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Quantitative expression of oestrogen receptor in breast cancer: Clinical and molecular significance

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ABSTRACT

Background: Oestrogen receptor (ER) positive breast cancer (BC) patients are eligible for endocrine therapy (ET), regardless of ER immunohistochemical expression level. There is a wide spectrum of ER expression and the response to ET is not uniform. This study aimed to assess the clinical and molecular consequences of ER heterogeneity with respect to ET-response.

Methods: ER expression, categorised by percentage and staining intensity in a large BC cohort (n = 7559) was correlated with clinicopathological parameters and patient ET response. The Cancer Genome Atlas Data BC cohort (n = 1047) was stratified by ER expression and transcriptomic analysis completed to better understand the molecular basis of ER heterogeneity.

Results: The quantitative proportional increase in ER expression was positively associated with favourable prognostic parameters. Tumours with 1–9% ER expression were characteristically similar to ER-negative (<1%) tumours. Maximum ET-response was observed in tumours with 100% ER expression, with responses significantly different to tumours exhibiting ER at < 100% and significantly decreased survival rates were observed in tumours with 50% and 10% of ER expression. The Histochemical-score (H-score), which considers both staining intensity and percentage, added significant prognostic value over ER percentage alone with significant outcome differences observed at H-scores of 30, 100 and 200. There was a positive correlation between ER expression and *ESR1* mRNA expression and expression of ER-regulated genes. Pathway analysis identified differential expression in key cancer-related pathways in different ER-positive groups.

Conclusion: ET-response is statistically proportionally related to ER expression with significant differences observed at 10%, 50% and 100%. The H-score adds prognostic and predictive information.

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1. Introduction

Oestrogen receptor (ER) influences breast cancer (BC) development, progression and resistance to therapy through regulation of molecular pathways involved in BC cell proliferation and invasion [1]. ER status is a well-established prognostic and predictive marker in BC patients. ER-positive BC is defined as ER protein expression in ≥ 1% of invasive tumour cells, using immunohistochemistry (IHC), regardless of the absolute level and intensity of staining [2]. This definition has been agreed following considerable debate regarding the lowest cut-off for ER positivity at which patients are expected to exhibit a significant response to endocrine therapy (ET) [2–6].

All patients with ER-positive BC, as currently defined, are candidates for ET. However, response to ET is variable [7–10] which may be attributable to intra-tumoural heterogeneity. This heterogeneity is reflected in the morphological and molecular variability that may be observed within a tumour and reflects the underlying genetic variation [11] and differing patterns of cellular differentiation, proliferation, architectural features and cancer-associated biomarker expression [12]. Tumours with heterogeneous ER expression (<100% ER positive cells) harbour a population of ER-negative tumour cells that may exhibit different biological behaviour and response to therapy to the ER-positive component. Progression of ET-resistant clones within a heterogeneous tumour can ultimately dominate tumour behaviour and response to ET, and contribute to treatment resistance [13,14]. Clinical evaluation of individual tumour ER heterogeneity and its role in BC behaviour, is challenging [15]. Furthermore, while the clinical definition of ER-positivity has been agreed [16], the minimum ER-expression required to accurately predict patient benefit from ET and/or the need for adjuvant chemotherapy remains poorly understood [12,17]. This suggests that the present binary classification of ER status, as either being ER-positive or ER-negative, may be over-simplified and should be replaced by a graded system to reflect the functional complexity of differences in ER expression, and how this influences response to ET [6, 18,19]. For this reason, this study investigated how ER expression in BC influences tumour behaviour and response to ET. We aimed to refine the prognostic and predictive stratification of ER-positive BC based on a more precise assessment of ER expression considering both percentage and intensity of expression.

2. Materials and methods

2.1. Study cohorts

This study included two large BC cohorts comprising:

2.1.1. Nottingham cohort

A large well characterised consecutive BC cohort (n = 7559) from patients treated at Nottingham City Hospital, Nottingham, United Kingdom (UK) from 1990 to 2018, was included. Clinicopathological parameters, including patient age at diagnosis, tumour size, histological tumour grade and its components, histological subtype, lymphovascular invasion (LVI), lymph node (LN) status and Nottingham prognostic index (NPI), treatment regimens and follow-up data were collected from the data registry [20,21]. Assessment of ER status included documentation of both percentage and intensity of expression and histochemical scores (H-score) [22]. This information was retrieved from the original histopathology reports.

ER IHC staining was carried out according to standard protocol [23]. Briefly, antigen retrieval was carried out through pre-diluted tris-based buffer with a basic pH (Roche, Ventana) for 64 min at 95 °C. Slides were then incubated with the primary antibody (anti-ER Rabbit monoclonal antibody SP1 clone (Roche) for 16 min or EP1 anti-ER Rabbit monoclonal antibody (Dako, Ref- M3643) for 30 min, according to the availability). Diaminobenzidine (DAB) was used for peroxidase detection (8 min) followed by haematoxylin counterstaining (Roche, Ventana Haematoxylin II).

Data on progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67 scores were also available [24–28]. Hormone receptor and HER2 scoring were assessed following the UK guidelines and the American Society of Clinical Oncology and College of American Pathologists (ASCO CAP) guidelines [29–32]. Ki67 index > 14% was classified as high [33]. Oncotype Dx recurrence scores (RS), which included *ESR1* RNA levels, were available in a subset of tumours (n = 430). Cut-offs of < 11, 11–25 and > 25 were defined as low, intermediate and high RS, respectively [34]. BC-specific survival (BCSS), defined as the time from initial diagnosis to time of BC related death, and disease-free survival (DFS) representing the time from diagnosis to any event, were calculated at a 10-year endpoint. ET (Tamoxifen or aromatase inhibitors (AI)) was administered as the only adjuvant therapy in 51% of our cohort, 14% of patients received only chemotherapy (cyclophosphamide, methotrexate and 5-fluorouracil), while 16% received both ET and chemotherapy. Adjuvant systemic therapies were given following multidisciplinary team decision according to ER status, NPI, menopausal status and associated comorbidities. ER-positive BC patients with good prognostic NPI received Tamoxifen if postmenopausal or both Tamoxifen and AI if premenopausal. Chemotherapy regimens were given to ER-negative patients. ER+ BC patients with moderate and poor prognostic NPI (>4.4) received ET with combined chemotherapy, if patients were fit to tolerate chemotherapy.

2.1.2. The Cancer Genome Atlas (TCGA) breast cancer dataset

A BC cohort of 1047 cases was accessed from the publicly available TCGA data (https://tcga-data.nci.nih.gov/tcga/) to identify *ESR1* mRNA expression, PAM50 molecular subtypes and RNA-seq expression. Gene expression data expressed as counts were accessed for female primary BC tumour specimens were obtained from the NCI Genomics Data Commons data portal. Cases were stratified on the basis of ER expression and differential expression analysis completed using DESeq2 [35].

