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Original Research

Characterisation of luminal and triple-negative breast cancer with HER2 Low protein expression

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ABSTRACT

Background: Breast cancer (BC) expressing low levels of human epidermal growth factor receptor 2 (HER2 Low) is an emerging category that needs further refining. This study aims to provide a comprehensive clinico-pathological and molecular profile of HER2 Low BC including response to therapy and patient outcome in the adjuvant and neoadjuvant settings.

Methods: Two different independent and well-characterised BC cohorts were included. Nottingham cohort (A) (n = 5744) and The Cancer Genome Atlas (TCGA) BC cohort (B) (n = 854). The clinical, molecular, biological and immunological profile of HER2 Low BC was investigated. Transcriptomic and pathway enrichment analyses were performed on the TCGA BC cohort and validated through next-generation sequencing in a subset of Nottingham cases.

Results: Ninety percent of HER2 Low tumours were hormone receptor (HR) positive (HR+), enriched with luminal intrinsic molecular subtype, lacking significant expression of HER2 oncogenic signalling genes and of favourable clinical behaviour compared to HER2 negative (HER2-) BC. In HR+ BC, no significant prognostic differences were detected between HER2 Low and HER2- tumours. However, in HR- BC, HER2 Low tumours were less aggressive with longer patient survival. Transcriptomic data showed that the majority of HR- /HER2 Low tumours were of luminal androgen receptor (LAR) intrinsic subtype, enriched with T-helper lymphocytes, activated dendritic cells and tumour associated neutrophils, while most HR-/HER2- tumours were basal-like, enriched with tumour associated macrophages.

Conclusion: HER2 Low BC is mainly driven by HR signalling in HR+ tumours. HR-/HER2 Low tumours tend to be enriched with LAR genes with a unique immune profile.

1. Introduction

The binary categorisation of human epidermal growth factor receptor 2 (HER2) expression in breast cancer (BC) into HER2 positive (HER2+) tumours, that either overexpress HER2 protein on immunohistochemistry (IHC) or display borderline IHC expression with evidence

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of *HER2* gene amplification, and HER2 negative (HER2-) has evolved. With the introduction of antibody–drug conjugates (ADC) that target HER2 protein such as trastuzumab deruxtecan (T-DXd) [1,2], the clinical relevance of the traditional HER2 classification is changing. Patients with low levels of HER2 expression (HER2 Low), defined as either HER2 IHC score 1+ or IHC 2+ without evidence of *HER2* gene amplification, have proved to benefit from these new therapeutics [1–4]. As the HER2 Low category represents ~45–55% of BC [5,6], it is important to accurately identify this group of patients for clinical management and to gain further insight into the biology and behaviour of HER2 Low BC.

The success of T-DXd compared with chemotherapy in patients with HER2 Low metastatic BC [4,7] as well as trastuzumab–duocarmazine in patients with advanced HER2 Low BC [8,9] has led to the hypothesis that HER2 Low tumours may harbour biological characteristics distinct from HER2- luminal and triple negative BC (TNBC) with distinct clinical properties and potential for new targeted therapies.

The updated American Society of Clinical Oncology / College of American Pathologists (ASCO/CAP) guidelines [10] stated that there is currently no justification for a new designation of HER2 test results for patients with IHC HER2 low levels of expression. It was considered premature to create a new (HER2 Low) category as the implication would be that tumours with HER2 Low behave differently or should be treated differently than tumours that test HER2 IHC 0. However, current evidence based on Destiny-breast04 clinical trial [4] indicates that patients with HER2 Low BC respond to T-DXd therapy tumour. No clinical trial data to support the response of HER2- (score 0) to HER2-based ADC are available and previous *in vitro* studies have demonstrated a strong quantitative relationship between antigen expression level and intracellular exposure of ADCs in cancer cells [11,12]. In line with this, the United Kingdom HER2 guidelines [13] acknowledged the existence of HER2 Low category.

Data regarding the clinico-pathologic characteristics and the prognostic impact of HER2 Low BC are limited and the published reports are inconsistent. Some studies have reported that HER2 Low tumours are more often hormone receptor positive (HR+) and are associated with higher tumour histologic grade, higher proliferation rate and increased regional lymph nodal metastases compared to HER2- tumours [5,14–17]. In contrast, others have reported that HER2 Low tumours are associated with a low proliferation index and negative lymph node (LN) status and improved patient outcome [18,19].

The crosstalk between oestrogen receptor (ER) and HER2 signalling has attracted a great deal of attention and has been studied in several clinical trials [20–24] but it remains unclear whether ER or HER2 signalling is the dominant pathway in tumours expressing low levels of HER2. TNBC is a highly diverse and heterogeneous group of tumours associated with a higher risk of local and distant recurrence and poor patient survival [25,26]. At least six molecular subtypes of TNBC; basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem like (MSL) and luminal androgen-like (LAR) have been reported [27]. Among these, the basal-like subtype is the most aggressive and least differentiated while the LAR and IM subtypes are associated with a more favourable prognosis [27,28]. Chemotherapy is the main treatment option for patients with TNBC in addition to personalised therapies such as immune checkpoint inhibitors in TNBC in the neoadjuvant setting or in patients with programmed death ligand-1 (PDL-1) positive TNBC post-surgery [29,30]. However, the response to these agents is variable which has prompted researchers to perform further immunophenotyping and genotyping of Tumour infiltrating lymphocytes (TILs) in TNBC [31]. To date, no useful data have emerged regarding the significance of TILs in TNBC /HER2- compared with TNBC HER2 Low BC. We believe that further profiling of TNBC into either HER2 Low or HER2- may enable the provision of more effective and alternative personalised therapy for patients with these tumours.

In this study, we aimed to provide an in-depth understanding of the HER2 Low category of BC with emphasis on clinical and pathological characteristics, immunophenotypic signature and molecular profile in the luminal and TNBC subtypes.

2. Materials and methods

2.1. Study cohorts and data acquisition

Two independent datasets have been enroled in this study.

(A) **The Nottingham cohort** (n = 5744) was divided into.

(A1) A BC cohort from patients who had primary breast surgery (n = 5115), many of whom were diagnosed at the Nottingham University Hospitals. Documented clinico-pathological data included age at diagnosis, histologic tumour type, histological tumour grade, tumour size, axillary LN status, lympho-vascular invasion (LVI), Nottingham prognostic index (NPI) and HER2 IHC scores (0−3). HR status, including ER and progesterone receptor (PR), in addition to Oncotype DX score, where available, were retrieved from patient records. ER and PR positivity were defined according to ASCO/CAP guidelines which stipulate a requirement for positive immunohistochemical staining in ≥1% of the invasive tumour cell nuclei [32].

HER2 staining had been completed on the Ventana Benchmark ULTRA Immunohistochemistry Automated staining system using the Ventana PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal *ready to use* primary antibody in combination with Ventana detection kits. *HER2* gene amplification status, where available, was obtained from patient records. HER2 chromogenic ISH (CISH) was performed using the HER2 CISH PharmDx kit (Dako), as previously described [33,34], on a subset of HER2 non amplified BCs (n = 1366) to assess the significance of *HER2* gene copy number in HER2 Low and HER2- tumours.

The percentage of stromal TILs (sTILs) on haematoxylin and eosin stained whole slides was scored into three categories; score 1: <5%, score 2: 5–50% and score 3: >50%, according to the recommendations of the International TILs Working Group [35]. Long term follow-up data and treatment regimens were extracted from patients reports. The patients were treated uniformly according to local protocol. HR+ patients received hormone therapy with or without chemotherapy according to clinical risk or recurrence risk score. TNBC patients received chemotherapy and HER2 positive patients received anti-HER2 therapy. This cohort was used to profile the clinico-pathologic characteristics of HER2 Low tumours and patient outcome. None of the patients in the cohort received neoadjuvant therapy or T-DXd.

(A2): A BC cohort from patients who received neoadjuvant therapy (n = 517) was included to assess possible differences in pathological complete response (pCR) between those with HER2 Low compared with HER2- tumours. HR- patients received either anthracycline with taxanes or anthracycline only, while HR+ patients received neoadjuvant endocrine therapy with or without chemotherapy. None of the patients received T-DXd.

(A3): A subset of TNBCs (n = 112), tested using next-generation sequencing (NGS) on representative formalin-fixed paraffin-embedded tissue as previously published [36], was used to investigate differentially expressed genes in HR-/HER2 Low versus HR-/HER2- tumours.

(B): Publicly available dataset: A retrospective cohort of primary BCs extracted from the publicly available BC dataset generated by The Cancer Genome Atlas (TCGA) Research Network BC cohort (n = 854) was also studied. PAM50 intrinsic molecular subtypes of primary tumour samples and clinico-pathological data were downloaded from https://identifiers.org/cbioportal:brcatcga.

A flow chart of the cohorts enroled in this study is shown in Fig. 1.

In the Nottingham cohort (A), HER2 IHC stained slides were rescored by two pathologists (NA and MT) according to the published guidelines [37,38] and the analysis was carried out based on the final updated HER2 score. In the TCGA cohort (B), the HER2 IHC stained slides were not available for re-scoring, so the documented original score was considered in the analysis. A 3-tier HER2 scoring system was used in this study: (1) HER2- (HER2 IHC score 0), (2) HER2 Low (HER2 IHC score of

