THE CLINICAL AND BIOLOGICAL SIGNIFICANCE OF ESTROGEN RECEPTOR-LOW POSITIVE BREAST CANCER

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ABSTRACT

Estrogen Receptor (ER) status in breast cancer (BC) is determined using immunohistochemistry (IHC) with nuclear expression in ≥1% of cells defined as ER-positive. BC with 1-9% expression (ER-low positive), is a clinically and biologically unique subgroup. In this study, we hypothesized that ER-low positive BC represents a heterogeneous group with a mixture of ER-positive and ER-negative tumor, which may explain their divergent clinical behavior. A large BC cohort (n=8171) was investigated and categorized into three groups: ER-low positive (1-9%), ER-positive (≥10%) and ER-negative (<1%) where clinicopathological and outcome characteristics were compared. A subset of ER-low positive cases was further evaluated using IHC, RNAscope and RT-PCR. PAM50 subtyping and *ESR1* mRNA expression levels were assessed in ER-low positive cases within The Cancer Genome Atlas dataset. The reliability of image analysis software in assessment of ER expression in the ER-low positive category was also assessed. ER-low positive tumors constituted <2% of BC cases examined and showed significant clinicopathological similarity to ER-negative tumors. Most of these tumors were non-luminal types showing low *ESR1* mRNA expression. Further validation of ER status revealed that 45% of these tumors were ER-negative with repeated IHC staining and confirmed by RNAscope and RT-PCR. ER-low positive tumors diagnosed on needle core biopsy were enriched with false positive ER staining. BCs with 10% ER behaved similarly to ER-positive, rather than ER-negative or low positive BCs. Moderate concordance was found in assessment of ER-low positive tumors, and this was not improved by image analysis. Routinely diagnosed ER-low positive BC includes a proportion of ER-negative cases. We recommend repeat testing of BC showing 1-9% ER expression and

using a cut-off ≥10% expression to define ER positivity to help better inform treatment decisions.

INTRODUCTION

Estrogen receptor (ER) is a key oncogenic driver in the majority of breast carcinoma (BC) and is expressed in about 80% of BC [1]. It is an established prognostic and predictive biomarker for BC management decision-making [2]. In the clinical setting, BC with ER immunohistochemical (IHC) expression in ≥1% of invasive tumor cells' nuclei is considered ER-positive for endocrine therapy (ET) eligibility [3,4]. However, ER shows significant intratumoral heterogeneity and there remains evidence that ER expression levels are linearly correlated with ET response [5]. While ET significantly improves the overall survival of ER-positive BC patients, the ER-threshold for response to ET is uncertain and the eligibility of those patients with low ER expression levels to adjuvant ET and chemotherapy is still debated[6,7].

Historically, ER was assessed with ligand-binding assays. Meta-analysis of randomized trials showed that tamoxifen was beneficial where ER expression was as low as 10–19 fmol/mg cytosol protein [8]. However, there have been no large trials of ET in the context of the more widely used IHC assessment of ER expression. The 1% cut-off proposed for ER was proposed to be functionally equivalent to 10 fmol/mg cytosol protein as measured by ligand binding assays. the management of BC with low ER expression levels remains a challenge in routine practice, and there is debate whether BCs with low ER expression differ from ER-negative tumors. It is argued that patients with low ER tumors benefit from chemotherapy and most of them have overlapping genetic profiles with ER-negative tumors [9,10].

The latest American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) guidelines update for ER and progesterone receptor (PR) testing in BC [11], recommend that tumors with low ER expression level (1-10%) be reported as "ER-low positive" along with a comment about inadequate evidence of ET benefit. In these

guidelines, not only tumors with 10% were considered as ER-low positive, but also the requirement for repeat testing was limited to cases with a suspected analytical issue. Other measures include the use of validated quantitative digital image analysis to confirm interpretation [11]. The definition of ER-low expression also differs in literature from 1 to 9% [7,12-14] or 1 to 10% [15-18].

ER status is assessed in the clinical setting utilizing needle core biopsy (NCB), and it is repeated on surgical excision specimens in a limited number of cases [3,4]. One challenge in designating the ER status of BC tumor, particularly those with low ER expression in NCB, is the specificity and reproducibility of assessment in a limited tissue sample. Discrimination between false and true IHC staining is often challenging and requires strict adherence to high quality standards to minimize subjective factors influencing assessment.