2.2. Oestrogen receptor (ER) categorisation

In the Nottingham cohort, ER positivity was first defined when ≥ 1% of invasive tumour cells expressed ER-nuclear staining [2,29]. ER expression was further categorised according to ER percentage, intensity, and H-scores. ER percentages were grouped into 10 categories: negative (<1%), 1–9%, 10–29%, 30–49%, 50–59%, 60–69%, 70–79%, 80–89%, 90–99% and 100% expression. H-scores were calculated as per McCarty et al., where the percentage of ER-positive cells was multiplied by a score (1−3) based on staining intensity from weak (1) to strong (3) to yield a final score ranging from 0 to 300 [22]. H-scores were categorised by 30-H score increments (Supplementary Figure 1).

Outcome analysis was performed on ET treated patients to evaluate response to therapy in the different ER groups. Each group was compared with other groups to identify cut-offs associated with differential response and patient outcomes. In subsequent outcome analyses, combination into five groups: ER-negative (<1%), ER low positive (1–9%), ER low-moderate (10–49%), ER moderate-high (50–99%), and ER-high (100%) was carried out. Staining intensity was evaluated as weak, moderate, or strong.

In the TCGA cohort, ER expression categorised in 10% increments were available and, for meaningful statistical analysis, were also combined into five groups (ER-negative, ER 1–9%, ER 10–49%, ER 50–89% and ER 90–100%).

2.3. Identification of differentially expressed genes and enrichment pathway analysis

The comparison groups involved BCs expressing ER < 10% vs ER 10–49%, ER 10–49% vs ER 50–89%, and ER 50–89% vs ER 90–100% (the highest expression category). The selected groups were used to

further validate the cutoffs generated by clinicopathological and outcome analysis in Nottingham cohort. Significantly differentially expressed genes (DEGs) defined as possessing log2 fold change $> \pm 1$ and adjusted p-value < 0.05) and were selected for analysis. Pathway analysis was performed on SHINNY GO enrichment analysis server [36], where log2 fold change ($\geq \pm 1$) and false discovery rate (FDR= 0.1) were set for significance.

2.4. Statistical analysis

Statistical tests were performed using the Statistical Package for the Social Sciences (SPSS) v28 software, (Chicago, IL, USA). Chi square tests were used to identify associations in ER categories and clinicopathological parameters, and for the comparison of molecular subtyping in each category. Continuous variables were analysed using the Mann Whitney U test. Correlations were tested using the Spearman's rank correlation coefficient test. Kaplan Meier curves and Log-rank test were used for outcome analysis. A *p*-value (two-tailed) of < 0.05 was considered significant in all tests. The web tools ShinnyGO and ClustVis were used for data visualisation.

3. Results

3.1. Patient and tumour characteristics of the Nottingham cohort

ER percentage staining of the whole cohort ranged from 0% to 100% (mean = 72%), while the mean H-score was 157 (range 0–300). The frequency distribution of each percentage and H-score category is illustrated in Supplementary Figure 1. Tumours with an ER score of $\geq$ 1% constituted 81% of the cohort. The distribution of ER expression within different subgroups is presented in Table 1. Sixty-five percent of tumours were PR positive, 13% HER2 positive and 51% had a high Ki67 proliferation index.

3.2. Correlation of ER expression with the other clinicopathological parameters

There was a significant proportional association between the gradual increase in ER expression, assessed according to ER percentage and H-score, and favourable clinicopathological prognostic parameters including older age at diagnosis, smaller tumour size, lower tumour grade, lower NPI, lymph node stage 1, absence of LVI, higher PR positivity, lower Ki67 proliferation index and negative HER2 status (Table 1).

ER expression from 1% to 9% were similar in their clinicopathological characteristics to ER-negative tumours apart from increasing levels of PR expression in the ER 1–9% tumours. The first significant cut-off was observed at $\geq$ 10%. Increasing positive associations with parameters of good prognosis were observed between BCs with 1–9% ER versus BCs with 10–49% ER and also between tumours with 1–9% versus those with 10–19% ER positivity particularly with regard to histological grade and NPI scores (Table 2 **and** Supplementary Tables 1 to 5).

The second significant cut-off was observed at $\geq$ 50% with differences observed between BCs with 10–49% ER versus those with 50–99% ER. The third significant cut-off was observed at 100% (Tables 2 and 3) in which differences in the association with the clinicopathological parameters were observed between BCs with 100% ER expression and tumours with lower levels of ER expression, even at levels as high as 90–99%. In tumours with 100% ER positivity, there was a further positive association between staining intensity and parameters of good prognosis (Table 4).

3.3. Association of ER expression with Oncotype Dx recurrence score (RS)

A significant negative correlation was observed between Oncotype

Table 1
The association of oestrogen receptor (ER) continuous scores with the clinicopathological parameters.

