIL assessment method yields the strongest prognostic information for breast cancer in general or TNBC in particular. To enable clinical validation for this promising biomarker, the most optimal assessment approach in a prognostic context should be determined. This is an important prerequisite to generate maximum patient benefit [20].

The present study aimed to establish the optimal assessment method for immunohistochemically stained TILs in TNBC in relation to patient outcome. To allow accurate identification of the optimal immunohistochemical marker, multiplex immunohistochemistry (miHC) in combination with spectral image acquisition was used [21]. Tyramide signal amplification (TSA) was used for multiplex immunohistochemistry. Different subsets of TILs (CD8, FOXP3 and CD3 as an overall T-cell marker) in different areas of the tumour and its environment were studied. Advanced image analysis methods based on deep learning were used to generate objective and highly reproducible TIL assessment methods. For this study, we used a subset of a previous established TNBC cohort [8] as discovery cohort.

2. Materials and methods

2.1. Patients and tissue selections

As part of a previous study, a multicentre, retrospective cohort of stage I-III, non-neoadjuvantly treated TNBC from Eastern Netherlands was assembled using the Netherlands Comprehensive Cancer Registry (IKNL; a nationwide registry in which all malignancies in the Netherlands are registered) [5]. This resulted in a database with a total of 811 patients who were diagnosed between the years 2006 and 2014 in an academic hospital (Radboud University Medical Center (Radboudumc), Nijmegen) or in a general hospital (Canisius Wilhelmina Hospital, Nijmegen; Jeroen Bosch Hospital, ’s-Hertogenbosch; Bernhoven Hospital, Uden; Hospital Pantein, Boxmeer). Because of the laborious analysis methods used in the present study, a subcohort of 100 patients (Fig. 1) was selected from the previously established TNBC cohort (see further under statistical analysis).

One representative tissue block per tumour was selected based on inspection of archival tissue sections for the presence of invasive cancer with a transition from tumour to normal breast tissue being present [19]. All tumours underwent central histopathological revision for histological subtype and grade (MCAB, PB) according to the prevailing guidelines [23,24]. Both reviewers were blinded for clinicopathological variables and outcome measures (RFS and OS). Clinical and follow up data were retrieved from the Netherlands Comprehensive Cancer Registry (overall survival; OS) and from local patient files (relapse free survival; RFS).

RFS was defined as the time span between the date of diagnosis of TNBC via core needle biopsy/fin needle aspiration and the date of clinically and/or pathologically detected recurrence of TNBC. Hormonal receptor and/or HER2 positive breast cancer occurring after TNBC diagnosis were regarded as a new primary tumour and not as TNBC recurrence. Patients who did not develop a recurrence were censored at the date of last follow up. OS was defined as the interval between date of diagnosis of TNBC and date of death or moment of last follow up. The REMARK guidelines for reporting tumour marker prognostic studies were used [8] and the study was conducted according to the Standards for Reporting of Diagnostic Accuracy (STARD) guideline [22].

2.2. Ethical approval

The institutional review board of the Radboudumc waived the requirement for ethical approval (case number 2015–1711). The Dutch codes of conduct for the use of data in health research [23] and for dealing responsibly with human tissue in the context of health research [24] were adhered to.