We hypothesized that BC with ER-low positive expression as assessed on NCB are biologically and clinically heterogeneous and behave differently compared to tumors with higher ER expression, as a considerable percentage of these tumors are falsely interpreted as ER-positive. We thus aimed to: 1) characterize ER-low positive BC, as assessed on NCB, with an emphasis on its prevalence and association with different clinicopathological parameters and patient outcome using a large well-characterized BC cohort, 2) evaluate the spectrum of ER IHC staining using different techniques to distinguish ER-positive from ER-negative and to identify the proportion of cases remaining as ER-low positive, 3) identify the optimal cut-off to distinguish ER-low positive from ER-positive BC, and 4) determine the value of image analysis in tumors showing low ER expression to provide improved pathological assessment.

MATERIALS AND METHODS

This study included two cohorts, the Nottingham cohort (n=8171), and The Cancer Genome Atlas (TCGA) BC cohort (n=1047).

Nottingham Cohort

A large cohort of primary invasive BC from Nottingham City Hospital patients, Nottingham, UK from 1990 to 2018 was investigated, where 7559 cases had available relevant clinicopathological data. Clinicopathological data including age at diagnosis, tumor histologic grade and its components, tumor size, histological tumor type, ER, PR, Human epidermal growth factor receptor 2 (HER2) status, Ki67 index [19], lymph node (LN) stage, lymphovascular invasion and Nottingham Prognostic Index (NPI) were collected. Hormonal receptors and HER2 were assessed in accordance to published guidelines [11,20]. Receptors were assessed on the NCBs as part of the routine patient health care. ER positivity between 1-9% of invasive tumor cells represented 1.6% of cases (n=123). **Supplementary Figure 1** illustrates the Nottingham cohort characteristics. Although the Nottingham protocol for tissue processing of excision specimens ensured adequate fixation as demonstrated by the biomarkers' staining quality in previous publications [21-24], it was decided to further validate the ER expression level in the excision specimens using (i) IHC with two different antibody clones with and without casein blocking, (ii) *ESR1* mRNA expression using RNAscope and (iii) real time reverse transcription–polymerase chain reaction (RT-PCR). None of the patients included in this study received neoadjuvant therapy.

Immunohistochemical staining

The available formalin fixed paraffin embedded (FFPE) tissue blocks from NCB and excisional biopsies of 64 cases initially reported as ER-low positive BC were retrieved. In

addition, a subset of cases (n=39) with ER staining between 10-30% were used for comparison. Fresh hematoxylin and eosin (H&E) sections were prepared from the specimens. Excision specimens were further reviewed to select sections containing the largest tumor burden, for repeated staining. Sections were cut at 4µm thickness. IHC was carried out according to the standard in-house protocols. The slides were processed through pre-diluted tris-based buffer with a basic pH (Roche, Ventana) for 64 minutes at 95°C for antigen retrieval. Either anti ER Rabbit monoclonal antibody, SP1 clone (Roche) or EP1 anti-ER Rabbit monoclonal antibody (Dako, Ref- M3643), based on availability, was applied and incubated for 16 and 30 minutes, respectively. A small subset of cases was also stained individually with both ER antibody clones (EP1 and SP1). In addition, casein block (Roche, antibody diluent with casein) was also tested in a subset of slides to evaluate whether casein blocking could refine the scoring and differentiate false positive from true positive cases. External (a known ER high positive BC case) and internal (normal breast ductal epithelial cells) controls were used.

Estrogen receptor expression scoring

Available original ER IHC-stained slides in clinical settings were collected and reassessed by a pathologist (observer 1) and in case of discordance with the original reports, the case was independently assessed by another pathologist and the agreed scoring was used in the final analysis. In addition, all old and newly stained slides were independently scored by a consultant breast pathologist (observer 2) with >20 years' experience to assess the overall concordance. Where blocks or slides were unavailable, the score assigned at the initial assessment was used. The percentage of nuclear ER immunostaining, intensity, pattern (homogenous, granular (punctate) or mixed), subcellular localization, stromal and

background staining were evaluated. Intensities ranged from very weak to strong staining, using the internal (if present) or external positive controls as a benchmark.