Characteristics	ER percentage			ER H-score		
	Mean Rank	N (%)	*p*-value	Mean Rank	N (%)	*p*-value
Age at diagnosis (years)	3055	1967	<	2786	1957	<
	4035	(26)	**0.001**	4095	(26)	**0.001**
< 50		5592			5550	
≥ 50		(74)			(74)	
Tumour size (cm)	4012	4695	<	4003	4664	<
< 2	3396	(62)	**0.001**	3342	(62)	**0.001**
≥ 2		2862			2841	
		(38)			(38)	
Tumour grade	4718	1276	<	4691	1275	<
1	4494	(17)	**0.001**	4450	(17)	**0.001**
2	2610	3322		2546	3301	
3		(44)			(44)	
		2952			2922	
		(39)			(39)	
Mitotic count	4586	4035	<	4609	4019	<
1	3635	(53)	**0.001**	3516	(54)	**0.001**
2	2348	1357		2281	1340	
3		(18)			(18)	
		2158			2139	
		(29)			(28)	
Nuclear pleomorphism	4302	124	<	4013	123	<
	4697	(2)	**0.001**	4708	(2)	**0.001**
1	3170	2901		3123	2895	
2		(38)			(39)	
3		4525			4480	
		(60)			(59)	
Tubule formation	4625	570	<	4637	568	<
1	4166	(8)	**0.001**	4062	(8)	**0.001**
2	3519	2021		3519	2010	
3		(27)			(27)	
		4959			4920	
		(66)			(65)	
Nottingham Prognostic Index	4579	2975	<	4578	2964	<
	3335	(39)	**0.001**	3297	(40)	**0.001**
Good Prognostic Group	2779	3612		2709	3576	
		(48)			(48)	
Moderate Prognostic Group		929			925	
		(12)			(12)	
Poor Prognostic Group						
Histological types	3430	4836	<	3400	4794	<
No special type (NST)	4398	(64)	**0.001**	4408	(64)	**0.001**
	4132	837		4302	837	
Lobular	4466	(11)		4363	(11)	
Other special types[a]		452			451	
		(6)			(6)	
Mixed NST and other tumour types		1423			1414	
		(17)			(19)	
Axillary nodal status	3873	5021	<	3866	4985	<
	3535	(67)	**0.001**	3471	(67)	**0.001**
Negative		2499			2484	
Positive		(33)			(33)	
Lymph node stage	3873	5021	<	3866	4985	<
1 (Negative)	3709	(67)	**0.001**	3634	(67)	**0.001**
2 (1–3 positive)	2935	1938		2910	1925	
3 (>3 positive)		(26)			(26)	
		561			559	
		(7)			(7)	
Lymphovascular invasion	3386	5908	<	3920	5865	<
	3872	(78)	**0.001**	3130	(78)	**0.001**
Negative		1639			1631	
Positive		(22)			(22)	
Progesterone receptor	2177	2560	<	2159	2537	<
	2390	(35)	**0.001**	4364	(35)	**0.001**
Negative		2647			4629	
Positive		(65)			(65)	
Human epidermal growth factor receptor 2	3767	6225	<	2738	6195	<
	2390	(87)	**0.001**	2410	(87)	**0.001**
		945			633	
Negative		(13)			(13)	
Positive						

(*continued on next page*)

Table 1 (*continued*)

Characteristics	ER percentage			ER H-score		
	Mean Rank	N (%)	*p*-value	Mean Rank	N (%)	*p*-value
Ki67 index	1857	1541	<	1863	1538	<
Low (≤14%)	1334	(48)	**0.001**	1332	(48)	**0.001**
High (>14%)		1642 (51)			1639 (52)	
Oncotype Dx recurrence score	233	81	<	251	81	**0.002**
Low (<11)	225	(19)	**0.001**	215	256	
Intermediate (11–25)	173	257 (60)		184	92	
High (>25)		92 (21)				
Endocrine therapy	2244	2408	<	2407	2017	**<0.001**
No	4323	(33)	**0.001**	2845	4426	
Yes		4860 (67)				
Chemotherapy	4114	5132	<	4088	5120	**<0.001**
No	2546	(70)	**0.001**	2582	2161	
Yes		2165 (30)				

Significant *p*-values are in bold.

[a] Special histological tumour types include mucinous, tubular, metaplastic, papillary, adenoid cystic and cribriform carcinomas.

Dx RS and ER expression ($r = -0.26$, $p < 0.001$) and H score ($r = -0.19$, $p < 0.001$) (Table 1). Oncotype Dx RS was high in 50% of tumours with 1–50% ER compared with 19% in the > 90% ER group ($p = 0.01$). Fifty-five percent of tumours with a H-score < 100 had high RS compared to 17% with a H-score > 200 ($p = 0.006$).

3.4. Outcome analyses in Nottingham cohort

Survival analysis of ET-treated patients was evaluated with respect to ER expression categories **(**Supplementary Figure 2**)**. Compared to patients with ER-negative tumours, those with 1–9% ER-positive tumours did not show a significant difference in outcome, while a significantly favourable outcome was observed in the 10–19% ER-positive group ($p = 0.03$; Supplementary Figure 3**)**.

In ET-treated patients with 10–49% ER-positive tumours, no significant outcome differences were observed between subgroups defined according to 10% incremental increase. Similarly, in patients with 50–99% ER positive tumours, no significant outcome difference was observed between subgroups according to 10% incremental increase. Patients with 50–99% ER-positive tumours had a significantly better outcome than those with 10–49% ER-positive tumours ($p = 0.01$) and worse outcome than patients with 100% ER-positive tumours.

BC patients with 100% ER-positive tumours had the most favourable outcome in terms of longer survival ($p = 0.002$) compared to those with BCs expressing ER in < 100% of cells, including tumours with 90–99% ER positivity **(**Fig. 1**,** Supplementary Figure 4**)**.

There was a significant correlation between the proportional increase in ER staining intensity and prolonged survival ($p < 0.001$) in patients with tumours showing the same percentage of ER positive tumour cells **(**Fig. 1**)**. Patients with 90–99% ER-positivity and strong intensity, had a more favourable outcome than those with 100% ER-positive tumours with low staining intensity ($p = 0.01$; Supplementary Figure 5).

BC patients with H-score ≥ 30 had more favourable outcome in terms of BCSS ($p = 0.01$) and DFS ($p = 0.02$) than those with H-scores less than 30. Notably, BC with H-scores > 0 and < 30 (n = 161) comprised of a mix of tumours; 55% scored as < 10% staining with weak intensity, 7% staining in < 10% with strong intensity, and 38% staining in ≥ 10% with weak intensity.

A second significant association with favourable outcome was observed in tumours with a H-score of 100/300. No outcome differences were shown between subgroups of tumours within score range 100 to

Table 2
The association of oestrogen receptor (ER) 1–9%, ER 10–49% and ER 50–99% tumours with clinicopathological parameters.