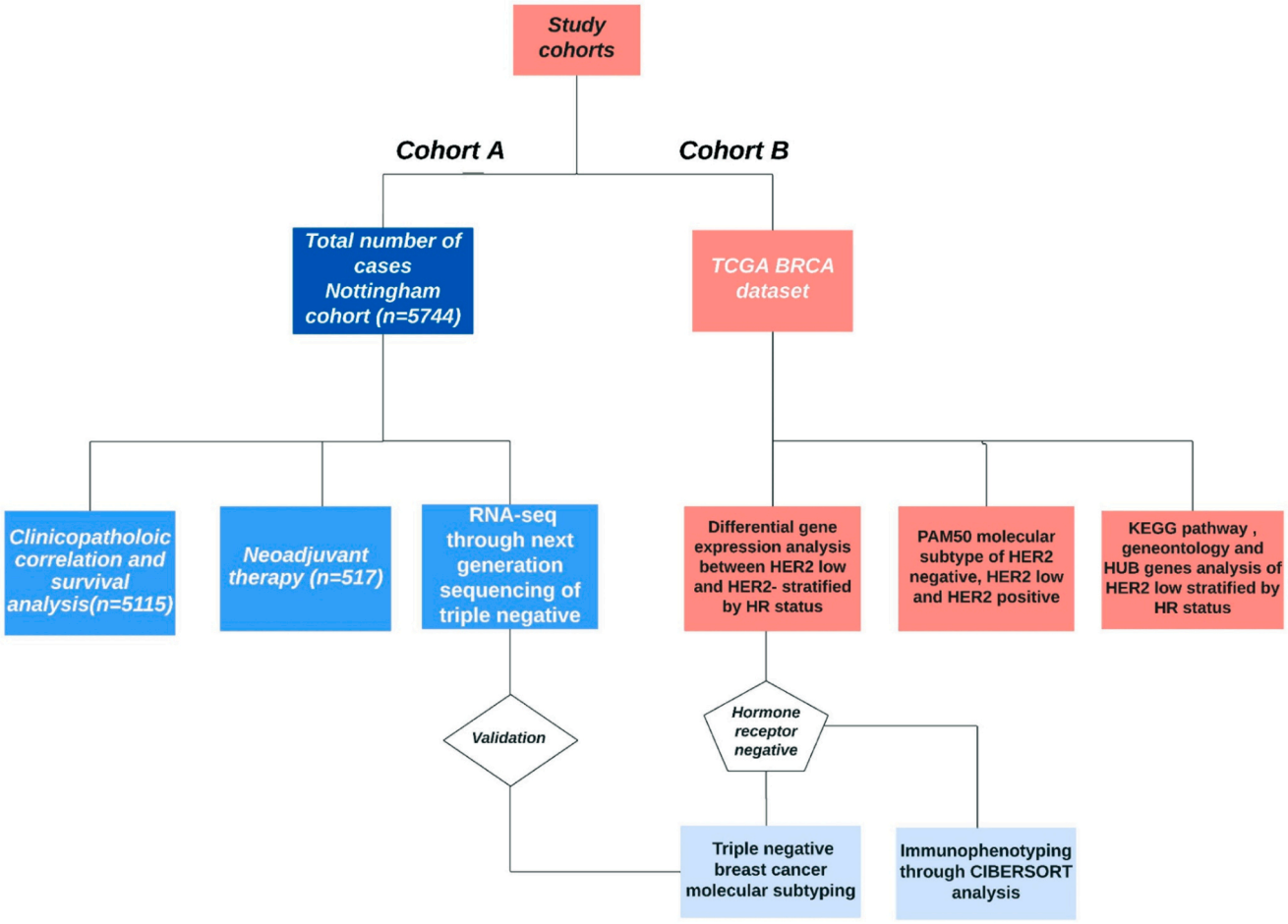


Fig. 1. Flowchart summarising cohorts enroled in the study.

1+, or 2+ with a non-amplified ISH), and (3) HER2+ (HER2 IHC score of 3+ and 2+ with an amplified ISH score). The clinico-pathologic characteristics of HER2 Low tumours were compared with those of the HER2- and HER2+ tumours. The Nottingham cohort (A) was then stratified for HR status into HR+ HER2 Low, HR+ HER2-, HR- HER2 Low and HR-/HER2- and subsequent correlations were performed.

2.2. Differential gene expression analysis (DGE) and pathway enrichment analysis

Differential expression analysis of genes expressed in HER2 Low tumours was performed with R 3.1.1 Bioconductor package DESeq2 using RNA-seq counts obtained from the TCGA-BRCA RNAseqV2 dataset [39] and from the in-house validation cohort [40,41]. The following workflow was carried out for the comparison of HER2 Low versus HER2- tumours, each stratified by HR status. Raw read counts were normalised by DESeq2. The magnitude (log2 transformed fold change) and significance (P-value) of differential expression between groups were calculated, and genes with false discovery rate adjusted P-values <0.05 and log2 fold change $\pm \geq 1$ were considered significant. Further validation of the results was performed on the Nottingham NGS TNBC cohort. Details are explained in Supplementary materials 1.

2.3. HER2 Low tumours: molecular signature and subtyping

The enrichment of *HER2* oncogenic signalling pathway genes and PAM50 intrinsic molecular subtype genes identified from literature [28,42], within the three HER2 IHC classes, stratified for HR status, was evaluated on the TCGA cohort. To further decipher the molecular differences between HR- /HER2 Low and HR- /HER2- tumours, differential expression of the six TNBC molecular subtypes, BL1, BL2, IM, M, MSL and LAR genes, in these two groups was investigated on the TCGA and validated on the Nottingham cohort (A3). TNBC subtype genes were pre-identified as previously published [27].

2.4. Immunologic profile and immunophenotyping of HER2 Low tumours

To investigate the role of sTILs in HR-/HER2 Low versus HR-/HER2- BC, Cell Type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) immunophenotype analysis was carried out to identify differences in immune cell phenotypes using the TCGA dataset (http://cibersort.stanford.edu/). Further details are outlined in Supplementary materials 2.

2.5. Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses of DEGs

To explore the KEGG signalling pathways and GO of HER2 Low tumours, Gene Set Enrichment Analysis (GSEA) was performed using The Database for Annotation, Visualization and Integrated Discovery https://david.ncifcrf.gov/ software for HR+/HER2 Low versus HR+/HER2- and HR-/ HER2 Low versus HR-/ HER2- BCs in the TCGA cohort, with a cut-off criterion of adjusted $p < 0.05$. KEGG is an encyclopaedia of genes and genomes, which may be used for pathway enrichment analysis of lists of genes [43]. GO annotation contains the three sub-ontologies, biological process, cellular component and molecular function, that can identify the biological properties of genes and gene sets for all organisms [44].

2.6. Protein-protein interaction (PPI) network construction and hub genes selection

The Search Tool for the Retrieval of Interacting Genes (STRING); https://string-db.org/; version 10.5) was applied to construct the PPI network. Acknowledgement of interactions between proteins may provide further understanding of the complex mechanisms of tumour development. A combined score of >0.4 was considered to indicate statistical significance. Cytoscape (version 3.9.1) [22] was used for visualising PPI.

2.7. Validation of hub genes

For validation of preidentified hub genes, IHC staining of AR, which had the highest rank among other hub genes, was performed on a subset of the Nottingham cohort (n = 1117) using BC tissue microarray sections which were constructed using the Grand Master® (3D HISTECH®, Budapest, Hungary) [32]. A primary antibody specific for AR (AR-N20, sc-816, Santa Cruz Biotechnology) was validated by western blotting using human cell lines MCF-7 and T47D. The AR antibody was used at a 1:10000 dilution which showed a band at ~110 kDa. AR was considered positive on nuclear expression. H-scores were calculated as previously reported by McCarty et al [45]. AR H-score was divided into high and low using the median as a cut-off. H-scores were validated by a second reader, who independently assessed 10% of cases. Details on AR IHC staining are summarised in Supplementary material 3.

2.8. Statistical analysis

Statistical package of social science (IBM-SPSS) statistical software v. 28.0 (SPSS, Chicago, IL, USA) was used to carry out the statistical analysis. Correlations between HER2 IHC scores and clinico-pathologic parameters were analysed using Chi-square ($\chi 2$) test and Kruskal-Wallis where appropriate. Univariate and multivariable logistic regression analyses were performed to investigate the association of each variable with HER2 status and the effect of other confounders and to assess factors affecting pCR. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated for each variable. The difference in patient survival between those with HER2- versus HER2 Low tumours using BC specific survival (BCSS) and distant metastasis free survival (DMFS) was evaluated using Kaplan–Meier curves and the log-rank test. The univariate and multivariate Cox regression analysis was carried out on the entire cohort and when tumours were adjusted for HR status. For all analyses, a P value of <0.05 (two-tailed) was considered statistically significant.

3. Results

3.1. Patients and tumour characterisation

Within the Nottingham cohort (**A1**) 2196 tumours (43%) were HER2 Low, 2262 (44%) were HER2- and 657 (13%) were HER2+.

In the HER2 Low category, 91% of tumours were ER+ compared to 81% in the HER2- and 64% in the HER2+ groups. The mean ± SD of *HER2* gene copy number in the HER2 Low group was 2.2 ± 1.04 copies compared to 2.0 ± 0.34 and 7.6 ± 2.9 copies for HER2- and HER2+, respectively.

The patient's mean age was 61 years (range 25–95) in the overall HER2 Low group. 22% of patients in the HR- /HER2 Low group were premenopausal compared with 33% in the HR-/HER2- group. Further details are summarised in Table 1 and Supplementary Table 1.

In the neoadjuvant treated patient cohort (A2), 362 of 517 had HER2 Low tumours and 155 had HER2- tumours. HR- patients (n = 143, 28%) received either anthracycline with taxanes (80%) or anthracycline only (20%), while HR+ (n = 374, 72%) patients received neoadjuvant endocrine therapy with or without chemotherapy. None of the patients received T-DXd. Data regarding response to therapy was available for 296 patients of whom 53 (18%) achieved pCR. In cohort (A3), 90 patients had HER2- tumours and 23 had HER2 Low tumours.

In the TCGA cohort (B) (n = 854), 47% (405) were HER2-, 41% (342) were HER2 Low and 12% (107) were HER2+.

3.2. Clinico-pathologic characteristics of HER2 Low BC in cohort A1

HER2 Low tumours were significantly associated with HR positivity and favourable tumour biological behaviour compared to HER2- BC. HER2 Low status was significantly associated with a high ER H-score compared to HER2- and HER2+ (Supplementary Fig. 1). When the cohort was stratified according to HR status, the favourable prognostic significance of HER2 Low status was maintained in HR- tumours. However, in HR+ BC, no significant prognostic difference was detected between HER2 Low and HER2- status except for the absence of LVI in HER2 Low tumours ($p < 0.001$). There was no significant difference in the Oncotype DX recurrence scores between HER2 Low and HER2- BC in this group.

Overall, HER2 Low status was significantly associated with low TILs score ($p = 0.02$) and the association was maintained in HR- subgroups but not in HR+ tumours (Table 1). A multivariate logistic regression analysis performed to analyse which clinico-pathologic parameters are independently associated with HER2 Low status, revealed that only ER positivity was an independent predictor of HER2 Low tumours ($p < 0.001$).

No significant association was detected between *HER2* gene copy number as determined by the CISH assay and HER2 protein expression level in HER2 Low or HER2- tumours.

3.3. Difference in patient outcome between HER2 Low and HER2- tumours according to HR status

In cohort A1, HER2 Low BC patients had prolonged BCSS and DMFS ($p = 0.004$ and $p = 0.009$, respectively) compared to HER2- patients. In luminal tumours, this significance was lost (Fig. 2A) while in HR- BC, HER2 Low tumours were associated with improved patient outcome ($p = 0.04$ for both BCSS and DMFS). In multivariate Cox regression analysis, HER2 Low patients had prolonged DMFS compared to those with HER2- BC, adjusted for the significant parameters in univariate analysis (LN status, LVI, and NPI) (Table 2B). Within the group of patients with HR-/ HER2 Low BC, negative LN status was associated with prolonged patient survival ($p = 0.008$ and $p = 0.006$ in BCSS and DMFS respectively). In patients with HR+ BC, no significant difference in the factors affecting survival between HER2 Low and HER2- was detected.