2.3. Multiplex IHC staining procedure

Of every TNBC tissue block, one section was cut to perform the multiplex staining and one for a conventional H&E staining. A panel of six antibodies was composed for the multiplex stainings, from which the results of three antibodies were used in this study (CD3, CD8, FOXP3). The most optimal order and dilutions of antibodies were tested on formalin fixed paraffin embedded (FFPE) tissue sections of a randomly selected invasive ductal breast cancer from the Radboudumc which was not part of the TNBC cohort. miHC was optimized by performing a duplex staining consisting of FOXP3 (Opal 620) with CD8 (Opal 650), followed by a triplex staining with the addition of CD3 (Opal 570). All miHC experiments were performed by repeating staining cycles in series, with microwave treatments in between each cycle and at the end of the staining, protein blocking was performed using TBS-Tween 1% BSA (A7034, Sigma-Aldrich). Primary antibodies FOXP3 (clone 236A/E7; Ebioscience), CD8 (clone C8/144B; DAKO) and CD3 (clone SP7; Thermo Fisher) were incubated for 1 h at room temperature. After
several times rinsing in PBS/BSA/Tween, slides were incubated with BrightVision poly-HRP-anti-Mouse/Rabbit/Rat IgG (DPVO999HRP; ImmunoLogic) at room temperature for 30 min. The Opal seven-colour IHC Kit (NEL797B001KT, PerkinElmer) which contains fluorophores DAPI, Opal 520, Opal 540, Opal 570, Opal 620, Opal 650 and Opal 690 (NEL703001KT; PerkinElmer) was used to visualize mHIC results. In order to remove the antibody/mHIC complex, a microwave treatment with Tris-EDTA buffer (pH 9) was performed between each staining cycle. Single stain slides were finished with microwave treatment and counterstained with DAPI for 5 min and were enclosed in Fluoromount-G (010010; SouthernBiotech). Of every tissue block one extra slide was cut, directly adjacent to the

Fig. 1. Flow diagram showing the initial number of 811 triple negative breast cancer patients as retrieved from the Dutch Cancer Registry from which 100 patients were selected. During the process of collecting archival tissue blocks and detailed follow up information, 6 patients were excluded from the study. Numbers of excluded patients are listed per factor in descending order.

Fig. 2. Schematic representation of a part of the TILs assessment work flow. Upper left: for each case a conventional H&E and a mHIC stained section were made and imaged. The different channels in the mHIC image were separated and in the individual images the positive cells were detected for each marker (blue: CD3; red: CD8; yellow: FOXP3). In the H&E image, the epithelial tumour and stroma were segmented for the intratumoural measures (ITA, ITT, ITS). Finally, the mHIC and H&E images were registered which enabled to calculate the density of positive lymphocytes in different areas.
slide used for mIHC, and stained for H&E in the Radboudumc pathology department according to routine practice.

2.5. Imaging and image co-registration

Resulting mIHC slides were imaged using the Vectra spectral imaging system version 2.0.7 (PerkinElmer, Waltham, MA). The Vectra system can acquire mIHC images by recording images at a range of wavelengths, using a spectral camera. Subsequently, the iForm Advanced Image Analysis software (iForm 2.1.1; PerkinElmer) was used to apply spectral unmixing, to reproduce the individual IHC signals in the mIHC staining (Fig. 2, upper part). To be able to perform spectral unmixing, a library of reference signals was built on the basis of each individual single staining of the primary antibodies used in the mIHC and a single staining of DAPI. For background removal, an unstained slide was imaged. Corresponding H&E sections were scanned on a Pannoramic 250 Flash II slide scanner (3DHistech, Hungary) at a spatial resolution of 0.12 μm/pixel. Resulting pairs of H&E and mIHC whole slide images (WSI) were co-registered (i.e. images were aligned such that there is a pixel level correspondence between two images) based on a previous developed algorithm [25,26], which consists of nonlinear registration of whole-slide images.

2.6. Image analysis

TIL densities were assessed in different areas of the tumour and its environment, comparable to methods published previously [19,27,28]. To facilitate such analyses, we used H&E WSIs to identify different components within the invasive tumour (stroma, malignant epithelium) and its neighbouring environment (invasive margin) in which TIL densities were measured. In every digitized H&E slide, the invasive tumour was outlined by an experienced observer (MCAB) (Fig. 3A) who was blinded for clinicopathological variables and outcome measures. To allow for TIL measurements in the invasive margin of the tumours, the annotated tumour bulk outline was dilated on both sides by either 500 μm or 1 mm, resulting in an effective invasive margin of 1 mm and 2 mm, respectively (Fig. 3B and C).