Characteristics	ER IHC expression				
	ER 1–9% N (%)	ER 10–49% N (%)	X^2 (*p*-value)[b]	ER 50–99% N (%)	X^2 (*p*-value)[c]
Age at diagnosis (years)	42 (34)	84 (37)	0.46 (0.5)	670 (35)	0.6 (0.4)
< 50	82 (66)	140 (63)		1250 (65)	
≥ 50					
Tumour size (cm)	59 (48)	136 (61)	5.6 (**0.01**)	1197 (62)	0.23 (0.6)
< 2	65 (52)	88 (40)		723 (38)	
≥ 2					
Tumour grade	4 (3)	22 (10)	16.8 (**<0.001**)	348 (18)	42.5 (**<0.001**)
1	23 (20)	80 (36)		947 (49)	
2	89 (77)	121 (54)		625 (33)	
3					
Mitotic count	19 (16)	75 (34)	12.3 (**0.002**)	1138 (59)	54.1 (**<0.001**)
1	34 (29)	61 (27)		346 (18)	
2	63 (54)	87 (39)		436 (23)	
3					
Nuclear pleomorphism	2 (2)	1 (1)	15.2 (**<0.001**)	34 (2)	23.2 (**<0.001**)
1	11 (9)	58 (26)		780 (40)	
2	103 (89)	164 (74)		1106 (58)	
3					
Tubule formation	2 (2)	16 (7)	7 (**0.03**)	164 (9)	4.7 (0.09)
1	21 (18)	54 (24)		581 (30)	
2	93 (80)	153 (69)		1175 (61)	
3					
Nottingham Prognostic Index	10 (9)	58 (26)	15.7 (**<0.001**)	806 (42)	21.9 (**<0.001**)
Good Prognostic Group	83 (73)	120 (54)		863 (45)	
Moderate Prognostic Group	20 (18)	43(20)		250 (13)	
Poor Prognostic Group					
Histological types	101 (87)	158 (65)	11.1 (**0.01**)	1066 (55)	20 (**<0.001**)
No special type (NST)	4 (3)	18 (11)		287 (15)	
Lobular	3 (3)	12 (5)		129 (7)	
Other special types	8 (7)	35 (18)		438 (23)	
Mixed NST and other tumour types					
Axillary nodal status	75 (66)	130 (59)	1.7 (0.1)	1208 (63)	1.6 (0.2)
Negative	39 (34)	92 (41)		711 (37)	
Positive					
Lymph node stage	75 (66)	130 (60)	1.7 (0.4)	1208 (63)	2.5 (0.2)
1 (Negative)	28 (25)	66 (29)		540 (28)	
2 (1–3 positive)	11 (9)	26 (11)		171 (9)	
3 (>3 positive)					
Lymphovascular invasion	95 (83)	159 (72)	5.6 (**0.01**)	1450 (76)	1.6 (0.2)
Negative	19 (17)	63 (28)		470 (24)	
Positive					
Progesterone receptor	83 (82)	98 (45)	38.5 (**<0.001**)	370 (20)	70.2 (**<0.001**)
Negative	18 (18)	119 (55)		1480 (80)	
Positive					
Human epidermal growth factor receptor 2	74 (69)	145 (68)	0.02 (0.8)	1603 (87)	59.2 (**<0.001**)
Negative	34 (31)	69 (32)		231 (13)	
Positive					
Ki67 index	3 (14)	40 (41)	5.4 (**0.02**)	449 (55)	6.5 (**0.01**)
Low (≤14%)	18 (86)	57 (59)		363 (45)	
High (>14%)					

Significant *p*-values are in bold.
[b] Comparison of ER1–9% and ER 10–49%
[c] Comparison of ER 10–49% and ER 50–99%

< 200, while significantly improved outcome was noted in BC patients with H-scores ≥ 200/300 (Fig. 1).

Regarding chemotherapy treated BC patients (high risk patients), significantly favourable outcome differences were shown with higher compared to lower ER expression. However, highly ER expressing BC patients were shown to have a notable increase in the event rate in long-term outcomes (8–10 years), where they experienced survival events equivalent to patients with ER-negative BC (Supplementary Figure 6). Additionally, survival outcomes were tested in chemo-naive patients (low risk patients), stratified according to ER levels, with favourable outcomes associated with high ER expression, both short and long term (Supplementary Figure 7).

Multivariate Cox Regression analysis revealed that higher ER percentage expression was an independent predictor of improved patient outcome when adjusted for histological grade, tumour size and LN status. With every 10% increase in ER percentage the risk of death from BC decreased by 5% (HR=0.95, 95% CI=0.938–0.968, $p < 0.001$) and disease recurrence by 4% (HR=0.96, 95% CI= 0.955–0.978, $p < 0.001$) (Table 5A). Similar findings were observed when H-score was evaluated as an independent prognostic indicator for outcome, where BC death risk decreased by 4% (HR=0.96, 95% CI=0.96–0.975, $p < 0.001$) and disease recurrence by 3% (HR=0.97, 95%CI=0.968–0.979, $p < 0.001$) with every 10 unit increase in H-score (Table 5B). However, when both ER percentage expression and H-score were tested together, H-score emerged as an independent prognostic marker of BCSS and DFS (Table 5C).

3.5. Association of ER expression with PAM50 molecular subtyping and ESR1 mRNA expression and ER-related genes

Using the TCGA-BRCA dataset, there was no significant difference in the distribution of intrinsic molecular subtypes between tumours in the ER 1–9% category and ER negative tumours. Non-luminal BC subtypes were predominant in the 1–9% expression category (p=0.008) compared with the 10–49% subgroup. ER 10–49% tumours were significantly different from ER 50–89% tumours ($p < 0.001$; Supplementary Table 7), with 30% of ER 10–49% presenting as a non-luminal subtype compared to 4% in the ER 50–89% category. PAM50 molecular subtyping of each ER category is presented in Supplementary Figure 8.

ESR1 mRNA expression was positively correlated with IHC ER expression. Significant differences in mRNA expression were demonstrated between ER 1–9% versus 10–49% ($p = 0.005$), ER 10–49% versus 50–89% ($p = 0.03$) and ER 50–89% versus 90–100% ($p < 0.001$; Supplementary Table 7). Investigation of expression of genes involved in ER-signalling pathway (ER related/responsive genes) [37–41] demonstrated increasing ER expression associated with higher mean RNA-seq values of ER related genes (Supplementary Table 8), as shown in the plotted heatmap (Fig. 2).

3.6. Differential gene expression, enrichment pathway analysis and gene ontologies

The differential gene expression analysis between tumours expressing ER < 10% and those expressing ER in 10–49% of tumour cells in TCGA-BRCA dataset identified 5129 DEGs including 1722 with increased expression and 3407 with reduced mRNA expression in the ER 10–49% group compared to ER < 10%. Over-expressed genes involved *ESR1, TFF1, PGR, FOXA1, GATA3, AGR3, TFF3, KCNJ3, SCUBE2, NAT1* and *RIMS4* genes which are known to be ER-target genes. Pathway enrichment analysis revealed involvement in oestrogen signalling pathway, PPAR, IL-17 and cAMP signalling pathways (Supplementary Figure 9). Furthermore, 176 were over-expressed and 1146 were under-

Table 3
The clinicopathological differences between tumours with oestrogen receptor (ER) 50–99% and ER 90–99% and tumours with ER 100%.