In the neoadjuvant setting (cohort A2), HER2- tumours were significantly associated with high pCR rates compared to HER2 Low tumours (74% versus 26%), $p < 0.001$. In HR- BC, patients with HER2- tumours had significantly higher pCR than those with HER2 Low tumours, $p = 0.037$ following treatment with neoadjuvant chemotherapy. This difference was not observed in HR+ patients. In the multivariate regression model, HER2 status was an independent predictor of pCR (HR: 3.31, 95%CI:1.7–6.4, $p < 0.001$) when adjusted for HR status, tumour grade and type of neoadjuvant therapy (Table 2A).

3.4. HER2 Low molecular signature

In TCGA dataset (cohort B), a positive significant correlation between *ERBB2* and *ESR1* gene ($p < 0.001$, $r = 0.4$) was observed, while *ERBB2* and *MKI67* were negatively correlated to each other ($p < 0.001$) (Supplementary Fig. 1).

There was no significant difference between HER2 Low and HER2- tumours regarding the expression of HER2 oncogenic signalling pathway genes regardless of HR status, unlike HER2+ tumours that were enriched for HER2 oncogenic signalling pathway genes (Supplementary Fig. 2). PAM50 intrinsic molecular subtyping revealed that 57% of HER2

Table 1
Correlation between clinico-pathologic parameters and human epidermal growth factor receptor 2 (HER2) immunohistochemistry scores.

Parameter	All Cohort					HR positive					HR Negative				
	HER2-	HER2 Low	HER2+	P value[a]	P value[b]	HER2-	HER2 Low	HER2+	P value[a]	P value[b]	HER -	HER2 Low	HER2+	P value[a]	P value[b]
	N (%)	N (%)	N (%)			N (%)	N (%)	N (%)			N (%)	N (%)	N (%)		
Age at diagnosis (years)															
< 50	551 (24)	439 (20)	211 (32)	X^2=12.3	X^2=42.0	410 (22)	394 (20)	142 (34)	X^2=3.4	X^2=38.0	141 (33)	45 (22)	69 (29)	X^2=9.3	X^2=3.4
≥50	1711 (76)	1757 (80)	446 (68)	**<0.001**	**<0.001**	1430 (78)	1594 (80)	280 (66)	0.06	**<0.001**	281 (67)	163 (78)	166 (71)	**0.002**	0.07
Menopause															
Pre	647 (29)	573 (26)	233 (36)	X^2=3.5	X^2=21.9	505 (27)	527 (27)	162 (38)	X^2=0.4	X^2=3.7	142 (34)	46 (22)	71 (30)	X^2=8.9	X^2=3.7
post	1615 (71)	1623 (74)	424 (64)	0.06	**<0.001**	1335 (73)	1461 (73)	260 (62)	0.54	0.06	280 (66)	162 (78)	164 (70)	**0.003**	0.07
Tumour size (cm)															
< 2.0	1475 (65)	1470 (67)	417 (64)	X^2=1.4	X^2=2.7	1262 (69)	1362 (69)	277 (66)	X^2=0.02	*X2=1.3*	213 (51)	108 (52)	140 (60)	X^2=0.12	X^2=2.6
≥2.0	787 (35)	726 (33)	240 (36)	0.22	0.1	578 (31)	626 (31)	145 (34)	0.97	*0.25*	209 (49)	100 (48)	95 (40)	0.73	0.12
Tumour grade															
1	449 (20)	444 (20)	18 (2.7)			442 (24)	443 (22)	16 (4)			7 (2)	1 (0.5)	2 (1)		
2	1048 (46)	1169 (53)	220 (34)	X^2=30.3	X^2=335	1011 (55)	1127 (57)	175 (42)	X^2=1.8	X^2=224	37 (9)	42 (20)	45 (19)	X^2=17.7	X^2=0.3
3	765 (34)	583 (27)	419 (64)	**<0.001**	**<0.001**	387 (21)	418 (21)	231 (55)	0.42	**<0.001**	378 (90)	165 (79)	188 (80)	**<0.001**	0.9
Tubule formation															
>75%	210 (9)	207 (9)	11 (1.7)			209 (11)	206 (10)	9 (2)			1 (0.2)	1 (0.5)	2 (1)		
25–75	603 (27)	590 (27)	118 (18)	X^2=0.06	X^2=76.7	557 (30)	558 (28)	82 (19)	X^2=4.1	X^2=51.6	46 (11)	32 (15.4)	36 (15)	X^2=2.9	X^2=0.2
<25%	1449 (64)	1399 (64)	528 (80)	0.97	**<0.001**	1074 (58)	1224 (62)	331 (79)	0.12	**<0.001**	375 (89)	175 (84.1)	197 (84)	0.09	0.85
Pleomorphism															
1	33 (1.5)	15 (1.0)	0 (0.0)			32 (2)	15 (1)	0 (0.0)			1 (0.2)	0 (0.0)	0 (0.0)		
2	951 (42)	1033 (47)	78 (12)	X^2=16.3	X^2=272	936 (50)	1027 (52)	74 (18)	X^2=7.7	X^2=170	15 (3.8)	6 (3)	4 (2)	X^2=0.5	X^2=0.7
3	1278 (57)	1148 (52)	579 (88)	**<0.001**	**<0.001**	872 (48)	946 (47)	348 (83)	**0.02**	**<0.001**	406 (96)	202 (97)	231 (98)	0.49	0.4
Mitosis score															
1	1369 (61)	1491 (68)	207 (32)	X^2=38.3	X^2=281	1330 (72)	1456 (73)	171 (41)	X^2=0.4	X^2=179	39 (9)	35 (17)	36 (15)	X^2=14	X^2=6.7
2	341 (15)	330 (15)	189 (29)	**<0.001**	**<0.001**	273 (15)	283 (14)	110 (26)	0.8	**<0.001**	68 (16)	47 (23)	79 (34)	**<0.001**	**0.03**
3	552 (24)	375 (17)	261 (40)			237 (13)	249 (13)	141 (33)			315 (75)	126 (61)	120 (51)		
LN status															
Negative	1568 (70)	1520 (70)	376 (58)	X^2=0.02	X^2=32.0	1278 (70)	1387 (70)	251 (60)	X^2=0.1	X^2=16.7	290 (70)	133 (66)	125 (54)	X^2=1.1	X^2=6.1
Positive	684 (30)	665 (30)	276 (42)	0.97	**<0.001**	557 (30)	595 (30)	169 (40)	0.83	**<0.001**	127 (30)	70 (34)	107 (46)	0.31	**0.02**
Histologic tumour type															
IDC NST	1381 (42)	1329 (61)	579 (88)			1003 (40)	1139 (57)	359 (85)			378 (90)	190 (91)	220 (93)		
ILC	269 (12)	294 (13)	17 (3)	X^2=0.06	X^2=176	266 (15)	287 (14)	13 (3)	X2=4.2	X2=117	3 (1)	7 (3)	4 (2)	X^2=0.8	X^2=0.19
Other types	612 (26)	573 (26)	61 (9)	0.97	**<0.001**	471 (45)	462 (29)	50 (12)	0.06	**<0.001**	41 (9)	11 (6)	11 (5)	0.36	0.75
TILS score															
<5%	445 (70)	993 (76)	183 (56)			390 (79)	921 (78)	139 (63)			55 (38)	72 (53)	44 (41)		
5–50%	121 (19)	207 (16)	92 (28)	X^2=7.0	X^2=52.3	74 (15)	171 (15)	51 (23)	X^2=1.3	X^2=25.4	47 (33)	36 (26)	41 (38)	X^2=5.9	X^2=4.4
>50%	69 (11)	112 (8)	54 (16)	**0.02**	**<0.001**	27 (6)	83 (7)	31 (14)	0.4	**<0.001**	42 (29)	29 (21)	23 (21)	**0.02**	0.11
LVI															
No	1829 (81)	1880 (86)	473 (72)	X^2=18.0	X^2=64.9	1496 (81)	1713 (86)	312 (74)	X^2=16.7	X^2=38.8	333 (79)	167 (80)	161 (69)	X^2=0.16	X^2=8.0
Yes	433 (19)	316 (14)	184 (28)	**<0.001**	**<0.001**	344 (19)	275 (14)	110 (26)	**<0.001**	**<0.001**	89 (21)	41 (20)	74 (31)	0.69	**0.005**
NPI risk group															
Low	1001 (44)	1074 (49)	141 (22)			968 (53)	1055 (53)	117 (28)			33 (8)	19 (9)	24 (10)		
Intermediate	1017 (45)	900 (41)	377 (58)	X^2=9.9	X^2=168	714 (40)	761 (38)	233 (56)	X^2=0.1	X^2=94.4	303 (73)	139 (69)	64 (28)	X^2=1.2	X^2=2.05
High risk	234 (10)	211 (10)	134 (21)	**0.007**	**<0.001**	153 (8)	166 (8)	70 (17)	0.9	**<0.001**	81 (19)	45 (22)	64 (28)	0.55	0.36
Oncotype DX score risk groups															
Low	20 (20)	30 (20)				64 (63)	104 (68)								
Intermediate	55 (54)	100 (65)		X^2=5.5		11 (11)	26 (17)		X^2=5.5						
High	26 (26)	23 (15)	NA	0.09	NA	26 (26)	23 (15)	NA	0.06	NA	NA	NA	NA		
ER status															
Negative	422 (19)	208 (9)	235 (36)	X^2=77.6	X^2=266										
Positive	1840 (81)	1988 (91)	422 (64)	**<0.001**	**<0.001**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PR Status															
Negative	767 (34)	496 (23)	376 (58)	X^2=71.6	X^2=293										
Positive	1484 (66)	1697 (77)	273 (42)	**<0.001**	**<0.001**										

LN: lymph node**; IDC NST:** Invasive duct carcinoma non-special type**; ILC:** Invasive lobular carcinoma; **TILS**: Tumour infiltrating lymphocytes; **LVI:** lymphovascular invasion**; NPI:** Nottingham prognostic index; **ER:** Oestrogen Receptors**; PR:** Progesterone receptor; **NA**: Not applicable **TNBC**: triple negative breast cancer. Significant p values shown in bold, $X^{2:}$ Chi square test value.
Some cases are missing within each parameter as they were collected from different datasets.

[a] p value across HER- and HER2 low.

[b] p value of significance between HER2+ and HER2 low.