Assessment of *ESR1* mRNA expression level using RNAscope and RT-PCR

ESR1 mRNA expression was evaluated using RNAscope [25]. For each case, *ESR*, *PPIB* (positive control) and *DapB* (negative control) were tested. Additionally, for each run the following controls obtained from Advanced Cell Diagnostics (ACD, BioTechne Ltd) were employed: (i) HeLa cell pellets were used as assay controls to confirm inter-run consistency, (ii) ACD triple negative BC (TNBC) sample as an ER-negative control, and (iii) ACD BC tissue as a ER-positive control. Briefly, tissue sections of 4µm thickness were deparaffinized and dehydrated. The slides were incubated in Pre-treat 1 (Ready-To-Use (RTU) endogenous blocker) followed by the boiling Pre-treat 2 (citrate buffer 10 nmol/L, pH 6) for 15 minutes. Then, Pre-treat 3 (10 g/mL protease, Sigma-Aldrich, St. Louis, MO) was added and incubated at 40°C for 30 minutes in a HybEZ hybridization oven. Subsequently, in the hybridization step, the tissue was placed at 40°C with the following solutions: target probes in hybridization buffer A [6X SSC (1X SSC is 0.15 mol/L NaCl, 0.015 mol/L Na-citrate), formamide 25%, 0.2% lithium dodecyl sulphate, blocking reagents] for 3 hours; preamplifier (2 nmol/L) in hybridization buffer B (20% formamide, 5X SSC, 0.3% lithium dodecyl sulphate, 10% dextran sulphate, blocking reagents) for 30 minutes; amplifier (2 nmol/L) in hybridization buffer B for 15 minutes at 40°C; and label probe (2 nmol/L) for 15 minutes in hybridization buffer C (5X SSC, 0.3% lithium dodecyl sulphate, blocking reagents). Signals were detected using a Fast Red chromogen, which was followed by hematoxylin counterstaining. RNAscope semiquantitative scoring was based on the average number of signal dots detected per invasive tumor cell throughout the entire slide as follows: negative=

0–1 dot/tumor cell or 2–3 dots/tumor cell in <50% of tumor cells, 1=2–3 dots/tumor cell in >50% of tumor cells, 2=4–10 dots/tumor cell, 3=>10 dots/tumor cell in >50% of tumor cells, and 4=>10 dots/tumor cell in >50% of tumor cells with clusters of the signal [26] (**Supplementary Figure 2**).

Real time reverse transcription–polymerase chain reaction (RT-PCR)

Ribonucleic acid (RNA) was purified from FFPE tissue sections using RNeasy FFPE Kit (Qiagen), according to manufacturer`s instructions. To assess RNA quantity and purity a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc, US) was used. Appropriate *ESR1* primers were designed by using PubMed and the Primer 3 software (web version 4.0.0) http://primer3.ut.ee/ (forward primer: TGGGCTTACTGACCAACCTG, reverse primer: CCTGATCATGGAGGGTCAAA). Complementary deoxyribonucleic acid (cDNA) was synthesized by reverse transcription of RNA, with reverse transcriptase (RT)+ and RT- reactions for every sample. After adding 2.5 µl of 0.5 µg random hexamers (Thermo Scientific 50142 200 ng/µl) to 1 µg RNA, the samples were made up to a total volume of 15 µl of H_2O and were incubated at 70°C for 5 minutes. Samples were immediately placed on ice for 5 minutes and then centrifuged. A master mix was prepared as follows: 5 µl M-MLV RT Buffer 5X (Promega), 1.25 µl dNTP (10 mM) (Thermo Scientific), 1 µl M-MLV Reverse Transcriptase (Promega 200 U/µl) and 5.25 µl water. 10 µl of appropriate master mix was added to each sample to reach the total volume of 25 µl. Next, samples were incubated at 37°C for 1 hour followed by 95°C for 10 minutes. After that, samples were diluted 1:10 and stored at -20°C. RT-negative samples in which RNase-free water were included instead of reverse transcriptase enzyme were prepared to ensure the purity of the RNA extracted sample from remains of genomic DNA.

RT-PCR was analyzed using comparative ΔΔCt method which quantifies RNA expression in the sample relative to a reference sample [27]. The cut-off point was selected depending on the average of the fold changes of negative cases relative to the pooled calibrator of negative ER expression cases. The cut-off point was 1.00 (1.006± 0.100).