Within the manually outlined tumour bulk area in the H&E section, we applied a previously developed epithelium-stroma segmentation algorithm [29]. This deep learning algorithm was trained on a large number of manually annotated breast cancer cases and is capable of differentiating between regions consisting of...
epithelial, stromal and fatty tissue. Applying the algorithm resulted in a subdivision of pixels within the tumour bulk as either belonging to the stroma or epithelium class (Fig. 2, lower part, and Fig. 3D). The above described delineation of the tumour and segmentation of the intratumoural area resulted in the following areas in which the TIL density was assessed for CD3, CD8 and FOXP3, as well as ratios between these markers: density of positive cells in the intratumoural area (ITA); density of positive cells in the epithelial tumour regions within the intratumoural area (ITT); density of positive cells in the stromal regions within the intratumoural area (ITS); density of positive cells in a margin of 500 μm on both sides of the boundary of the tumour area (IM1); density of positive cells in a margin of 1 mm on both sides of the boundary of the tumour area (IM2); ratio between the density of positive cells in the 500 μm inner and outer boundary of the tumour area (IM110); ratio between the density of positive cells in the 1 mm inner and outer boundary of the tumour area (IM210).

A previously developed convolutional neural network to detect individual lymphocytes, positive for the respective markers [30], was applied on the unmixed images (Fig. 2, upper part). Finally, to enable calculation of the density of positive lymphocytes in different areas of the tumour and its neighbouring environment, the detections of positive lymphocytes were projected on the H&E images (Fig. 2, lower right image).

2.7. Statistical analysis

For the present study, we selected 100 cases of a previously described cohort as follows [8]. After ranking all patients by incidence date (date of diagnosis with TNBC by either histology or cytology), the first 100 patients were included. To study representativeness of the sub-cohort used in this study against the total TNBC cohort from which cases were taken, the distribution of clinicopathological variables and the of number of events were compared between these two using cross tabulation. No significant differences were observed (p > 0.05; data not shown) using Pearson Chi-Square test. Also, independent samples T tests showed no significant differences (p > 0.05; data not shown) in mean time to events between the cohort used in this study and the total TNBC cohort.

Univariable Cox regression analysis with bootstrapping (5000 bootstraps) was performed for all TIL measures with RFS and OS as primary outcomes. To correct for tumour size, TILs were expressed per 1 mm² area. The number of CD3 and CD8 positive lymphocytes were evaluated per increment of 100 positive cells. Because of the low number of FOXP3 positive cells, this marker was evaluated per increment of 10 positive cells. TIL ratio measures were calculated based on the absolute number of positive cells. All TIL measures were analysed as continuous variables. For all analyses, confidence intervals were set at the 95% level. The baseline alpha level to test for statistical significance was 0.05. This baseline alpha value was not adjusted for multiple comparisons as the aim of this study was comparison of different assessment methodologies without studying the absolute prognostic value of the assessed TIL features. To investigate the prognostic value of TILs in the intratumoural areas more closely, Kaplan Meier curves were produced and Log rank tests were performed. For this, TIL count per increment of 100 positive cells (CD3 and CD8) and per increment of 10 positive cells (FOXP3) per 1 mm² area was dichotomized using the median value of the individual TIL markers as a cut-off. All analyses were performed using statistical software SPSS (version 25.0; IBM, Chicago, USA).

3. Results

3.1. Patient demographics and tumour characteristics

The Netherlands Comprehensive Cancer Registry provided a cohort of 811 patients who underwent surgery for primary breast cancer between 2006 and 2014 in the 5 participating hospitals from Eastern Netherlands. After ranking patients by incidence date in chronological order, we selected the first one hundred patients for this study. After applying the exclusion criteria and retrieval of archival tissue blocks, 94 tumours remained (Fig. 1) which were stained using miHC. Table 1 provides an overview of patient and tumour characteristics in the TNBC cohort. The majority of patients were 50 years or older at the time of diagnosis (61.7%). Less than half of the tumours were smaller than 2 cm (43.6%) in size. The prevailing histological subtype was invasive carcinoma of no special type (invasive carcinoma NST) (92.6%). About one in four patients developed a recurrence of TNBC (23.4%) and one in three patients deceased during the follow up period (33.0%). For the patients who were confronted with a recurrence, the median time...
for developing a clinically detected recurrence was 27.0 months after primary TNBC diagnosis.