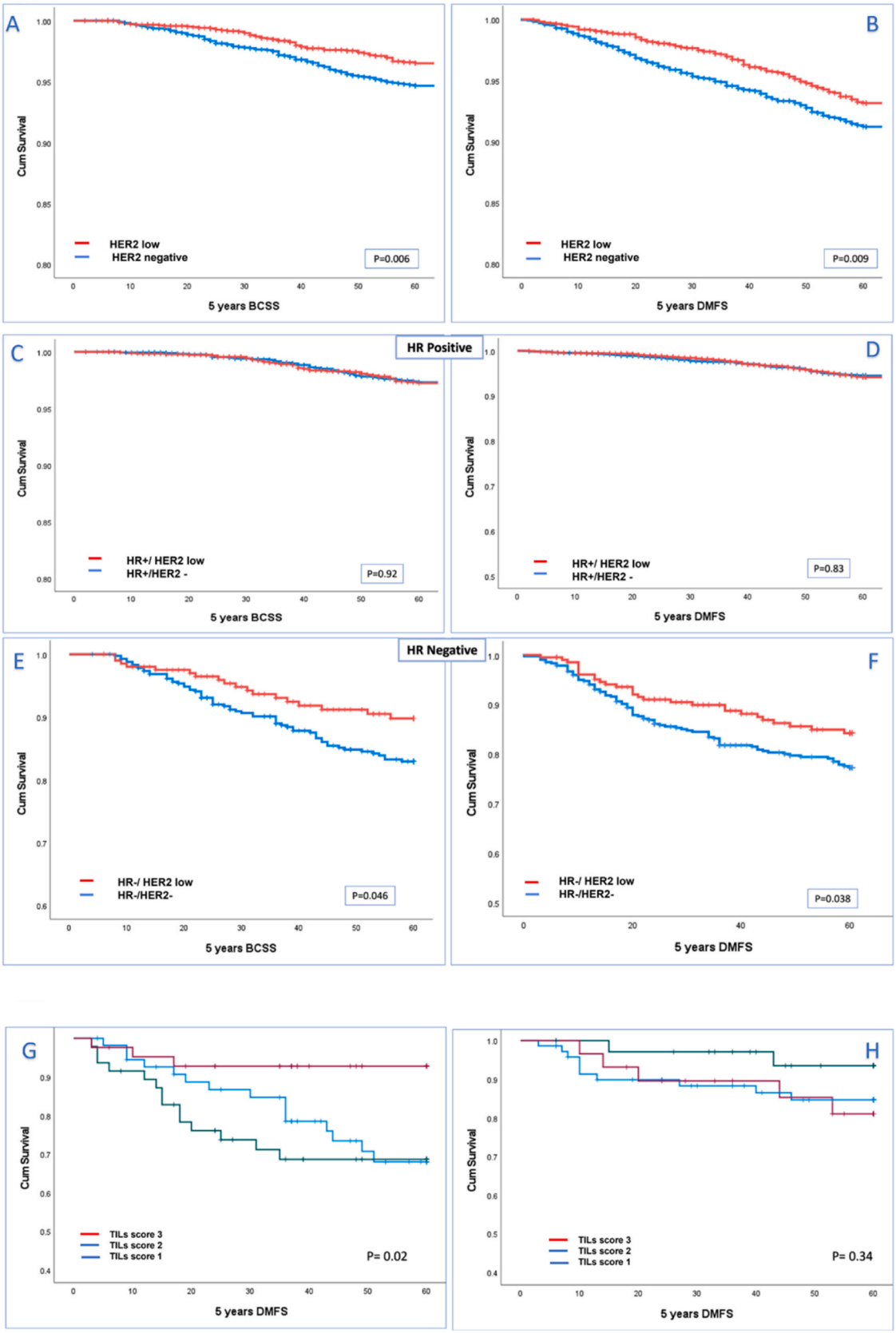


Fig. 2. Kaplan Meier's curve showing differences in BC specific survival (BCSS) and distant metastasis-free survival between human epidermal growth factor receptor 2 (HER2) Low and HER2- generally (A&B) and stratified by hormone receptor (HR) status. C&D: No significant difference in survival between HER2 Low and HER2- stratified in HR positive patients: E&F: HER2 Low patients had significantly better outcome than HER2- in HR negative tumours. TILs score and patients outcome in G: HER2- and H: HER2 Low.

Table 2A
Factors affecting pathologic complete response among HER2 low and HER2- breast cancer (BC) patients.

	Univariate regression analysis			Multivariate regression analysis		
	Odd's ratio	95% CI	P value	Odd's ratio	95% CI	P value
HER2 status						
Negative	3.31	1.7–6.4	**<0.001**	2.31	1.14–4.7	**0.02**
Low	1			1		
ER status						
Negative	3.32	1.8–6.2	**<0.001**	1.9	0.96–3.81	0.07
Positive	1			1		
Tumour grade						
1	0.1	0.1		0.1	0.1	0.06
2	0.34	0.2–0.62	**0.002**	0.5	0.23–0.84	
3	1			1		
Neoadjuvant therapy						
Chemotherapy only	0.76	0.15–3.75	0.6	NA	NA	NA
Endocrine + chemotherapy	1					

ER: Oestrogen receptor; **CI:** Confidence interval; Significant values if $p<0.05$ are in bold

Table 2B
Difference in patients' outcome between HER2 low and HER2- hormone receptor negative BC.

	5 years breast cancer specific survival						5 years distant metastasis free survival					
	Univariate Analysis 5 years			Multivariate Analysis 5 years			Univariate Analysis 5 years			Multivariate Analysis 5 years		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
HER2 status												
HER2-	1.73	1.02–2.9		0.6			1.5	1.0–2.3		0.52		
HER2 Low	1		**0.04**	1	0.95–2.72-	0.05	1		**0.04**	1	0.99–2.3	**0.049**
Grade												
1	0.0	0.0					0.0	0.0				
2	0.45	0.1–1.1					0.70	0.3–1.37				
3	1.0		0.22	NA	NA	NA	1.0		0.55	NA	NA	NA
LVI												
Negative	0.26	0.17 – 0.41		0.35	0.21–0.57		0.38	0.21–0.44		0.41		
Positive	1.0		**<0.001**	1.0		**<0.001**	1.0		**<0.001**	1.0	0.26–0.45	**<0.001**
LN status												
Negative	0.41	0.26–0.63		0.94			0.41	0.29–0.59		0.91	0.45–1.44	
Positive	1.0		**<0.001**	1.0	0.45–1.35	0.87	1.0		**<0.001**	1.0		0.46
TILS score												
Score 1	1.4						1.9					
Score 2	1.02	0.56–3.4					1.8	0.84–4.1				
Score 3	1.0	0.35–3.1	0.65	NA	NA	NA	1.0	0.7–4.3	0.27	NA	NA	NA
NPI												
Good	0.20	0.06–0.66		0.27			0.19	007–0.52				
Moderate	0.36	9.23–0.56		0.57	0.05–1.36		0.38	0.26–0.56		0.29	0.08–1.08	
poor	1		**<0.001**	1	0.27–1.2	0.2	1		**<0.001**	0.644	0.36–1.16	0.14
Chemotherapy												
No	1.3	0.47–3.7					1.1	0.73–1.6				
Yes	1		0.61	NA	NA	NA	1		0.72	NA	NA	NA

HR: Hazard ratio; **CI**: Confidence interval; **LN:** lymph node**; TILS**: Tumour infiltrating lymphocytes; **LVI:** lymphovascular invasion**; NPI:** Nottingham prognostic index; Significant p values shown in bold.

Low tumours were luminal A intrinsic molecular subtype and 20% were luminal B. In HR- tumours, 13% were HER2 enriched, compared to 0.5% in the HR+/ HER2 Low group (Fig. 3A).

The differential expression of normalised RNA-Seq reads of PAM50 genes revealed that HR+/HER2 Low and HR+/HER2- tumours were enriched for luminal A and luminal B genes as expected. HR-/HER2- tumours were significantly enriched for basal-like genes, while HR-/HER2 Low tumours were enriched for luminal and normal-like intrinsic subtypes genes (*GPR160, MLPH, PGR, KRT14, FOXA1, TMEM45B* and *ESR1*). *ERBB2* gene differential expression was not significantly different in HER2 Low versus HER2- tumours regardless of ER status (Fig. 3 B&C, Supplementary Table 3).

For further deciphering the molecular differences between HR-/HER2 Low and HR-/HER2- tumours, differential expression of the six TNBC molecular subtypes genes in those two classes was carried out on TCGA dataset and validated on cohort A3. This revealed that HR-/HER2 Low BC tumours are mainly of LAR subtype with significant expression of *AR, APOD, PIP, SPDEF, UGT2B28, SORD, GUCY1A1, HPGD, ELOVL5, and AZGP1* genes in both cohorts (Fig. 3 D&E, Supplementary Table 4A and B) while HR-/HER2- TNBC are mainly basal-like subtypes.

3.5. CIBERSORT Immunophenotyping analysis

Within the group of HR-/HER2 Low patients, sTILs score had no significant association with patient survival in contrast to the HR-/HER2- group where high sTILs scores were associated with prolonged 5-year DMFS (Supplementary Table 2 and Fig. 2B).

HR-/HER2 Low tumours contained a higher proportion of anti-tumorigenic T-helper lymphocytes, natural killer (NK) activated cells and tumour associated neutrophils, while HR- /HER2- tumours were enriched with pro-tumorigenic M0 and M1 macrophages, T cell gamma and delta, activated mast cells, cytotoxic T lymphocytes regulatory T cells (Tregs) and memory CD4 T lymphocytes (Fig. 4).