Image analysis

A subset of cases (n=98) was assessed using QuPath software version 0.3.2 [28]. The slides were scanned as high-resolution digital images at 20x magnification using a 3D Histech Panoramic 250 Flash II scanner (3DHISTECH Ltd., Budapest, Hungary). Images were imported into QuPath, tumor areas were manually annotated, and positive cell detection command was applied (**Supplementary Figure 3**). Cell detection settings were followed as Berben et al. [29], Several parameters have been explored for setting optimal thresholds. Hematoxylin OD was selected, a single intensity threshold was chosen and set at 0.1 and maximum background intensity was set to 2. The percentage of positive tumor cells detected divided by the overall tumor cell detection was considered. Scoring was carried out blindly and compared to visual estimates.

The Cancer Genome Atlas (TCGA) BC dataset analysis

A BC cohort of publicly available TCGA data (n=1047) with available ER data was used to identify *ESR1* mRNA expression and PAM50 subtyping of ER-low positive in comparison to higher ER expression. ER percentages were available as categories and ER IHC categories of 1-9% (n=17) and 10-19% (n=25) were analyzed. *ESR1* mRNA Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values were used and divided into quartiles for descriptive purposes.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) v26 for Windows (Chicago, IL, USA) was used. Chi-square test was used to compare categorical groups, while Mann-Whitney test was used for continuous variables. Inter-observer agreement was determined using the intra-class correlation coefficient (ICC). Kaplan-Meier curves and log-rank test were used for survival

analysis including BC specific survival (BCSS) as time from initial diagnosis to death related to BC, distant metastasis free survival (DMFS) defined as time from initial diagnosis to date of DM, and disease-free survival (DFS) which describes length of time to any recurrence event of disease. A *p*-value of less than 0.05 (two-tailed) was considered significant in all the statistical tests.

RESULTS

ER-positive tumors (≥1%) represented 78% of the Nottingham cohort including 123 cases (1.6%) which were initially reported as ER-low positive (1-9%) based on NCB assessment in the clinical setting. The median percentage of ER positivity in the 1-9% group was 4% (mean: 3.6±1.9%). 167 cases had ER expression in 10-30%.

Clinicopathological characteristics of ER-low positive BC

Most of the clinicopathological parameters of tumors initially reported as ER-low positive (1-9%) were similar to the ER-negative tumors but showed significant differences to ER-positive tumors (≥10%) in most clinicopathological parameters (**Table 1**). When ER 1-9% BCs were compared with the tumors showing ER expression in 10-30% of the cells, they were more often higher grade, larger size, and showed higher NPI, higher Ki67 index, and were more likely to be PR negative and HER2 positive (**Supplementary Table 1**).

Outcome analysis

Compared to ER-negative patients, the ER 1-9% group showed no differences in BCSS, DMFS or DFS (*p*=0.9, *p*=0.3 and *p*=0.2 respectively) despite the difference in ET given. In contrast, ER 1-9% showed significantly shorter BCSS compared to ER-positive ≥10% BC patients (*p*=0.002) (**Supplementary Figure 4**).

Breast cancer with 10% ER expression

As the number of cases scored as 10% ER was relatively high (n=47), taking in consideration the lack of consensus regarding 10% as ER-low positive or ER-positive, we next repeated the analysis considering tumors with 1-10% as ER-low positive cases. ER-low positive group (1-10%) showed lower tumor grade and grade components compared to ER-negative tumors (**Supplementary Table 2**). Outcome analysis revealed that patients with ER 1-10% had both longer DMFS and DFS compared with ER-negative patients (p=0.03 and p=0.02, respectively) (**Figure 1**).

As a group, tumors with 10% ER expression were significantly lower grade, better NPI and more PR positive than tumors with ER 1-9%, while they did not show significant difference from tumors with ER 11-30% **(Supplementary Table 3**). BCs with ER 10% have not shown significant outcome difference from ER 11-30% BC patients.