Characteristics		ER IHC expression			
	ER 50–99% N (%)	ER 90–99% N (%)	ER 100% N (%)	X^2 (*p*-value)[d]	X^2 (*p*-value)[e]
Age at diagnosis (years)	670 (35)	321 (29)	624 (17)	241.8 **(<0.001)**	12.1 **(<0.001)**
< 50	1250 (65)	786 (71)	3135 (83)		
≥ 50					
Tumour size (cm)	1197	715 (65)	2554 (68)	17.8 **(<0.001)**	0.46 (0.4)
< 2	(62)	392 (35)	1205 (32)		
≥ 2	723 (38)				
Tumour grade	348 (18)	213 (19)	872 (23)	81.7 **(<0.001)**	0.34 (0.8)
1	947 (49)	569 (51)	2070 (55)		
2	625 (33)	325 (30)	817 (22)		
3					
Mitotic count	1138	697 (63)	2635 (70)	95 **(<0.001)**	2.1 (0.3)
1	(59)	171 (17)	631 (17)		
2	346 (18)	219 (20)	493 (13)		
3	436 (23)				
Nuclear pleomorphism	34 (2)	18 (2)	75 (2)	75.6 **(<0.001)**	0.63 (0.7)
1	780 (40)	469 (42)	1976 (53)		
2	1106 (58)	620 (56)	1708 (45)		
3					
Tubule formation	164 (9)	104 (9)	374 (10)	3.1 (0.2)	1 (0.6)
1	581 (30)	327 (30)	1141 (30)		
2	1175 (61)	676 (61)	2244 (60)		
3					
Nottingham Prognostic Index	806 (42)	499 (45)	1966 (53)	81.5 **(<0.001)**	0.58 (0.7)
Good Prognostic Group	863 (45)	479 (43)	1509 (40)		
Moderate Prognostic Group	250 (13)	129 (12)	271 (7)		
Poor Prognostic Group					
Histological types	1066	611 (55)	2132 (57)	3.2 (0.3)	2.8 (0.4)
No special type (NST)	(55)	162 (15)	497 (13)		
Lobular	287 (15)	83 (7)	251 (7)		
Other special types	129 (7)	251 (23)	879 (23)		
Mixed NST and other tumour types	438 (23)				
Axillary nodal status	1208	710 (64)	2647 (71)	55.3 **(<0.001)**	0.1 (0.7)
Negative	(63)	397 (36)	1099 (29)		
Positive	711 (37)				
Lymph node stage	1208	710 (64)	2647 (71)	48.8 **(<0.001)**	0.66 (0.7)
1 (Negative)	(63)	306 (28)	926 (25)		
2 (1–3 positive)	540 (28)	91 (8)	173 (4)		
3 (>3 positive)	171 (9)				
Lymphovascular invasion	1450	857 (77)	3096 (82)	37.2 **(<0.001)**	2.5 (0.1)
Negative	(76)	250 (23)	663 (18)		
Positive	470 (24)				
Progesterone receptor	370 (20)	203 (19)	617 (17)	7.2 **(0.007)**	0.02 (0.8)
Negative	1480 (80)	858 (81)	3000 (83)		
Positive					
Human epidermal growth factor receptor2	1603	942 (90)	3359 (94)	59.1 **(<0.001)**	0.07 (0.7)
	(87)	110 (10)			
	231 (13)				

(*continued on next page*)

Table 3 (*continued*)

Characteristics		ER IHC expression			
	ER 50–99% N (%)	ER 90–99% N (%)	ER 100% N (%)	X^2 (*p*-value)[d]	X^2 (*p*-value)[e]
Negative			231		
Positive			(6)		
Ki67 index	449 (55)	293 (56)	861	6	0.1
Low (≤14%)	363 (45)	232 (44)	(60)	(**0.01**)	(0.6)
High (>14%)			576		
			(40)		

Significant *p*-values are in bold.
[d] Comparison of ER 50–99% and ER 100%
[e] Comparison of ER 90–99% and ER 100%

expressed in the ER 50–89% category compared to the 10–49% category. Significant involvement in PI3K/AKT Signalling in Cancer and DNA methylation pathways was shown (Supplementary Figure 10). The comparison between the ER 90–100% group and the lower category showed 3094 over-expressed and 195 under-expressed genes. DEGs were involved in several pathways including oestrogen-dependent gene expression, ESR-mediated signalling, and cell cycle pathways. (Supplementary Figure 11). The top 10 over- and under-expressed DEGs from each comparison are illustrated in Supplementary Table 9.

4. Discussion

The clinical decision to recommend adjuvant ET to patients with primary BC is informed by ER status as determined by IHC. All BCs with at least 1% ER-positivity are currently considered positive and potentially responsive to ET. Previous studies have classified ER as negative (0%), low (1–9%) or high (≥ 10%) [3,42–46], with the "high ER" group encompassing a wide range of ER expression values. It is unclear if there is a distinct cut-off that defines optimum patient benefit from ET or if ER expression is a continuous spectrum with corresponding various response levels [47]. It has been shown that ER quantification is related to prognosis with high ER expression associated with improved prognosis and response to therapy [6,48–50]. Additionally, response to adjuvant chemotherapy is reduced in patients with tumours expressing high numbers of ER positive cells (>50%) [51]. To the best of our knowledge, this is the first study that examines IHC ER expression as a continuous variable, in large well characterised BC cohorts, with clear demonstration of an association with prognostic and predictive parameters.

Tumours with diffuse and homogeneous ER staining (100%) were associated with favourable prognostic parameters and significantly improved survival compared to those with less homogenous ER expression (as high as > 90%), reflecting the impact of even minor ER heterogeneity on biological behaviour. BC patients with tumours showing at least 50% ER positivity had considerably better outcomes compared with patients whose tumours displayed less than 50% ER positivity, in agreement with Goldhirsch et al., who proposed that ≥ 50% BC ER positivity is required for response to ET [52]. Zhang et al., who categorised ER expression into five groups (< 1%, 1–10%, 11–50%, 51–70% and > 70%), reported that BCs with ER positivity in > 70% of tumour cells represented a distinct biological category. Although those authors did not find significant differences in pathological features between patients in the ER 11–50% and ER 51–70% subgroups, this was likely a reflection of the small sample size of the cohort (340 patients) [53]. The lower ER threshold of 50% observed in our study may reflect cohort dependent differences. Yi and colleagues also used a quantitative approach for assessment of ER expression (<1%, 1–39%, 40–59%, 60–79%, and 80–100%) and reported an inverse correlation between increasing ER expression and BC-related death [54].

A significantly favourable DFS was observed in patients with tumours showing with ≥ 10% ER positivity compared with tumours with

Table 4
The association of oestrogen receptor (ER) staining intensity with clinicopathological parameters.