### 3.2. TIL assessment

For every patient, the digitized H&E tissue section was co-registered (i.e. images were aligned such that there was a pixel level correspondence between two images) with the digitized mIHC tissue section (Fig. 2). Fig. 3 shows the different regions and measures for the TIL assessment. Different tumours showed a marked different immune response (two examples shown in Figs. 4 and 5).

#### 3.3. Effect of individual TIL markers

Table 2 shows that the presence of CD3, CD8 and FOXP3 all correlated with an improved survival. CD3 seemed to be slightly more prognostic than FOXP3 and CD8, in terms of lower p-values, with CD8 being least favourable. Generally, correlation between TILs and relapse free survival was weaker than with overall survival. In contrast to the use of individual markers, the ratios between markers generally correlated poorly with prognosis. Supplementary table 1 shows p-values of the Log rank tests of Kaplan Meier curves when analysing TIL markers in the intratumoural areas (ITA, ITT, ITS) as a dichotomous variable using the median as a cut-off. For both RFS and OS, a binary classification of CD3 and CD8 counts yielded significant differences in survival between high and low TIL counts. In these analyses, a high TIL count correlated with improved survival.

#### 3.4. Effect of region of TIL assessment

Assessment of TILs intratumourally or in the invasive margin showed comparable correlation with survival, with some variations between the different markers: higher TIL densities in these areas were associated with a survival benefit (RFS HR varying between 0.777 (CD8, IM2) and 0.915 (CD3, ITS); OS HR varying between 0.722 (FOXP3, ITT) and 0.908 (CD3, ITA)). We did not observe clear differences between intratumoural measurement overall versus in tumour nests only or stroma only: for instance the HR for RFS for CD3 overall in the tumour is 0.899 while limiting the analysis to either tumour (HR is 0.888) or stroma (HR is 0.915) did not markedly change the HR. The same phenomenon is seen for CD8 and FOXP3 and the OS. Also, the size of the invasive margin in which TIL density was assessed (1 mm vs 2 mm) did not affect the prognostic value. In addition, the ratio between the inner and outer margin of the invasive margin (IM1IO, IM2IO) did not yield any relation with survival, except from FOXP3 for RFS.

### 4. Discussion

In this study, we explored different methods to objectively...
assess TILs in immunohistochemically stained sections of TNBC and to relate this to patient outcome. We studied three TIL markers (CD3, CD8 and FOXP3) in various regions within the tumour and in its adjacent environment. To objectively assess TILs, we used automated analysis based on deep learning, which can detect each individual positive lymphocyte. Our results showed that in general the abundance of TILs was negatively correlated with RFS and OS, with minor differences between used markers or definition of measurement region (e.g. intratumourally, tumour periphery, etc.). Using ratios between markers (e.g. the CD3/CD8 ratio) was found to be poorly prognostic and should therefore be avoided.

Characterization of the tumour associated immune infiltrate in breast cancer has gained widespread attention of the scientific community in the last decade. Many studies concluded that there is prognostic value of TILs for breast cancer. A uniform and well-established assessment method, however, is currently still lacking and a variety of methods to assess TILs have been published [19,27,28,34]. As a result, published studies are difficult to compare and guidance for larger, prospective validation studies is lacking. Typically, protocols for visual TIL assessment take into account feasibility (should be executable within a limited amount of time and budget, by a sufficiently trained human) and are often based on understanding of (hypothesized) underlying pathological processes. For instance, the International TIL Working Group (ITWG) has published several guidelines to assess TILs in breast cancer [19,32]. Their proposed method consists of visually estimating the percentage of mononuclear inflammatory cells in the intratumoural stromal area in an H&E section. The reason behind this is that using only H&E reduces costs (compared to more specific TIL markers applying IHC), but makes it difficult for humans to recognize TIL’s within tumour nests [32,33]. Therefore, only TILs in tumour stroma are counted. In particular for HER2 positive and TNBC, they correlated a higher stromal TIL presence to an improved survival [18,33], which, combined with the ease of use, makes it a strong candidate for inclusion in guidelines. However, a datadriven approach in which the optimal protocol for biomarker assessment against the desired outcome (e.g. overall survival) is studied might lead to stronger biomarkers. In the present study, computational pathology techniques were instrumental in enabling such analysis, as it is not possible to assess a larger number of quantitative descriptors in a large series of cases.