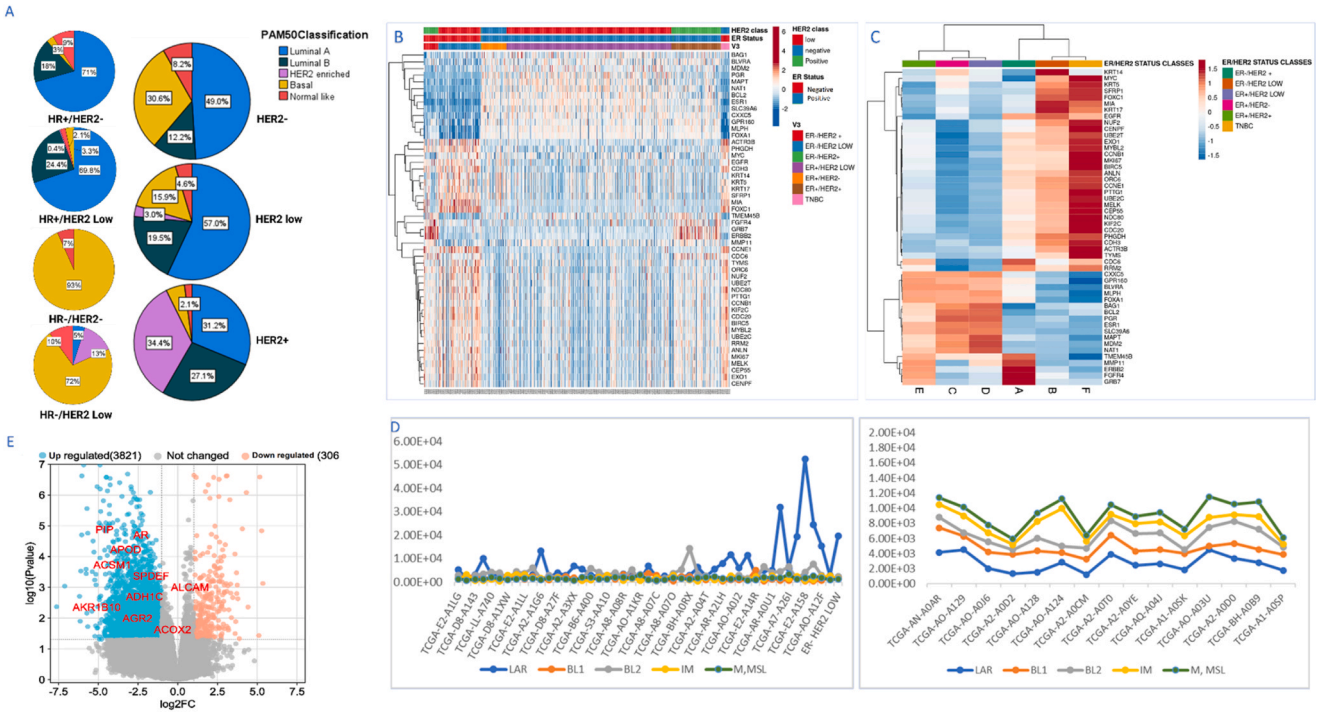


Fig. 3. Distribution of PAM50 molecular subtypes and triple negative breast cancer molecular subtypes between HER2 categories stratified by hormonal receptor status. A: Differences between HER2 categories regarding PAM50 intrinsic molecular subtype. B, C: Heatmaps illustrating expression of PAM50 genes among HER2 categories stratified by HR status. D: A correlogram summarising distribution of TNBC molecular subtypes between HER2 low (left) and HER2- (right). Luminal Androgen subtype (LAR) is more predominant in HER2 low cases compared to HER2- where other TNBC subtypes are most dominant. E: Volcano plot showing differentially expressed genes between HR-/HER2 low versus HR-/HER2- BC. Blue dots stand for significantly upregulated genes in HR-/HER2 low tumours, while orange dots stand for the significant downregulated genes. Luminal Androgen-like genes are highlighted. BL1: Basal like 1, BL2: Basal like 2, IM: Immunomodulatory, M,MSL: Mesenchymal, Mesenchymal stem like.

3.6. GSEA and GO

Pathway enrichment analysis showed that the top five significantly enriched pathways between HR-/HER2 Low and HR-/HER2- tumours are ascorbate and aldarate metabolism, steroid hormone biosynthesis, pentose and glucuronate interconversions, tyrosine metabolism and PPAR signalling pathway. No significantly enriched pathways were detected between HR+/HER2 Low and HR+/HER2–. Further details on pathway enrichment and GO functions in HR+ and HR- categories are summarised in Fig. 5 and Supplementary Fig. 3.

3.7. PPI network and hub genes

In HR- /HER2 Low tumours, the top ten up-regulated hub genes demonstrated by radiality, betweenness and connectivity degree in the PPI network were AR, OPN5, SHH, LEP, PGR, HNF4A, PTGS2, CAV1, CYP3A4 and POMC. Relevant results and further details are shown in Fig. 5 and Supplementary Fig. 3.

3.8. Immunohistochemical expression of AR

Inter-observer concordance of AR H score was 0.9. The median AR H-score in the overall immunostained cohort was 125 (5–280). This corresponded to 90 (range 5–275) in the HR-/HER2 Low group and 10 (range 5–275) in the HR-/HER2- group. High AR nuclear expression was significantly correlated with HER2 Low compared to HER2- status ($p = 0.012$). Within HR- /HER2 Low tumours, high AR expression was associated with a worse outcome in HER2 Low patients both in univariate ($p = 0.02$) and multivariate analyses ($p = 0.03$) when adjusted for LN status (Fig. 6). Details regarding patients and tumour characteristics are summarised in Supplementary Table 5.

4. Discussion

HER2 Low BC accounts for 40–50% of all BCs [2] and appears to be a heterogeneous disease [5,46]. The recent development of novel HER2 targeting ADCs, shown to have significant clinical benefits for patients with these tumours, will dramatically revolutionise the treatment landscape of some BCs classified as HER2- using the traditional classification system. Our understanding of this potential new BC category continues to evolve with the ongoing debate regarding its status as a distinct biological entity with a predictable clinical course, specific prognostic and therapeutic implications, and the potential interactive impact of HR status [47–49]. The current study aimed to thoroughly profile the HER2 Low category of tumours with particular emphasis on the potential impact on clinical behaviour, molecular signature and the interaction with and influence of HR status in a large BC series.

Some studies have reported that HER2 Low tumours are associated with a more favourable clinical outcome than HER2- tumours [18,19,50–54] while other authors have demonstrated that HER2 Low BC is not prognostically different from HER2- tumours [2,55]. Our results indicate that, when considered in isolation, HER2 Low tumours are associated with a more favourable clinical outcome, lower histological grade, less mitotic activity and a lower incidence of lymph node metastases compared to HER2–. These tumours also appear to be less responsive to neoadjuvant chemotherapy compared to true TNBC (HER2-), in agreement with previous studies [15,18,53,56].

In this study, HER2 Low tumours represented 43% of the overall study tumour population, 90% of which were HR+. The ER H score was higher in HER2 Low tumours compared to both HER2- and HER2+, as observed in previous studies [16,18,55,57]. Our results showed that HER2 Low tumours are associated with prolonged survival compared to HER2- tumours in unselected patients' population as well as in the HR- BC subgroup. These results were in line with multiple previous studies

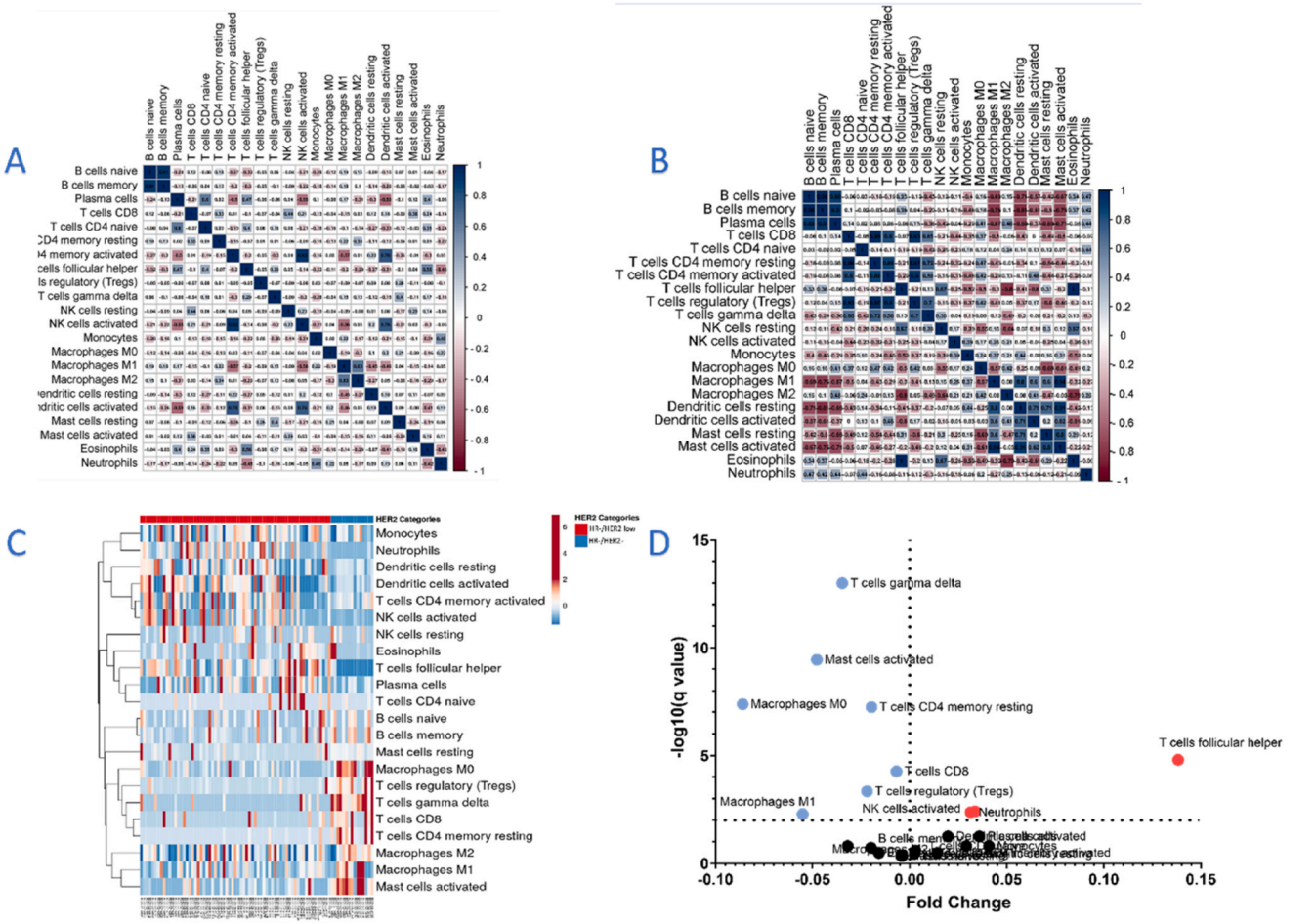


Fig. 4. Immunophenotyping of HR-/ HER2 low (A) and HR-/HER2- breast cancer (B) using CIBERSORT analysis. C: unsupervised clustering between HER2 low and HER2- immunophenotypes. D: Volcano plot showing the distribution of significant immunophenotypes within each HER2 class. Red dots refer to immunophenotypes significantly enriched in HER2 low BC, while blue dots refer to immunophenotypes significantly enriched in HER2- BC. Black dots refer to immunophenotypes with non-significant adj p value.