Characteristics	ER staining intensity				
	Weak N (%)	Moderate N (%)	X^2 (*p*-value)[f]	Strong N (%)	X^2 (*p*-value)[g]
Age at diagnosis (years)	670 (35)	248 (17) 1230 (83)	78.4 (**<0.001**)	123 (8) 1397 (92)	52.3 (**<0.001**)
< 50	1250 (65)				
≥ 50					
Tumour size (cm)	492 (65)	1002 (68) 476 (32)	2.2 (0.1)	1060 (70) 460 (30)	1.3 (0.2)
< 2	269 (35)				
≥ 2					
Tumour grade	153 (19)	337 (21) 829 (56)	18 (**<0.001**)	382 (25) 855 (56)	4 (0.1)
1	386 (50)	401 (23)			
2	312 (31)			283 (19)	
3					
Mitotic count	458 (60)	1041 (70) 251 (17)	24.9 (**<0.001**)	1136 (75) 217 (14)	7 (**0.02**)
1	163 (22)	186 (13)			
2	140 (18)			167 (11)	
3					
Nuclear pleomorphism	22 (2) 326 (41)	26 (2) 772 (53) 680 (45)	19 (**<0.001**)	27 (2) 878 (58) 615 (40)	9.5 (**0.009**)
1	413 (57)				
2					
3					
Tubule formation	64 (9) 261 (34)	150 (10) 438 (30) 890 (60)	5.8 (0.05)	160 (11) 442 (29) 918 (60)	0.19 (0.9)
1	436 (57)				
2					
3					
Nottingham Prognostic Index	353 (46) 327 (43) 80 (11)	766 (52) 606 (41) 100 (7)	12.2 (**0.002**)	874 (56) 576 (38) 91 (6)	4.7 (0.09)
Good Prognostic Group					
Moderate Prognostic Group					
Poor Prognostic Group					
Histological types	437 (55) 97 (15) 38 (7) 189 (23)	483 (57) 199 (13) 89 (6) 347 (24)	1.5 (0.6)	852 (56) 201 (13) 124 (8) 343 (23)	5.2 (0.1)
No special type (NST)					
Lobular					
Other special types					
Mixed NST and other tumour types					
Axillary nodal status	494 (65) 266 (35)	1030 (70) 442 (30)	5.7 (**0.01**)	1123 (74) 391 (26)	6.5 (**0.01**)
Negative					
Positive					
Lymph node stage	494 (65) 221 (29) 45 (6)	1030 (70) 373 (25) 69 (5)	5.9 (0.05)	1123 (74) 332 (22) 59 (4)	6.6 (**0.03**)
1 (Negative)					
2 (1–3 positive)					
3 (>3 positive)					
Lymphovascular invasion	547 (72) 214 (28)	1200 (81) 278 (19)	25.4 (**<0.001**)	1349 (89) 171 (11)	33.6 (**<0.001**)
Negative					
Positive					
Progesterone receptor	128 (18) 601 (82)	242 (17) 1189 (83)	0.14 (0.7)	247 (17) 1210 (83)	0.001 (0.9)
Negative					
Positive					
HER2 status	686 (94) 84 (6)	1314 (93) 103 (7)	0.39 (0.5)	1359 (94) 80 (6)	3.5 (0.06)
Negative					
Positive					

(*continued on next page*)

Table 4 (*continued*)

Characteristics	ER staining intensity				
	Weak N (%)	Moderate N (%)	X^2 (*p*-value)[f]	Strong N (%)	X^2 (*p*-value)[g]
Ki67 index	295	318 (59)	0.09	248	2.4
Low (≤14%)	(58)	222 (41)	(0.7)	(64)	(0.1)
High (>14%)	214			140	
	(42)			(36)	

Significant *p*-values are in bold.
[f] Comparison of weak and moderate intensity
[g] Comparison of moderate and strong intensity

0–9% ER. In previous guidelines, a cut-off of 10% was suggested as the threshold for ER positive status [46,55–57]. However, a meta-analysis of 20 clinical trials using data on ET response after 5-years of tamoxifen treatment reported that ER status is the only independent factor for response with no significant difference observed in tumours with higher (ER ≥ 200 fmol/mg) compared with lower levels of expression (ER 10–19 fmol/mg) [4,37,58–62]. In most of these studies, ER was measured by ligand-binding assays which may not accurately reflect IHC ER levels. The European Commission Initiative on Breast Cancer (ECIBC) Guideline Development Group (GDG) [63] recommended 1% IHC staining as the threshold for ER-positive status and suggested monitoring low (1–9%) and high (10% and above) ER-positivity in relation to patient outcome to better assess ER thresholds for treatment. In the most recent ASCO/CAP update, breast tumours with ER levels of 1–10% are designated ER *low* positive [16]. In the guidelines, a recommendation was made for such cases with 1–10% ER expression to acknowledge the more limited data on endocrine responsiveness in this group and overlapping features with ER negative cancers.

Increasing BC H-score values have been shown to correlate with longer survival [54,64] where the 10-year survival of ET-treated patients with tumour H-score > 50 was 71% compared to 41% below this cut point [64]. More detailed stratification was performed in the present study with significantly improved survival outcomes observed at cut-off H-scores of 30, 100 and 200. Overall, BC death risk decreased by 4% with every 10 unit increase in H-score compared with an 8% decrease