In the present study we focused on TNBC, applying the most common TIL markers using measuring approaches as published by different research groups [19,27,28]. We could, for instance, show that only including the tumour stroma and ignoring TILs within tumour nests, as prescribed by the ITWG, does not reduce prognostic value as compared to counting TILs within the entire tumour region. The finding that the choice of marker or measurement region has minor effect on prognostic value implies that in general TILs are a robust and reliable biomarker for this group of patients, as the marker is not overly sensitive to methodological variables.

Although the main focus of the Immunoscore consortium is

Fig. 5. Example of one of the TNBC tumours in the cohort with a sparse lymphocytic infiltrate. A: Overview of the tumour in H&E. The region in the magenta coloured rectangle is shown on a higher magnification in D. B: Result of CD3 staining of the tumour. For visualization purposes, the original immunofluorescent image is displayed in conventional bright field colours. The region in the magenta coloured rectangle is shown on a higher magnification in E. C: Heat map based on the density of CD3 positive lymphocytes with a circle shaped sliding window with an area of 1 mm². Yellow: intermediate density; green: low density. D: Selected area (magenta rectangle in A and B) at high magnification in which a sparse lymphocytic infiltrate is present. E: Selected area (magenta rectangle in B) at high magnification in which a low density of CD3 positive cells is present.
Table 2
Univariable analyses for relapse free survival and overall survival for the triple negative breast cancer cohort (5000 bootstraps).

| Relapse free survival HR (95% CI) | p value | Overall survival HR (95% CI) | p value |
|----------------------------------|---------|-----------------------------|---------|
| CD3 | | | |
| ITA | 0.899 (0.771–0.974) | 0.065 | 0.890 (0.782–0.959) | 0.019 |
| ITT | 0.888 (0.721–0.969) | 0.089 | 0.883 (0.758–0.956) | 0.032 |
| ITS | 0.915 (0.822–0.978) | 0.046 | 0.908 (0.829–0.964) | 0.011 |
| IM1 | 0.914 (0.847–0.969) | 0.008 | 0.901 (0.833–0.954) | 0.002 |
| IM2 | 0.888 (0.803–0.955) | 0.009 | 0.878 (0.787–0.946) | 0.004 |
| IM1IO | 0.461 (0.150–0.921) | 0.081 | 0.987 (0.423–1.153) | 0.969 |
| IM2IO | 0.597 (0.254–0.984) | 0.134 | 1.001 (0.555–1.408) | 0.995 |
| CD8 | | | |
| ITA | 0.826 (0.457–0.969) | 0.204 | 0.791 (0.515–0.922) | 0.073 |
| ITT | 0.824 (0.345–0.975) | 0.257 | 0.788 (0.464–0.926) | 0.115 |
| ITS | 0.826 (0.546–0.969) | 0.131 | 0.805 (0.592–0.930) | 0.041 |
| IM1 | 0.826 (0.651–0.942) | 0.036 | 0.801 (0.637–0.913) | 0.013 |
| IM2 | 0.777 (0.551–0.919) | 0.045 | 0.756 (0.557–0.894) | 0.014 |
| IM1IO | 0.508 (0.188–0.975) | 0.113 | 0.836 (0.356–1.344) | 0.596 |
| IM2IO | 0.654 (0.286–1.234) | 0.181 | 0.854 (0.483–1.198) | 0.491 |
| FOX3 | | | |
| ITA | 0.694 (0.416–0.837) | 0.020 | 0.728 (0.545–0.845) | 0.005 |
| ITT | 0.705 (0.396–0.958) | 0.090 | 0.722 (0.505–0.853) | 0.013 |
| ITS | 0.749 (0.517–0.968) | 0.019 | 0.781 (0.624–0.875) | 0.003 |
| IM1 | 0.846 (0.640–0.945) | 0.083 | 0.901 (0.694–0.982) | 0.157 |
| IM2 | 0.794 (0.549–0.909) | 0.061 | 0.860 (0.631–0.962) | 0.095 |
| IM1IO | 0.457 (0.196–0.753) | 0.031 | 0.997 (0.455–1.176) | 0.989 |
| IM2IO | 0.625 (0.356–0.850) | 0.032 | 1.005 (0.645–1.121) | 0.960 |
| CD8/CD3 ratio | | | |
| ITA | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| ITT | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| ITS | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM1 | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM2 | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM1IO | 0.451 (0.077–1.844) | 0.292 | 0.242 (0.045–0.815) | 0.049 |
| IM2IO | 0.551 (0.142–1.706) | 0.338 | 0.264 (0.064–0.825) | 0.037 |
| FOX3/CD3 ratio | | | |
| ITA | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| ITT | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| ITS | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM1 | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM2 | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM1IO | 0.780 (0.281–1.728) | 0.491 | 0.581 (0.454–1.752) | 0.575 |
| IM2IO | 0.857 (0.441–1.449) | 0.318 | 0.835 (0.575–1.770) | 0.681 |
| CD8/FOX3 ratio | | | |
| ITA | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| ITT | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| ITS | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM1 | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM2 | 0.000 (0.000–∞) | NA | 0.000 (0.000–∞) | NA |
| IM1IO | 0.982 (0.362–1.781) | 0.956 | 1.109 (0.640–2.032) | 0.662 |
| IM2IO | 0.925 (0.500–1.384) | 0.736 | 1.064 (0.722–1.714) | 0.703 |