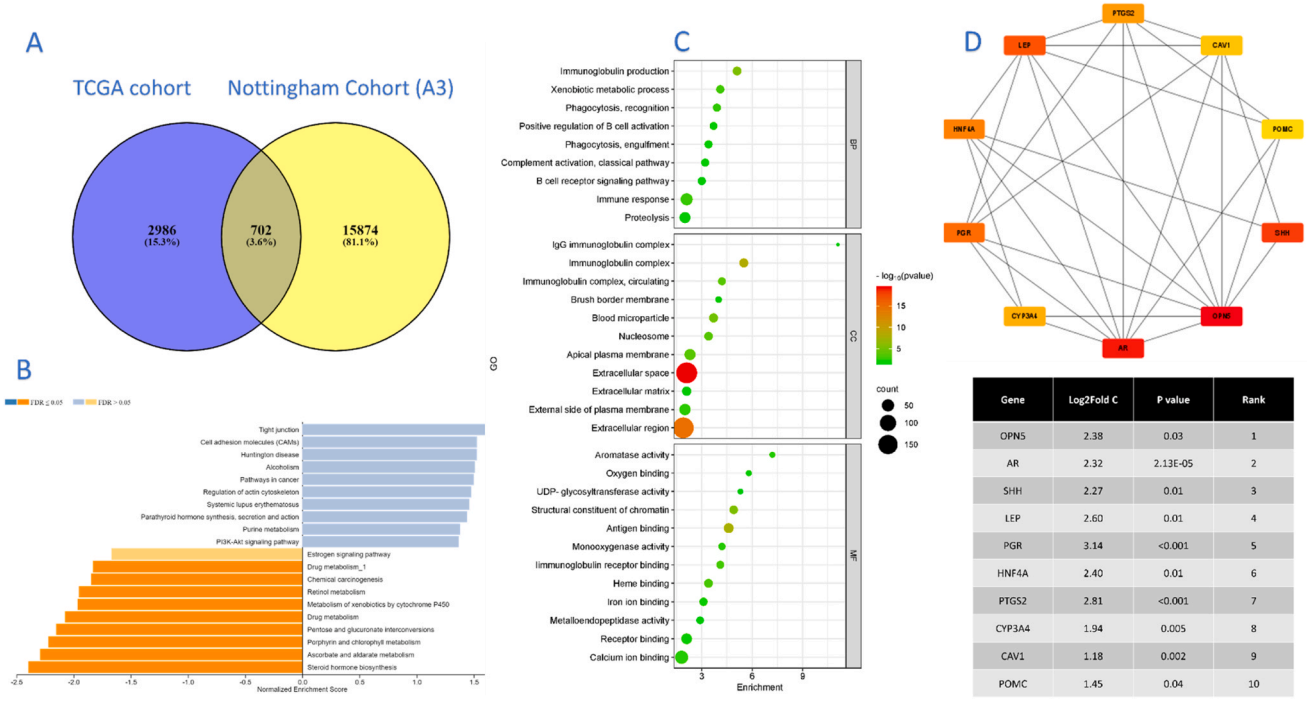
[18,52,58–61]. However, this trend was not maintained when results were adjusted for HR positivity, where HER2 Low tumours lost their prognostic significance in terms of the clinic-pathologic parameters or patients' outcome. These results were also demonstrated by some authors [2,15,16]. These findings indicate that the better prognosis of HER2 Low tumours is dependent on HR activity as demonstrated by the lack of prognostic significance in HER2 Low tumours in HR+ BC and the association between HER2 Low and luminal phenotype in the HR- BC subgroup. Therefore, we conclude that although HER2 Low tumours are a unique therapeutic class of BC that can be treated with specific HER2 based ADC [4], they are not biologically or prognostically a unique class of BC.

HER2 plays an important role in normal cell growth and differentiation. In normal breast epithelial cells, HER2 is expressed at low levels (two copies of the *HER2* gene and up to 20,000 HER2 receptors) [62]. This may indicate that tumours that maintain low levels of HER2 protein are more differentiated while deviation from the normal expression level, either through positive protein expression (IHC 3+), gene amplification in *HER2*+ tumours or complete loss of expression in HER2- BC, is associated with increased tumour aggressiveness. This deviation is more frequent in HR- tumours, while in luminal subtypes HR is the dominant oncogenic driver of BC regardless of HER2 levels.

HER2 Low tumours do not appear to be driven by the HER2 oncogenic signalling pathway. PAM50 molecular subtyping and DGE revealed that neither the *ERBB2* gene nor other HER2 oncogenic signalling pathway genes were significantly enriched in HER2 Low tumours regardless of HR status.

On the contrary, HER2 Low tumours tend to be of luminal molecular subtype if HR + and normal-like subtype if HR-, in agreement with other studies [6,16,55].

Regarding the molecular differences between HER2 Low and HER2- BC in the HR- tumours, our study showed that HER2 Low tumours exhibit a distinct molecular profile. This was evident through the DGE and pathway enrichment analysis which revealed that HR-/HER2 Low tumours are mainly of LAR subtype, with upregulation of luminal androgen-like pathway genes, in particular *AR*, which was also one of the top 10 PPI hub genes and being enriched with fatty acid and steroid hormone metabolism pathways that was previously reported in the LAR TNBC subtype [27]. This accords with the experience of other researchers who observed that HER2- and HER2 Low BC exhibit different molecular properties. *Yam et al.* [63] investigated the molecular differences between HER2- and HER2 Low tumours in TNBC patients and demonstrated that HER2 Low was associated with increased AR expression and upregulation of genes associated with fatty acid and steroid hormone metabolism [63]. Other study showed differences in resistance drivers between HER2- and HER2 Low tumours mainly in the HR- group [64]. An association between AR and HER2 expression has been reported previously [65]. Our results revealed that high AR protein expression was significantly associated with HR- /HER2 Low tumours and was an independent predictor of worse outcome, in accordance with previous studies that demonstrated that AR expression was more frequent in HER2 Low than in HER2- BCs [66–68]. These findings



Gene	Log2Fold C	P value	Rank
OPN5	2.38	0.03	1
AR	2.32	2.13E-05	2
SHH	2.27	0.01	3
LEP	2.60	0.01	4
PGR	3.14	<0.001	5
HNF4A	2.40	0.01	6
PTGS2	2.81	<0.001	7
CYP3A4	1.94	0.005	8
CAV1	1.18	0.002	9
POMC	1.45	0.04	10

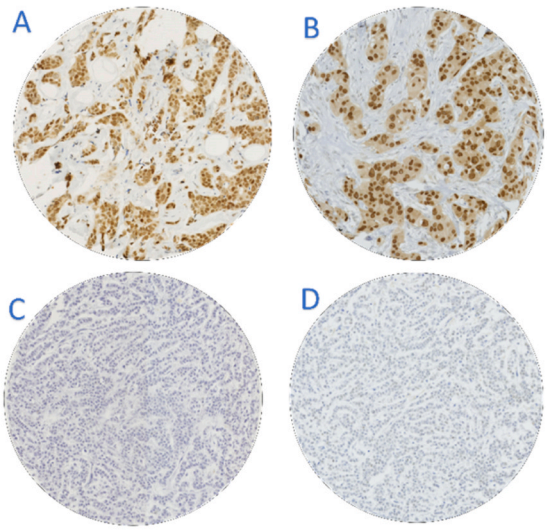
Fig. 5. Bioinformatics analysis carried out in HR-/HER2 Low BC cases. A: Venn diagram of genes overlapping upregulated genes between The Cancer Genome Atlas and Nottingham cohort A3. B: Kyoto Encyclopaedia of Genes and Genomes pathways enriched in HR-/HER2- BC against HR-/HER2 low, significant upregulated pathways in HR-/HER2 low are presented in deep orange with FDR <0.05. C: Diagram illustrating gene ontology (Molecular function (MF), Cellular component (CC) and biological processes (BP)) of the upregulated genes in HR-/HER2 low BC. D: Protein-protein interaction HUB genes of upregulated genes in HR-/HER2 low with their rank, log2 fold changes and adj p value. Red colour means high rank with the most significant adj p value. FDR: The False Discovery Rate, GO: Gene-ontology.

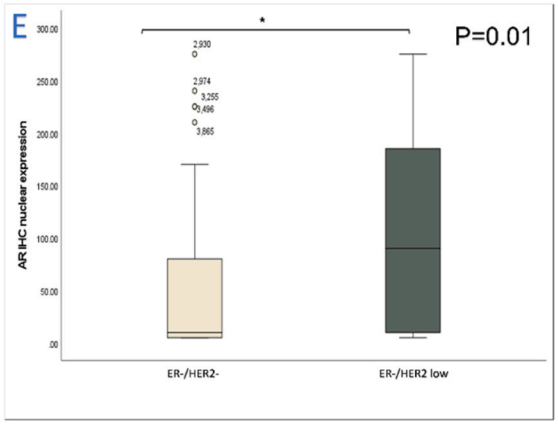
support the hypothesis that HER2 Low expression is associated with luminal differentiation in TNBC, in keeping with *Zhang et al* [16] who also concluded that HER2 Low tumours are less likely to be of basal TNBC subtype. The role of anti-AR inhibitors, e.g. enzalutamide in the treatment of AR+ BC, has been investigated but these have not yet been implemented as therapeutic targets [62,69–71]. Triana et al [70] reported that enzalutamide demonstrated clinical activity and was well tolerated in patients with advanced AR+ TNBC, but with a clinical benefit rate of only 33%. Based on our findings, the association between AR and HER2-low merits further investigations to evaluate the combination of AR inhibitor and novel anti-HER2 ADC targeted therapy.

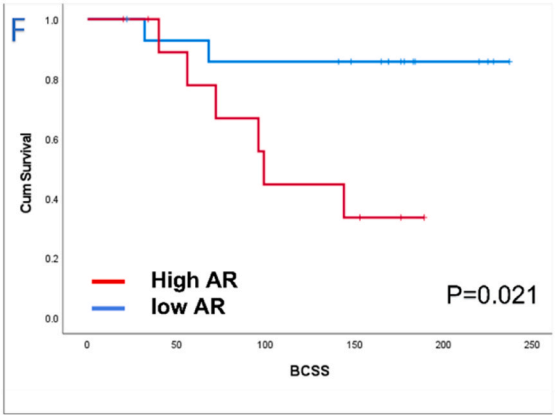
The dynamic nature of HER2 expression is an important issue when tumour heterogeneity and low level of expression are considered [72]. In a previous study of 1119 HR+/HER2- patients who participated in the phase 3 PENOLOPE-B study and were offered neoadjuvant chemotherapy, *Denkert and colleagues* [73] reported 14% shift from HER2- to HER2 Low and a 23% shift from HER2 Low to HER2- in patients who had residual tumours. The heterogeneity of HER2 Low status was also addressed by *Geukens T and coauthors* who demonstrated the presence of intra-patient, inter-metastases heterogeneity of HER2 Low status in the form of coexistence of HER2 Low and HER2- metastases [74]. Change of HER2 status between primary tumours and matched relapses in either local recurrences or distant metastases was also reported. Recent research findings indicate an increase in HER2 Low tumours during advanced stages. Additionally, they found correlations between these changes and factors such as the time of recurrence [75]. *Bar and colleagues* highlighted the issue of HER2 heterogeneity in repeated biopsies among patients with TNBC and concluded that performing a repeat biopsy at the time of disease progression can enhance the likelihood of obtaining a HER2 Low result [76]. These findings will influence our perspective regarding the reliability of assessing HER2 status in a single tumour sample as a predictive marker for T-DXd treatment, particularly the Destiny-breast04 clinical trial [4] have included patients with any HER2 Low sample even if other samples are negative.