***ESR1* mRNA expression and PAM50 classification**

The association between the ER protein expression and *ESR1* mRNA expression obtained from the TCGA-BRCA study revealed no difference in *ESR1* mRNA expression and PAM50 molecular subtypes between ER-negative and ER 1-9% (p=0.1 and p=0.2 respectively). ER 1-9% had significantly lower *ESR1* mRNA expression than the ER 10-19% group (p=0.005) and significant difference in PAM50 classification (p=0.007). **Supplementary Tables 4** and **5** show ESR*1* and PAM50 classification of each ER category. ER-negative group had *ESR1* expression in the lower quartile in 92% of cases. ER 1-9% cases showed lower quartile expression in 59% of cases compared to only 20% of 10-19%. ER-negative tumors showed non-luminal molecular profiling in 94% of cases compared to 81% of 1-9% group, while 10-19% group predominantly expressed luminal subtyping (76%).

Validation of ER-low positive expression

In the 64 originally reported ER-low positive group (1-9%), 30 cases (47%) were negative on repeated IHC staining on the excisional specimens (**Figure 2**), 16 cases remained in the ER

1-9% group, while 18 were more strongly positive (≥10%) (**Supplementary Figure 5**). After re-staining, RNAscope was also negative in 82% of negative cases in addition to RT-PCR which yielded the same result. All these cases were also PR negative. Among cases that remained positive, RNAscope confirmed positivity by 93% and RT-PCR was positive in 64% of cases tested. Most of these cases (71%) were PR negative.

Of the ER-positive cases (10-30%), RNAscope were positive in all cases while RT-PCR was negative in 20% of cases. In that group (ER 10-30%), PR was positive in 32% of cases. The subset of cases which have been initially reported as 10%, positive expression was present in 70% when repeated (**Tables 2** & **3)**.

Staining patterns

In 64% of cases an unusual staining pattern of ER was observed which was characterized by very weak to weak granular/punctate staining that was mostly associated with non-specific staining either cytoplasmic or background staining (**Figure 3**). In ER 1-9% cases, 56% of cases with the unusual pattern were negative on the repeat (**Supplementary Figure 6**). Cases of ER 10-30% that showed negative ER status on repeat displayed the same staining pattern in the NCB (**Supplementary Figure 7**). Regarding the staining intensity, 72% of ER-low positive cases showed very weak to weak intensity. Over half of cases (56%) showed non-specific staining: stromal (34%), cytoplasmic (29%), both stromal and cytoplasmic (25%) or background non-specific staining (12%) (**Supplementary Figure 8**).

Comparison between different ER antibody clones

There was strong agreement in assessment between different ER antibody clones tested (ICC =0.98). Cases with the unusual granular staining pattern did not show differences using both clones. When the casein block step was employed in IHC, non-specific staining was reduced in 66% of cases and these cases showed a high concordance between observers (ICC=0.88). However, some of the true positive but weak intensity ER staining (40%) were classed as ER-

negative when the casein blocking step was used. Therefore, casein was not used in the remaining cases to differentiate true from false staining. (**Supplementary Figure 9**).

Concordance in ER-low positive staining assessment

The concordance between three pathologists` visual scoring (observers 1 and 2, in addition to the original clinical scoring retrieved from reports) was assessed and revealed moderate agreement (ICC=0.5). Furthermore, the same level of agreement was observed between visual estimate and QuPath scoring (ICC=0.5). The highly discordant cases were associated with either background cytoplasmic staining that were considered positive by QuPath or very weak staining that was not detected as positive cells by QuPath. Contrasting this, when ER-positive cases (>50% ER positivity) (n=40) were used for calibration, excellent concordance (ICC=0.9) was obtained.

DISCUSSION

ER expression has largely been considered a binary variable where ER is either expressed or not. This simplified dichotomization of ER expression facilitates patient risk stratification and therapeutic management. However, semiquantitative rather than qualitative assessment of ER has a confounding impact on treatment strategy as the threshold for response to ET remains uncertain[5]. The ASCO/CAP guidelines defining ER-positivity as low as 1% have led to those tumors expressing ER at low levels being considered as “ER-low positive BC”. However, whether the ER-low group represents a distinct entity as opposed to either true ER-positive or ER-negative remains uncertain. This can either deprive patients from receiving chemotherapeutic agents, probable benefit from ET, or combination therapies [30,31]. Moreover, several issues relating to ER-low positive BC remain to be addressed including whether these ER-low tumors more closely resemble ER-negative are related to being low but genuine expressers or a mixture of both ER-positive and negative tumors. ER-low

positive tumors in a limited NCB could represent the low percentage (<1%) of ER-stained cells in higher tumor cellularity of an excisional specimen or due to enrichment with false positive ER staining. Indeed, it remains uncertain whether cases with 10% ER expression should be included in the ER-low positive or ER-positive category. The benefits of image analysis as a reliable tool to assess such cases more accurately than a pathologist visual estimate is also yet to be established.