Abbreviations.
CI: confidence interval.
HR: hazard ratio.
NA: not applicable; NST: no special type.
ITTA: Density of positive cells in the intratumoural area.
ITTA: Density of positive cells in the epithelial tumour regions within the intratumoural area.
ITTS: Density of positive cells in the stromal regions within the intratumoural area.
IM1: Density of positive cells in a margin of 500 µm on both sides of the boundary of the tumour area.
IM2: Density of positive cells in a margin of 1 mm on both sides of the boundary of the tumour area.
IM1IO: Ratio between the density of positive cells in the 500 µm inner and outer boundary of the tumour area.
IM2IO: Ratio between the density of positive cells in the 1 mm inner and outer boundary of the tumour area.
* per increment of 100 positive lymphocytes.
† per increment of 10 positive lymphocytes.
‡ No longer significant after correction for multiple comparisons for alpha = 5%.

Colon cancer, the methodology of this research group has been of great influence in the field of TILs in cancer [27]. The Immunoscore method consists of assessment of CD3 and CD8 densities both in the tumour and invasive margin regions (yielding 4 scores). These are subsequently translated into percentiles and averaged, after which the average is translated into a so-called ‘immunoscore’ (high, intermediate and low). It is not straightforward to compare the extensive immnoscore protocol with results from the present study. We have observed that both intra-tumoural TILs and TILs at the tumour margin contain prognostic information. We have also found that combining multiple markers (by calculating ratios) does not yield prognostic information, making it questionable whether more than one single marker is needed for TIL assessment in TNBC.

In melanoma research, the ratio between the intratumoural and peritumoural T cell density (I/P ratio) was shown to correlate with the survival [28] for patients who had been diagnosed with distant
metastases of their melanoma. Patients with a higher I/P ratio had a longer survival. In the present study, the intratumoral to peri-tumoural ratio in our TNBC cohort (IM1IO, IM2IO) was generally not prognostic. This may be attributed to different immune-regulation mechanisms for different types of malignancies.