Cytotoxic chemotherapy is currently the mainstay of treatment for patients with primary TNBC. In patients with metastatic TNBC with immunohistochemical PD-L1 expression on tumour-associated immune cells, the addition of anti-PD-L1 immunotherapy has been shown to be superior to chemotherapy alone regarding patient survival [77,78]. The clinical response of patients to these treatments is variable and characterisation of the molecular and immunophenotypic differences between HER2 Low TNBC and pure TNBC may offer insight into the heterogeneity of tumour response. Our study showed that HER2 Low tumours had significantly lower sTILs scores compared to HER2- tumours. This has also been observed by van den Ende *et al* [17] who reported a significant association between HER2 Low status and lower density TILs, despite no significant association between HER2 Low status and clinic-pathologic features in ER- tumours. Regarding immunophenotype, HER2 Low tumours featured aberrant, heterogeneous immune infiltration in patients with HR- BC with helper T lymphocytes, activated NK cells and cancer associated neutrophils being the dominant immunophenotype in contrast to HER2- tumours that were enriched for macrophages M0 and M1. Previous studies have demonstrated the role of NK and T helper cells in BC patients with improved survival [79–81]. These findings may have clinical relevance and inform the future development of personalised therapy for patients with HER2 Low BC.

This study has several strengths. It benefits from a substantial clinical cohort, re-assessment of HER2 status in the larger patient cohort, and comprehensive data on patient response to treatment with long-term survival outcome. Additionally, the study focus on immunophenotyping and subtyping of TNBC highlights the distinctions between HER2 Low and HER2- BC (true TNBC), with a particular emphasis on exploring the role of AR and its potential therapeutic applications. The study has some limitations. The research is constrained by the historical nature of the patient cohort, with no patients having received T-DXd therapy. Additionally, the study did not include assessment of HER2 status in the recurrence samples, which may have provided valuable insights into the dynamics of HER2 status and heterogeneity of HER2 expression with







Univariate and multivariate analysis of AR H score in HR-/HER2 low BC specific survival

HR-/HER2 low	Univariate analysis BCSS			Multivariate analysis BCSS		
	OR	95% CI	P value	OR	95% CI	P value
Lymph node status						
Negative	1		**0.005**	1		
Positive	3.1	1.4 – 7.0		3.65	0.83-16.0	0.086
AR H Score						
low	1			1		
High	4.67	1.1-19.7	**0.036**	4.7	1.1-20.2	**0.03**

Fig. 6. The significance of androgen receptor (AR) in HR-/HER2 Low BC. A&B: AR immunohistochemical expression in HER2 Low while C&D are its expression in and HER2- BC. E: Difference in AR expression between HER2 Low and HER2- shows significant association with HER2 Low breast cancer. F: Kaplan Meier curve of BCSS between high and low AR H score in HER2 Low patients. Table showing the role of AR in HER2 Low BC patients' survival both in univariate and multivariate analysis.

disease progression.

5. Conclusion

HER2 Low tumours are frequently HR+. HR+/HER2 Low tumours exhibit no significant differences from HR+/HER2- tumours regarding tumour behaviour and patients' outcome. In HR- BC, HER2 Low tumours have improved outcomes but appear to be less responsive to neo-adjuvant chemotherapy. HR- /HER2- tumours are enriched with unique immunophenotypes associated with aggressive tumour behaviour. AR is predictive of poor outcome in HER2 Low TNBC patients and may be a potential therapeutic target.

Ethics approval and consent to participate

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.

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CRediT authorship contribution statement

Concept and Design: N.A., E.R., Collection of Data: N.A., M.T, MA. Identifying and reviewing the images: N.A., M.T., Analysis of Data: N.A., NM., MA., MH., Interpretation of Results: All Authors. ER conceived and supervised the study, and participated in its design, interpretation, and analysis, including drafting. All authors contributed to drafting and reviewing the paper and approved the submitted and final version.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

All data used in this study are available and can be accessed upon reasonable request. The following publicly available datasets were used on https://identifiers.org/cbioportal:brca_tcga;

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.113371.

References

[1] Rinnerthaler G, Gampenrieder SP, Greil R. HER2 directed antibody-drug-conjugates beyond T-DM1 in breast cancer. Int J Mol Sci 2019;20(5):1115.

[2] Tarantino P, Hamilton E, Tolaney SM, Cortes J, Morganti S, Ferraro E, et al. HER2-low breast cancer: pathological and clinical landscape. J Clin Oncol 2020;38(17):1951–62.

[3] Banerji U, van Herpen CM, Saura C, Thistlethwaite F, Lord S, Moreno V, et al. Trastuzumab duocarmazine in locally advanced and metastatic solid tumours and HER2-expressing breast cancer: a phase 1 dose-escalation and dose-expansion study. Lancet Oncol 2019;20(8):1124–35.

[4] Modi S, Jacot W, Yamashita T, Sohn J, Vidal M, Tokunaga E, et al. Trastuzumab deruxtecan in previously treated HER2-low advanced breast cancer. N Eng J Med 2022;387(1):9–20.

[5] Marchiò C, Annaratone L, Marques A, Casorzo L, Berrino E, Sapino A, editors. Evolving concepts in HER2 evaluation in breast cancer: heterogeneity, HER2-low carcinomas and beyond. Seminars in cancer biology; 2021. Elsevier.

[6] Schettini F, Chic N, Brasó-Maristany F, Paré L, Pascual T, Conte B, et al. Clinical, pathological, and PAM50 gene expression features of HER2-low breast cancer. NPJ Breast Cancer 2021;7(1):1–13.

[7] Li Z, Guo S, Xue H, Li L, Guo Y, Duan S, et al. Efficacy and safety of trastuzumab deruxtecan in the treatment of HER2-low/positive advanced breast cancer: a single-arm meta-analysis. Front Pharmacol 2023;14:1183514.

[8] Banerji U, van Herpen CML, Saura C, Thistlethwaite F, Lord S, Moreno V, et al. Trastuzumab duocarmazine in locally advanced and metastatic solid tumours and HER2-expressing breast cancer: a phase 1 dose-escalation and dose-expansion study. Lancet Oncol 2019;20(8):1124–35.

[9] Modi S, Park H, Murthy RK, Iwata H, Tamura K, Tsurutani J, et al. Antitumor activity and safety of trastuzumab deruxtecan in patients with HER2-low-expressing advanced breast cancer: results from a phase Ib study. J Clin Oncol 2020;38(17):1887–96.

[10] Wolff AC, Somerfield MR, Dowsett M, Hammond MEH, Hayes DF, McShane LM, et al. Human epidermal growth factor receptor 2 testing in breast cancer: ASCO–College of American Pathologists Guideline Update. J Clin Oncol 2023;41(22):3867–72.

[11] Bussing D, Sharma S, Li Z, Meyer LF, Shah DK. Quantitative evaluation of the effect of antigen expression level on antibody-drug conjugate exposure in solid tumor. AAPS J 2021;23(3):56.

[12] Sharma S, Li Z, Bussing D, Shah DK. Evaluation of quantitative relationship between target expression and antibody-drug conjugate exposure inside cancer cells. Drug Metab Dispos 2020;48(5):368–77.

[13] Rakha EA, Tan PH, Quinn C, Provenzano E, Shaaban AM, Deb R, et al. UK recommendations for HER2 assessment in breast cancer: an update. J Clin Pathol 2023;76(4):217–27.

[14] Miglietta F, Griguolo G, Bottosso M, Giarratano T, Lo Mele M, Fassan M, et al. Evolution of HER2-low expression from primary to recurrent breast cancer. NPJ Breast Cancer 2021;7(1):137.

[15] de Moura Leite L, Cesca MG, Tavares MC, Santana DM, Saldanha EF, Guimarães PT, et al. HER2-low status and response to neoadjuvant chemotherapy in HER2 negative early breast cancer. Breast Cancer Res Treat 2021;190(1):155–63.

[16] Zhang H, Katerji H, Turner BM, Audeh W, Hicks DG. HER2-low breast cancers: incidence, HER2 staining patterns, clinicopathologic features, MammaPrint and BluePrint genomic profiles. Mod Pathol 2022;35(8):1075–82.

[17] van den Ende NS, Smid M, Timmermans A, van Brakel JB, Hansum T, Foekens R, et al. HER2-low breast cancer shows a lower immune response compared to HER2-negative cases. Sci Rep 2022;12(1):12974.

[18] Denkert C, Seither F, Schneeweiss A, Link T, Blohmer J-U, Just M, et al. Clinical and molecular characteristics of HER2-low-positive breast cancer: pooled analysis of individual patient data from four prospective, neoadjuvant clinical trials. Lancet Oncol 2021;22(8):1151–61.

[19] Molinelli C, Jacobs F, Agostinetto E, Nader-Marta G, Ceppi M, Bruzzone M, et al. Prognostic value of HER2-low status in breast cancer: a systematic review and meta-analysis. ESMO Open 2023;8(4):101592.

[20] Beitsch P, Whitworth P, Baron P, Rotkis MC, Mislowsky AM, Richards PD, et al. Pertuzumab/trastuzumab/CT versus trastuzumab/CT therapy for HER2+ breast cancer: results from the prospective neoadjuvant breast registry symphony trial (NBRST). Ann Surg Oncol 2017;24(9):2539–46.

[21] Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. Lancet 2014;384(9938):164–72.

[22] Gianni L, Pienkowski T, Im Y-H, Roman L, Tseng L-M, Liu M-C, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol 2012;13(1):25–32.

[23] Schneeweiss A, Chia S, Hickish T, Harvey V, Eniu A, Hegg R, et al. Pertuzumab plus trastuzumab in combination with standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer: a randomized phase II cardiac safety study (TRYPHAENA). Ann Oncol 2013;24(9):2278–84.

[24] Swain S, Ewer M, Viale G, Delaloge S, Ferrero J-M, Verrill M, et al. Pertuzumab, trastuzumab, and standard anthracycline-and taxane-based chemotherapy for the neoadjuvant treatment of patients with HER2-positive localized breast cancer (BERENICE): a phase II, open-label, multicenter, multinational cardiac safety study. Ann Oncol 2018;29(3):646–53.

[25] Lehmann BD, Jovanović B, Chen X, Estrada MV, Johnson KN, Shyr Y, et al. Refinement of triple-negative breast cancer molecular subtypes: implications for neoadjuvant chemotherapy selection. PloS One 2016;11(6):e0157368.

[26] Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Investig 2011;121(7):2750–67.

[27] Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Investig 2011;121(7):2750–67.

[28] Chen X, Li J, Gray WH, Lehmann BD, Bauer JA, Shyr Y, et al. TNBCtype: a subtyping tool for triple-negative breast cancer. Cancer Inform 2012;11:147–56.

[29] Yang T, Li W, Huang T, Zhou J. Immunotherapy targeting PD-1/PD-L1 in early-stage triple-negative breast cancer. J Pers Med 2023;13(3):526.