To address these challenges, we used a large cohort to define the prevalence and clinicopathological characteristics of the ER-low category. Less than 2% of cases were ER-low positive. Previous reports have demonstrated a wide range in prevalence (<2% up to 9%) of ER-low cases in cohorts [2,32-34]. Consistent with previous reports [2,10,34,35], we found that most clinicopathological criteria and outcome of ER-low positive tumors were similar to ER-negative BC. Although the ER-low positive group showed higher PR positivity than the ER-negative tumors, PR was negative in those cases which were ER-negative following validation and repeat ER staining. PR is thought to be mainly regulated by ER through binding to the *PgR* promotor region [36].

Previous studies investigated the molecular subtyping of ER-low positive BC where >80% were either basal-like or HER2 enriched [9,13,37]. Although this may be the result of low ER protein expression and inactive downstream ER pathways, our study demonstrated by repeating ER IHC staining and validation using *ESR1* gene expression levels that a large proportion should be classified as ER-negative if strict criteria for ER assessment were used. The basis for false positive ER staining in NCBs in ER-low positive tumors were multifactorial and related to the weak intensity and unusual pattern of staining. A few cases were reassigned into the ER-negative (<1%) category due to the difference in the percentage of positive cells between NCB and the excision specimen and these were negative at the mRNA level. Importantly, ER-low positive tumors assessed on NCB which remained ER-low positive/

positive showed higher frequency of PR positivity and clinicopathological features that are different from ER-negative tumors. Therefore, we suggest repeating ER IHC staining in all ER-low positive tumors before assigning them as either ER-negative or positive, unlike the ASCO/CAP recommendation which limits repeat ER staining to cases with preanalytical/ analytical issues [38]. The observed weak granular staining pattern of ER was difficult to interpret. This unusual pattern showed a high probability of negativity on re-staining, even with cases showing higher ER expression (10-30%). These staining patterns can be identified by pathologists as clues for false positive staining, therefore we suggest reporting weak granular staining as ER-positive with a caveat that it may represent false-positive staining and recommend repeating staining on excisional specimens. Casein blocking can reduce the false positive rates, but at the same time it resulted in some cases classified as ER-negative despite true ER staining using the standard validated staining techniques. ER antibody clones used showed no difference in staining sensitivity and specificity in the detection of ER-low positive tumors.

At the molecular level, neither *ESR1* mRNA levels nor molecular subtyping were significantly different between ER-negative and ER 1-9% groups. This indicates that the ER 1-9% cases at IHC level may not represent a truly ER-positive BC and intermingled ER-negative cases are the reason for molecular resemblance. On the contrary, ER 10-19% cases had significantly higher tendency as being classified as luminal types with higher *ESR1* mRNA levels.

Pathologists tend to round scores, so a score of 10% may be more frequent than scores around this figure, when convincing staining is present. Consequently, we analyzed tumors expressing ER 10% which were associated with favorable parameters compared with ER-low positive tumors. Cases with exactly 10% ER positivity seemed to differ from ER-negative and ER low group (1-9%). A study showed that 75% of ER-positive tumors with 10% positivity were of the luminal A molecular subtype [9]. Based on a similar response of ER 1-9% patients

to ER-negative patients who received either neoadjuvant or adjuvant chemotherapy in previous studies, 10% was used to define ER-positive BC [39,40]. Additionally, BC patients with ER 1-10% showed better DMFS and DFS compared to ER-negative BC patients, whereas ER 1-9% tumors did not show any difference. It seems that adding 10% was the key parameter in this statistical difference, which supports that 10% ER score should be regarded as positive BC. Therefore, our results suggest that 10% would be a more reliable cut-off rather than the 1% currently proposed by ASCO/CAP.

The outcome of ER low BC is variable between studies. In a recent clinical trial, there was a significant difference between survival in patients with ER-low positive and positive BC with the former showing inferior outcome which was not observed when ER-low positive cases were compared with ER-negative cases [37]. This was consistent with our study. Similar results were reported in a meta-analysis conducted on 11 different studies [2,9,10,14,35,40-45]. Better outcome in ER-low positive compared to ER-negative tumors has been further documented, however, these studies had limited cases numbers [13,18].