In the present study we used multiplex IHC and deep learning, to prevent being hampered by the subjectivity of the human eye. Even though it may be felt that the use of IHC, whole slide imaging and machine learning will only be available in a limited number of diagnostic settings, we believe that the general trend of digitization of pathology diagnostics will result in wide-scale implementation of AI for histopathology. The results from the present study can be translated into essays based on straightforward single IHC staining protocols using DAB, making them available for any sufficiently equipped pathology laboratory.

Our study has several strengths. The patients within this study were selected from a multicentre TNBC cohort which consists of patients from 5 different hospitals, including both academic and general hospitals. To objectify TIL assessment, we used previously developed machine learning algorithms, preventing human intra- and interobserver variability. To optimize comparison between markers, we used mHIC combined with spectral imaging. This study is limited by the constraints of a retrospective analysis. However, a considerable effort was made to obtain high quality and complete follow up data. The extensive analysis with mHIC made it infeasible to study more than the currently included 100 patients.

We used an existing deep learning algorithm for automated delineation of regions containing epithelium, which was trained on common adenocarcinomas. Because TNBC tumours display a wider variation in morphological appearance as compared to general breast cancer, the delineation of tumour cells was unsatisfying for some tumours. As detailed manual outlining of individual tumour cells is not feasible, we had to take these segmentation results for granted, adding some noise to the ITT and ITS measures.

In conclusion, we studied the prognostic values of TILs in TNBC using completely automated assessment methods and IHC. The suggested prognostic value of TILs in our study are in line with previous research, and provide directions for optimizing TILs assessment methodology. This paper proposes a structured framework for optimizing automated TILs assessment which is preferred to application of a single (potentially suboptimal) method. Larger studies are needed to find out if one of the used image analysis algorithms is superior in predicting survival.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2021.02.007.

References

[1] Dutch Cancer Registry. Incidence numbers. cited 5 February 2018, https://www.cancerregistry.nl/selecties/incidencerate_borst/.
[2] Lakhani SR, Ellis IO, Schnitt SJ, Tan PH, van de Vijver MJ. World Health Organization classification of tumours: classification of tumours of the breast. fourth ed. Lyon: IARC Press; 2012.
[3] Ellis IO, Schnitt SJ. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
[4] Voduc KD, Cheang MC, Lydesley S, Selmon K, Nielsen TO, Kernecke H. Breast cancer subtypes and the risk of local and regional relapse. J Clin Oncol 2010;28:1564–91.
[5] Partridge AH, Hughes ME, Werner ET, Ottesen RA, Wong NY, Edge SB, et al. Subtype-dependent relationship between young age at diagnosis and breast cancer survival. J Clin Oncol 2016;34:3080–14.
[6] Dent R, Trudeau M, Pritchard KI, Sawka W, Teicher M, & the Canadian National Breast Cancer Center Group. Tumour-infiltrating lymphocytes in breast cancer patients. Cancer Res 2009;69:6865–71.
[7] Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating lymphocytes (TILs) are associated with improved pathological complete response at 6 weeks in hormone receptor-positive breast cancer. Breast Cancer Res Treat 2016;158:323–31.
[8] Rees AJ, Zeman A, Karanicolas E, Dairkee SH, Gubbi R, Pinder SE, et al. Tumour-infiltrating lymphocytes: a new functional biomarker to monitor the response to neoadjuvant therapy in triple-negative breast cancer. J Clin Pathol 2018;71:1–5.
[9] Balkenhol MCA, Bult P, Zelles D, Vreuls W, Cahn PC, Ciompi F, et al. Deep learning in diagnostic pathology: a review. Mod Pathol 2015;28:1684–91.
[10] Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, et al. Role of tumour microenvironment and molecular subtype in triple-negative breast cancer. J Clin Pathol 2017;70:46–52.
[11] Pruneri G, Vingiani A, Denkert C. Tumor infiltrating lymphocytes in triple-negative breast cancer. Breast 2018;37:207–14.
[12] Yeong J, Thike AA, Lim JC, Lee B, Li H, Wong SC, et al. Higher densities of FoxP3+ regulatory T cells are associated with better prognosis in triple-negative breast cancer. Breast Cancer Res Treat 2017;163:21–35.
[13] Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating cytotoxic lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011;29:1949–55.
[14] Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating cytotoxic lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011;29:1949–55.
[15] Ali HR, Provenzano E, Dawson SJ, Bloms FM, Liu B, Shah M, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. Ann Oncol 2014;25:1536–43.
[16] Oehlert WR, Kost SE, Martin SD, Milne K, Deleuwe RJ, Nelson BH, et al. Tumour-infiltrating FoxP3(−) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer 2013;108:155–62.
[17] Doei MV, Gaukler MC, Giavazzi R, Vente P, Delaloge S, Andre F, et al. Prognostic and predictive value of tumour-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. Ann Oncol 2015;26:1698–704.
[18] Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, et al. Assessing tumour-infiltrating lymphocytes in solid tumours: a practical review for pathologists and proposal for a standardized method from the international immunonomenclature biomarkers working group: Part 1: assessing the host immune response, TILs in invasive breast carcinoma and ductal carcinoma in situ, metastatic tumour deposits and areas for further research. Adv Anat Pathol 2017;24:235–51.
[19] McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Statistics reporting recommendations for tumour marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180–4.
[20] Voduc KD, Cheang MC, Roman KA, Hoyt CC. Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. Methods 2014;70:46–58.
[21] Stack EC, Wang C, Roman KA, Hoyt CC. Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. Methods 2014;70:46–58.
[22] Bossuyt PM, Reitsma JB, Bruins DE, Gatsonis CA, Glasziou PP, Irwig L, et al. STARD Group. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. BMJ 2015;351:h5527.
[23] Foundation federation of Dutch medical scientific societies. Code Of Conduct For Medical Research 2004, cited 2 May 2018, https://www.federa.org/codes-conduct.
[24] Foundation Federation of Dutch Medical Scientific Societies. Federa. Human
tissue and Medical research: code of conduct for responsible Use. 2011. cited 2 May 2018, https://www.federa.org/codes-conduct.