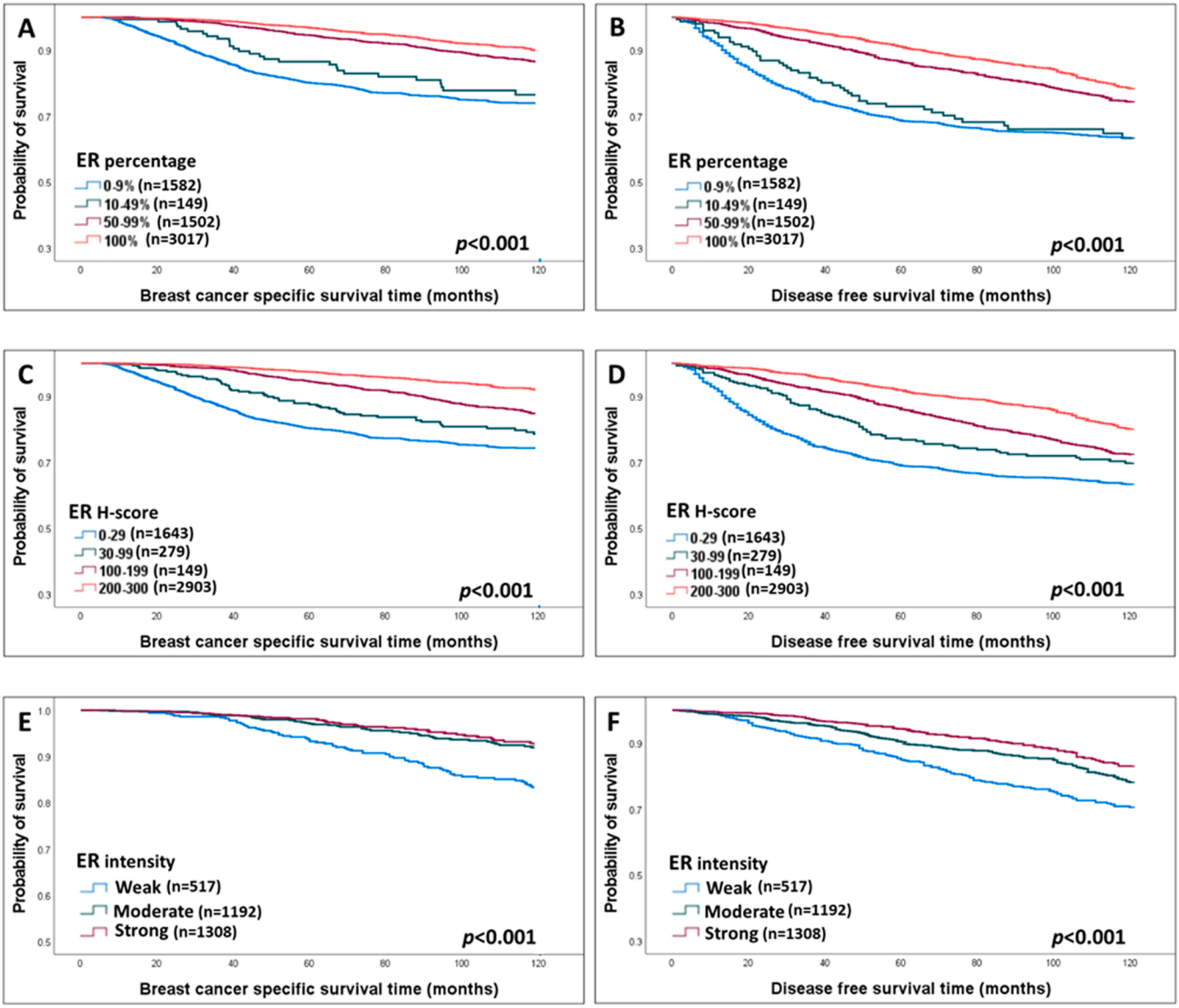

Fig. 1. A-D: Kaplan-Meier (KM) survival plots of endocrine-treated patients (patients received endocrine treatment regardless of additional chemotherapy administration) show favourable breast cancer-specific survival (BCSS) and disease-free survival (DFS) with higher ER expression categories based on ER percentage (A and B) and H-scores (C and D) (Note: patients with ER 0–9% and H score 0–29 did not receive endocrine therapy but included in this curve as a control). **E-F**: KM plots of BC patients showing ER expression in 100% of the tumour indicate favourable BCSS (E), and DFS (F) based on the intensity of staining.

Table 5
Multivariate Cox Regression analysis shows prognostic variables for breast cancer specific survival and disease free survival.

A					B					C				
Feature	BCSS		DFS		Feature	BCSS		DFS		Feature	BCSS		DFS	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
ER percentage (Continuous variable, per 10-unit increase)	0.9 (0.938–0.968)	**< 0.001**	0.9 (0.955 −0.978)	**< 0.001**	**H-score** (Continuous variable, per 10-unit increase)	0.9 (0.96–0.975)	**< 0.001**	0.9 (0.968–0.979)	**< 0.001**	**ER percentage** (Continuous variable, per 10-unit increase)	1 (0.963–1.05)	0.7	1 (0.994–1.05	0.1
Grade (1 and 2 vs 3)	0.4 (0.316–0.433)	**< 0.001**	0.6 (0.551–0.687)	**< 0.001**	**Grade** (1 and 2 vs 3)	0.4 (0.332–0.4)	**< 0.001**	0.7 (0.598–0.758)	**< 0.001**	**H-score** (Continuous variable, per 10-unit increase)	0.9 (0.945–0.984)	**< 0.001**	0.9 (0.950–0.977)	**< 0.001**
Lymph node status (negative vs positive)	0.4 (0.376–0.491)	**< 0.001**	0.6 (0.538–0.654)	**< 0.001**	**Lymph node status** (negative vs positive)	0.4 (0.374–0.497)	**< 0.001**	0.6 (0.531–0.655)	**< 0.001**	**Grade**(1 and 2 vs 3)	0.4 (0.331–0.469)	**< 0.001**	0.6 (0.5–0.7)	**< 0.001**
Tumour size (<2 cm vs ≥2 cm)	0.6 (0.503–0.661)	**< 0.001**	0.6 (0.577–0.704)	**< 0.001**	**Tumour size** (<2 cm vs ≥2 cm)	0.6 (0.512–0.686)	**< 0.001**	0.6 (0.569–0.705)	**< 0.001**	**Lymph node status** (negative vs positive)	0.4 (0.374–0.498)	**< 0.001**	0.5 (0.4–0.7)	**< 0.001**
-	-	-	-	-	-	-	-	-	-	**Tumour size** (<2 cm vs ≥2 cm)	0.6 (0.512–0.686)	**< 0.001**	0.6 (0.5–0.7)	**< 0.001**

BCSS, Breast cancer specific survival; DFS, Disease free survival; HR, Hazard ratio; CI, Confidence interval.
Significant *p*-values are in bold.