Response to ET in ER-low positive BC is of crucial clinical significance. However, available data are limited, and most studies conducted were retrospective due to the rarity of this group. Few studies reported benefits from ET in ER-low positive patients, however the use of different scoring methods and cut-offs combined with limited follow-up make such results less reliable [18,46,47]. A systematic review and meta-analysis included 6 studies on the benefit of ET in ER-low positive BC but did not find significant survival benefit, although it suggested a better prognosis than in case of ER-negative tumors [7]. The scoring method of these studies was variable, using either the Allred score [48] or combined ER/PR scores [14]. Landmann et al. reported that ER-low positive BCs achieved similar pathological complete response rates as ER-negative tumors which were significantly different from ER-positive tumors [10]. It was

suggested that ER-low positive BCs should be treated as ER-negative BC, rather than ER-positive tumors, based on their similarity to ER-negative BC in the response to neoadjuvant chemotherapy [41]. A prospective study on the benefit of ER-low positive BC patients from ET found better DFS after 5 years of tamoxifen [49]. The latest ASCO/CAP guidelines recommend a discussion with patients diagnosed with ER-low positive BCs particularly as there are limited data on ET benefit [11]. Our survival data were restricted due to the small number of ET ER-low positive patients with censoring limitation.

Although a strong concordance of ER status between NCB and surgical excision specimen was reported [50,51], with less than 1% of cases being reassigned from ER-positive to negative (5 out of 1249 [52], and 4 out of 465 [53]), spatial heterogeneity in different tumor areas [54] could account for the difference in ER status reported in NCB and excision specimens, which has been reported to range from 6% to 17% [52,53,55]. In a previous concordance study of 806 NCBs and the corresponding surgical excision specimens [56], only one ER-positive case in NCB (>10%) was subsequently reassigned to ER-negative on the excision specimen. Contrasting this, analysis of 23 ER-low positive cases reveals that while 11 (48%) were consistently assigned as ER-low in NCB and excision specimens, 8 cases (35%) were reassigned to ER-negative, and 4 cases (17%) were reassigned to ER-positive. These results are consistent with our results and demonstrate the importance of repeat staining of tumors with low positive ER status.

Using percentages as a cutoff is complicated by variation in tumor sampling and cellularity in core biopsies particularly in the low ER expression cases. Repeating the staining on the excisional specimens of cases reported as ER 1-9% in original NCBs will also allow a more accurate assessment and categorization of these cases where more tumor tissue is represented to overcome the heterogeneity and “denominator” effects.

Previous studies reported a significant lower mean of ER expression in excisional specimens compared to NCB, especially in the ER-low group, due to better fixation [57,58]. Adequate fixation of the excision specimen is essential to obtain reliable results. In our institution, a cruciate incision through excisional breast specimens is routinely carried out to ensure proper immediate fixation and proper internal and external controls are applied for each case. If fixation is suboptimal, the results of the core biopsy can be considered and a statement to highlight the limitation of the final results should be included in the pathology report.

Although methods for *ESR1* mRNA detection could be beneficial in these cases, reproducibility can be influenced by different RNA extraction assays, reagent, and probes selectivity, PCR protocols and RNA integrity. Contamination with non-malignant cells during RNA extraction is an additional potential cause of false negativity [59]. Thus, IHC remains the method of choice for ER testing [11].

High concordance in assessment of ER staining as either ER-positive/negative is reported [6,60], however, Reisenbichler et al showed discrepancy of ER scoring in ER-low positive tumors [61]. Only moderate agreement was revealed, and the majority of the discrepant cases showed very weak to weak intensities. Unlike a previous study [18] that showed all ER-low positive cases were weak, we found a few cases with moderate and strong intensities. This is noteworthy as the Allred score of 3 previously recommended by Harvey and colleagues representing 1 to 10% of weak positive cells, can also represent <1% of cells with moderate or strong intensity [6,61]. The tumor cells showing nuclear expression are defined as positive regardless of staining intensity, however, Caruana et al argued that low ER positivity seen in this group originated from low staining intensity. They examined the adjacent normal breast ducts and compared them to normal ducts in control cases, to find that they have lower

intensity and suggested to repeat IHC staining even in the presence of positive internal control [31]. The recent ASCO/CAP updates recommended the inclusion of an external positive control with known low ER expression such as tonsil and cervix as a quality assurance step while assessing ER expression in BC, especially the low-expressing tumors [11].