[25] Lotz J, Olesch J, Muller B, Polzin T, Galuschka P, Lotz JM, et al. Patch-based nonlinear image registration for gigapixel whole slide images. IEEE Trans Biomed Eng 2016;9:1812–9.

[26] Bulten W, Bandi P, Hoven J, van de Loo R, Lotz J, Weiss N, et al. Epithelium segmentation using deep learning in H&E-stained prostate specimens with immunohistochemistry as reference standard. Sci Rep 2019;9:864.

[27] Pagès F, Mlecnik B, Marliot F, Bindea G, Ou FS, Bifulco C, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. Lancet 2018;391:2128–39.

[28] Vasaturo A, Halilovic A, Bol KF, Verweij DI, Bloks WA, Punt CJ, et al. T-cell landscape in a primary melanoma predicts the survival of patients with metastatic disease after their treatment with dendritic cell vaccines. Canc Res 2016;76:3496–506.

[29] de Bel T, Hermens M, Smeets B, Hilbrands L, van der Laak J, Litjens G. Automatic segmentation of histopathological slides of renal tissue using deep learning. SPIE Proceedings 2018;Volume 10581:1058112. Medical Imaging 2018: Digital Pathology.

[30] Swiderska-Chadaj Z, Pinckaers H, van Rijthoven M, Balkenhol M, Melnikova M, Geessink O, et al. Convolutional neural networks for lymphocyte detection in immunohistochemically stained whole-slide images. Medical Imaging with Deep Learning 2018.

[31] Greene FL, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG, et al. AJCC cancer staging manual. sixth ed. New York: Springer; 2002.

[32] Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumour-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 2015;26:259–71.

[33] Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol 2014;25:1544–50.

[34] Kos Z, Robin E, Kim RS, Michiels S, Gallas BD, Chen W, et al. International Immuno-Oncology Biomarker Working Group. Pitfalls in assessing stromal tumour infiltrating lymphocytes (sTILs) in breast cancer. NPJ Breast Cancer 2020;6:17.