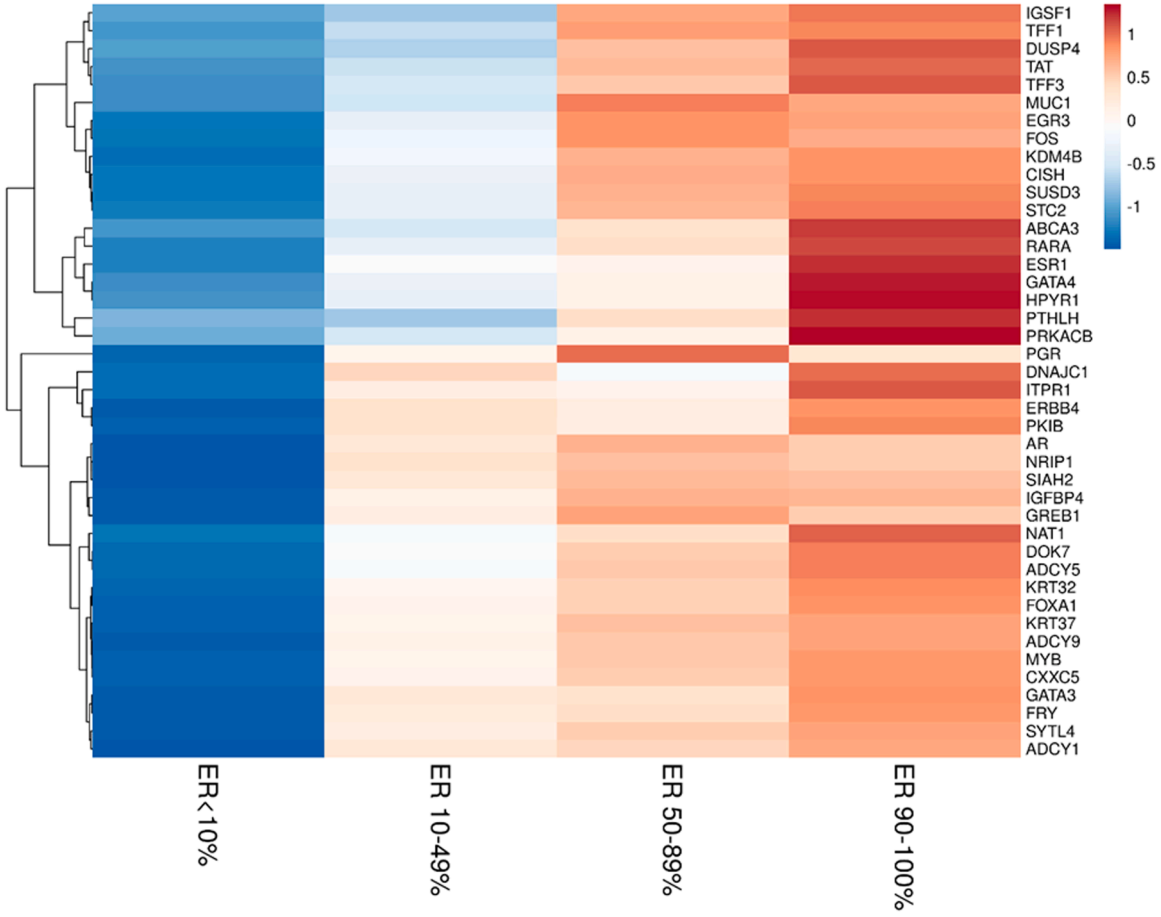

Fig. 2. A heatmap showing the expression of ER-related genes in relation to the level of ER protein expression.

for each 20 unit increase in H-score reported by Ma et al [54]. Hill and colleagues highlighted the importance of ER staining intensity as an independent prognostic marker [65]. This is supported by our finding that increased staining intensity in homogenous ER positive tumours was associated with favourable outcome. Patients whose tumours displayed strong ER staining intensity and low percentage cell expression were comparable in their survival to those with high percentage of ER positive cells but weak intensity. The Allred score [66], which also provides a combined measurement of intensity and proportion of stained cells, and was used more frequently in clinical trials shows good overall correlation to H-score [67] with some discordance in ER low tumours due to the way each score is calculated [68,69], and interobserver variability at such lower ER expression [6].

Increasing ER expression was associated with favourable clinicopathological characteristics especially at the extremes of positivity. Consistent with published data [53], higher ER expression correlated with low histological grade and higher PR expression, the latter an ER-regulated gene that is utilised as a predictor of functional ER (66–68). Ki67, a cell proliferation marker [70], was highly expressed in low rather than high expressing ER BCs, in keeping with the favourable effect of ER high positivity on cell cycle regulation. The inverse correlation between ER expression and the Oncotype Dx RS affirms the association of ER highly expressing tumours and lower risk of recurrence [71].

According to local treatment protocols, high risk ER-positive BC patients, assessed through NPI, are given chemotherapy. Our study demonstrated the favourable outcomes of high ER expressing tumours in chemotherapy treated patients in the early follow-up period. However, long-term follow up demonstrated outcome similarity between patients with high ER and ER-negative tumours. This may refer to the weak long-term response of high-risk ER-positive BC patients to chemotherapy. Therefore, studies on the impact of chemotherapy on ER-positive BC should address the long-term survival outcomes to avoid different time-dependant survival patterns.

The representation of non-luminal BC subtypes in the ER 1–9% group highlights the similarity of ER-low positive to ER-negative tumours. The presence of non-luminal tumours within some of the high ER categories tumours may reflect intra-tumoural molecular heterogeneity.

ER protein expression was positively correlated with *ESR1* mRNA expression, as shown previously [72,73]. Significantly different *ESR1* mRNA levels were noted between different ER categories, except for the ER-low positive and ER-negative groups. Although the complexity of downstream ER signalling and the impact of various co-regulators is poorly understood, ER-positive BC appear to be distinct from ER-negative tumours with regard to a range of ER related genes and the results of our study provide potential proof of the direct relationship between ER expression and target gene response [74,75]. Further characterisation of these genes will enhance our knowledge of the biological process guided by ER and the possible impact of ER negative clones on the activity of target genes in ER-positive tumours that may assist formulation of treatment regimes in the future [76,77].

The biological effect of ER was also evident in the DEGs, and pathways involvement identified between different ER groups. An enrichment of genes involved in cell cycle pathway has been observed. Although ER is an essential regulator of normal breast tissue growth, the mechanism by which it influences cell proliferation is unclear [78]. IL-17 signalling pathway, known as a promoter for tumour proliferation and metastasis [79,80], was enriched in our study, which was previously identified to have higher expression in ER-negative compared to ER-positive BCs [81]. A potential anti-tumour function for PPAR

signalling, one of the enriched pathways, has been reported [82]. Besides its role in controlling the expression of genes involved in cell proliferation, and apoptosis, a cross talk between ER and PPAR genes has been documented [83]. PI3K/AKT, a proliferative driver, also exhibited lower expression in tumours with high ER expression, supporting the favourable effect of ER.

We acknowledge that our study has some limitations. Despite utilising a large well characterised cohort, it is important to recognise the retrospective nature of the study when interpreting the results. Furthermore, ER scoring is subject to interobserver variability either in reporting ER percentage or H-scores, especially in ER-low positive tumours [23,84], which could have affected the cutoffs used in our study. The prognostic and predictive value of ER staining intensity need to be further validated on large cohorts using different ER antibody clones used in the clinical settings.

In conclusion, our results demonstrate that response to ET is related to the actual level of ER expression on a continuous scale. Consideration of the intensity of ER staining and use of the H-score adds further prognostic and predictive information.

Ethical approval

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 were fully anonymised. All procedures performed in the study were in accordance with the Declaration of Helsinki.

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CRediT authorship contribution statement

SM took the lead in writing the manuscript, data analysis and interpretation. CQ, MT, NPM and MA contributed to data analysis and reviewing the article. CSR, NA and AI helped in data interpretation and reviewing the article. ER conceived and planned the presented idea, data interpretation and reviewing the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.113473.

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