We previously reported nuclear staining granularity with the 6F11 anti-ER antibody [62]. However, in the present study, this punctate staining was also detected in SP1 and EP1 antibodies. When we compared cases stained with both antibodies, there was a high correlation between them which addresses the concerns regarding the high sensitivity of SP1 leading to misclassification of some very low ER expressing BC as ER-positive [63].

We also found that more than half of the ER-low positive cases had non-specific staining either stromal, cytoplasmic or both. False interpretation of non-specific staining may explain the interobserver discordance reported in this group. Casein block has been used to reduce non-specific staining [64]. The inter-pathologist agreement was improved on the cases that were stained using casein blocking, however, some cells lost their true nuclear positivity [65]. Therefore, we do not recommend using casein block during ER IHC staining.

ASCO/CAP recommend consideration of image analysis to validate the results of ER-low expression [11], however, no defined scoring criteria were recommended. There was only moderate agreement between our visual assessment and digital scores, suggesting that image analysis is not the key to improving concordance in ER-low positive scoring. We noted that positive cell detection was overestimated in cases of non-specific/background staining which makes its reliability questionable. The threshold of intensity at which the cells would be detected as positive, is controllable, however, the scoring of weakly stained cells would still be subjective depending on the threshold. A recent study on quantitative image analysis on a

large number of ER cases showed strong agreement with the pathologist`s score but most discordant cases were in the ER-low positive category [66].

In conclusion, ER-low positive BCs (1-9%) have pathological features and outcome more similar to ER-negative tumors than those with stronger ER expression. Our data suggests that 10% ER positivity should be regarded as true ER-positive BC. Repeat staining on excisional specimens is advised in all ER-low tumors identified in NCB. Weak granular ER staining should be interpreted with caution, and we recommend reporting this may represent a false-positive result. Image analysis in ER-low positive BC is not superior to the visual estimates and should be used cautiously.

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AUTHORS' CONTRIBUTIONS

SM scored the cases and took the lead in writing the manuscript, data analysis and interpretation. MA and AM performed the IHC and RT-PCR and helped in data analysis. MT contributed to data analysis, scoring, and reviewing the article. NM and AG helped in data interpretation and reviewing the article, AL scored as the second observer and reviewed the article. ER conceived and planned the presented idea, data interpretation and reviewing the article.

ETHICAL STATEMENT

This study was approved by the Yorkshire & the Humber - Leeds East Research Ethics Committee (REC Reference: 19/YH/0293) under the IRAS Project ID: 266925. Data

collected was fully anonymized. All procedures performed in the study were in accordance with the Declaration of Helsinki.

DATA AVAILABILITY

The data presented in the current study are available upon reasonable request.

COMPETING INTERESTS

The authors have declared no conflicts of interest.

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SUPPLEMENTARY INFORMATION

Supplementary information is available at Modern Pathology's website.

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FIGURE LEGENDS

Figure 1. Kaplan Meier survival plots of estrogen receptor (ER) low positive and ER-negative breast cancers (BCs). (A) Distant metastasis free survival (DMFS) plot showing insignificant difference between ER 1-9% (n=20) BC patients and ER-negative patients (n=611) (B) DMFS plot showing significant difference between ER 1-10% (n=35) and ER-negative patients (n=611). (C) Disease free survival (DFS) plot showing insignificant difference between ER 1-9% (n=20) BC patients and ER-negative patients (n=611). (D) DFS plot showing significant difference between ER 1-10% (n=35) BC patients and ER-negative patients (n=611).

Figure 2. Example of needle core biopsy with paired surgical excision specimen`s immunohistochemistry (IHC) and RNAscope. (A&B) core biopsy with low estrogen receptor (ER) expression (8%) (20x and 40x magnification), (C) negative IHC excision specimen and (D) negative RNAscope. (C&D, x20 magnification).

Figure 3. (A to D) showing examples of unusual estrogen receptor (ER) staining pattern with very weak to weak granular staining. (x100 magnification). (E and F) representing diagram showing difference between (E) homogenous staining pattern and (F) unusual staining pattern with very weak granular nuclear staining.