[30] Erber R, Hartmann A. Understanding PD-L1 testing in breast cancer: a practical approach. Breast Care 2020;15(5):481–90.

[31] Gomez-Macias GS, Molinar-Flores G, Lopez-Garcia CA, Santuario-Facio S, Decanini-Arcaute H, Valero-Elizondo J, et al. Immunotyping of tumor-infiltrating lymphocytes in triple-negative breast cancer and genetic characterization. Oncol Lett 2020;20(5):140.

[32] Allison KH, Hammond MEH, Dowsett M, McKernin SE, Carey LA, Fitzgibbons PL, et al. Estrogen and progesterone receptor testing in breast cancer: ASCO/CAP guideline update. J Clin Oncol 2020;38(12):1346–66.

[33] Rakha EA, Pinder SE, Bartlett JM, Ibrahim M, Starczynski J, Carder PJ, et al. Updated UK recommendations for HER2 assessment in breast cancer. J Clin Pathol 2015;68(2):93–9.

[34] Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ. Strategies for subtypes–dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 2011;22(8):1736–47.

[35] Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Annals of oncology: official journal of the European Society for. Med Oncol 2015;26(2):259–71.

[36] Alsaleem MA, Ball G, Toss MS, Raafat S, Aleskandarany M, Joseph C, et al. A novel prognostic two-gene signature for triple negative breast cancer. Mod Pathol 2020;33(11):2208–20.

[37] Rakha EA, Pinder SE, Bartlett JM, Ibrahim M, Starczynski J, Carder PJ, et al. Updated UK recommendations for HER2 assessment in breast cancer. J Clin Pathol 2015;68(2):93–9.

[38] Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JM, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. Arch Pathol Lab Med 2018;142(11):1364–82.

[39] Hoadley KA, Yau C, Wolf DM, Cherniack AD, Tamborero D, Ng S, et al. Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. Cell 2014;158(4):929–44.

[40] Alsaleem MA, Ball G, Toss MS, Raafat S, Aleskandarany M, Joseph C, et al. A novel prognostic two-gene signature for triple negative breast cancer. Mod Pathol 2020;33(11):2208–20.

[41] Ogden A, Bhattarai S, Sahoo B, Mongan NP, Alsaleem M, Green AR, et al. Combined HER3-EGFR score in triple-negative breast cancer provides prognostic and predictive significance superior to individual biomarkers. Sci Rep 2020;10(1):3009.

[42] Prat A, Pineda E, Adamo B, Galván P, Fernández A, Gaba L, et al. Clinical implications of the intrinsic molecular subtypes of breast cancer. Breast 2015;24(Suppl.2):S26–35.

[43] Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28(1):27–30.

[44] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25(1):25–9.

[45] McCarty Jr KS, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, et al. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. Cancer Res 1986;46(Suppl.8):4244s–8s.

[46] Buckley NE, Forde C, McArt DG, Boyle NE, Mullan PB, James JA, et al. Quantification of HER2 heterogeneity in breast cancer-implications for identification of sub-dominant clones for personalised treatment. Sci Rep 2016;6:23383.

[47] Lai H-Z, Han J-R, Fu X, Ren Y-F, Li Z-H, You F-M. Targeted approaches to HER2-low breast cancer: current practice and future directions. Cancers 2022;14(15):3774.

[48] Molinelli C, Jacobs F, Marchiò C, Pitto F, Cosso M, Spinaci S, et al. HER2-low breast cancer: where are we? Breast Care 2022;17(6):533–45.

[49] Nicolò E, Tarantino P, Curigliano G. Biology and treatment of HER2-low breast cancer. Hematol Oncol Clin 2023;37(1):117–32.

[50] Li Y, Abudureheiyimu N, Mo H, Guan X, Lin S, Wang Z, et al. In real life, low-level HER2 expression may be associated with better outcome in HER2-negative breast cancer: a study of the National Cancer Center, China. Front Oncol 2021;11:774577.

[51] Rosso C, Voutsadakis IA. Characteristics, clinical differences and outcomes of breast cancer patients with negative or low HER2 expression. Clin Breast Cancer 2022;22(4):391–7.

[52] Tan RSYC, Ong WS, Lee K-H, Lim AH, Park S, Park YH, et al. HER2 expression, copy number variation and survival outcomes in HER2-low non-metastatic breast cancer: an international multicentre cohort study and TCGA-METABRIC analysis. BMC Med 2022;20(1):105.

[53] Zhong G, Song D, Lou W, Wei B, Chen Y, Cui H, et al. Pathological complete response rate and clinical outcome after neoadjuvant therapy of HER2-low breast

cancer: a National Cancer Database Analysis. Eur J Surg Oncol 2023:S0748–7983(23)00563-2.
[54] Almstedt K, Heimes AS, Kappenberg F, Battista MJ, Lehr HA, Krajnak S, et al. Long-term prognostic significance of HER2-low and HER2-zero in node-negative breast cancer. Eur J Cancer 2022;173:10–9.
[55] Agostinetto E, Rediti M, Fimereli D, Debien V, Piccart M, Aftimos P, et al. HER2-low breast cancer: molecular characteristics and prognosis. Cancers 2021;13(11):2824.
[56] de Nonneville A, Houvenaeghel G, Cohen M, Sabiani L, Bannier M, Viret F, et al. Pathological complete response rate and disease-free survival after neoadjuvant chemotherapy in patients with HER2-low and HER2-0 breast cancers. Eur J Cancer 2022;176:181–8.
[57] Prat A, Bardia A, Curigliano G, Hammond MEH, Loibl S, Tolaney SM, et al. An overview of clinical development of agents for metastatic or advanced breast cancer without ERBB2 amplification (HER2-Low). JAMA Oncol 2022;8(11):1676–87.
[58] Ergun Y, Ucar G, Akagunduz B. Comparison of HER2-zero and HER2-low in terms of clinicopathological factors and survival in early-stage breast cancer: a systematic review and meta-analysis. Cancer Treat Rev 2023;115:102538.
[59] Lai HZ, Han JR, Fu X, Ren YF, Li ZH, You FM. Targeted approaches to HER2-low breast cancer: current practice and future directions. Cancers 2022;14(15).
[60] Molinelli C, Jacobs F, Marchiò C, Pitto F, Cosso M, Spinaci S, et al. HER2-low breast cancer: where are we? Breast Care 2022;17(6):533–45.
[61] Nicolò E, Tarantino P, Curigliano G. Biology and treatment of HER2-low breast cancer. Hematol Oncol Clin North Am 2023;37(1):117–32.
[62] Ross JS, Fletcher JA, Bloom KJ, Linette GP, Stec J, Clark E, et al. HER-2/neu testing in breast cancer. Am J Clin Pathol 2003;120:S53–71. Suppl:.
[63] Jin J, Li B, Cao J, Li T, Zhang J, Cao J, et al. Evolving perspectives in clinical landscape and molecular characteristics of HER2-low breast cancer and beyond. Evolving perspectives in clinical landscape and molecular characteristics of HER2-low breast cancer and beyond.
[64] Dai L, Ma D, Xiao Y, Jin X, Wu S -Y, Zhao Y -X, et al. Multiomics profiling characterizes distinct HER2-low breast cancer subgroups in the East Asian population; 2022. Available at SSRN 4181698.
[65] Lehmann-Che J, Hamy A-S, Porcher R, Barritault M, Bouhidel F, Habuellelah H, et al. Molecular apocrine breast cancers are aggressive estrogen receptor negative tumors overexpressing either HER2 or GCDFP15. Breast Cancer Res 2013;15(3):R37.
[66] Jacot W, Maran-Gonzalez A, Massol O, Sorbs C, Mollevi C, Guiu S, et al. Prognostic value of HER2-low expression in non-metastatic triple-negative breast cancer and correlation with other biomarkers. Cancers 2021;13(23):6059.
[67] Li Y, Tsang JY, Tam F, Loong T, Tse GM. Comprehensive characterization of HER2-low breast cancers: implications in prognosis and treatment. EBioMedicine 2023;91:104571.
[68] Yang M, Sun J, Liu L, Kong X, Lin D, Zhou H, et al. Clinicopathological characteristics of HER2-low breast cancer: a retrospective study. Sci Rep 2023;13(1):12382.
[69] Cochrane DR, Bernales S, Jacobsen BM, Cittelly DM, Howe EN, D'Amato NC, et al. Role of the androgen receptor in breast cancer and preclinical analysis of enzalutamide. Breast Cancer Res 2014;16(1):R7.
[70] Traina TA, Miller K, Yardley DA, Eakle J, Schwartzberg LS, O'Shaughnessy J, et al. Enzalutamide for the treatment of androgen receptor-expressing triple-negative breast cancer. J Clin Oncol 2018;36(9):884–90.
[71] Wardley A, Cortes J, Provencher L, Miller K, Chien AJ, Rugo HS, et al. The efficacy and safety of enzalutamide with trastuzumab in patients with HER2+ and androgen receptor-positive metastatic or locally advanced breast cancer. Breast Cancer Res Treat 2021;187(1):155–65.
[72] Miglietta F, Griguolo G, Bottosso M, Giarratano T, Lo Mele M, Fassan M, et al. HER2-low-positive breast cancer: evolution from primary tumor to residual disease after neoadjuvant treatment. NPJ Breast Cancer 2022;8(1):66.
[73] Denkert C, Martin M, Untch M, editors. Outcome analysis of HER2-zero or HER2-low hormone receptor-positive (HR+) breast cancer patients—characterization of the molecular phenotype in combination with molecular subtyping. San Antonio Breast Cancer Symposium Annual Meeting; 2022.
[74] Geukens T, De Schepper M, Richard F, Maetens M, Van Baelen K, Mahdami A, et al. Intra-patient and inter-metastasis heterogeneity of HER2-low status in metastatic breast cancer. Eur J Cancer 2023;188:152–60.
[75] Bergeron A, Bertaut A, Beltjens F, Charon-Barra C, Amet A, Jankowski C, et al. Anticipating changes in the HER2 status of breast tumours with disease progression—towards better treatment decisions in the new era of HER2-low breast cancers. Br J Cancer 2023;129(1):122–34.
[76] Bar Y, Dedeoglu AS, Fell GG, Moffett NJ, Boyraz B, Ly A, et al. Dynamic HER2-low status among patients with triple negative breast cancer (TNBC): the impact of repeat biopsies. Journal of Clinical Oncology 2023;41(Suppl.16):1005 (-).
[77] Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Eng J Med 2018;379(22):2108–21.
[78] Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2020;21(1):44–59.
[79] Abdel-Latif M, Youness RA. Why natural killer cells in triple negative breast cancer? World J Clin Oncol 2020;11(7):464–76.
[80] Chan IS, Ewald AJ. The changing role of natural killer cells in cancer metastasis. J Clin Investig 2022;132:6.
[81] Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4+ follicular helper T cell infiltration predicts breast cancer survival. J Clin Investig 2013;123(7):2873–92.