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Concordance between ER, PR, Ki67, and HER2-low expression in breast cancer by MammaTyper RT-qPCR and immunohistochemistry: implications for the practising pathologist

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Concordance between ER, PR, Ki67, and HER2-low expression in breast cancer by MammaTyper RT-qPCR and immunohistochemistry: implications for the practising pathologist

Background: There are limited data on the role of multigene tests and their correlation with immunohistochemistry (IHC), especially on core biopsy. MammaTyper is a quantitative conformite Europeeanne (CE) marked, National Institute for Health and Care excellence (NICE) approved, in *in vitro* diagnostic quantitative real-time polymerase chain reaction (RT-qPCR) test for assessment of mRNA expression of four biomarkers (*ESR1*, *PGR*, *ERBB2*, *MKI67*).

Methods: We evaluated the concordance of MammaTyper with oestrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67 by IHC on 133 core needle biopsies of breast cancer. HER2 was positive if IHC 3+ or 2+ and fluorescence *in situ* hybridization (FISH)-amplified. Global and hotspot Ki67 expression was analysed using a cutoff of ≥20% assessed manually and by digital image analysis. Agreements were expressed as overall percent agreement (OPA), positive percent agreement (PPA), negative percent agreement (NPA), and Cohen's kappa.

Results: RT-qPCR results of *ESR1* were highly concordant with IHC with OPA of 94.7% using 1% cutoff and 91.7% when the low ER-positive category was included. The PPA and NPA between RT-qPCR and IHC for PR was 91.5% and 88.0%, respectively, when using the 1% cutoff. For *ERBB2*/HER2, the OPA was 95% and the PPA was 84.6%. 40 of 72 HER2 IHC score 0 tumours were classified as *ERBB2* low. Best concordance between *MKI67* by MammaTyper and Ki67 IHC was achieved using hotspot digital image analysis (OPA: 87.2%, PPA: 90.6%, NPA: 80%).

Conclusion: RT-qPCR-based assessment of the mRNA expression of *ESR1*, *PGR*, *ERBB2*, and *MKI67* showed high concordance with IHC, suggesting that the MammaTyper test on core needle biopsies represents a reliable, efficient, and reproducible alternative for breast cancer classification and refining HER2 low categorisation.

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Abbreviations: CI, confidence interval; CISH, chromogenic in situ hybridisation; ER, oestrogen receptor alpha protein; ERBB2, ERB-B2 tyrosine kinase 2/human epidermal growth factor receptor 2 gene; ESR1, oestrogen receptor 1 gene; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescent in situ hybridisation; HER2, ERB-B2 tyrosine kinase 2/human epidermal growth factor receptor 2 protein; IHC, immunohistochemistry; Ki67, marker of proliferation Ki67 protein; MKI67, marker of proliferation Ki67 gene; MT, MammaTyper; PA, percent agreement; PGR, progesterone receptor gene; PR, progesterone receptor protein.

Keywords: breast cancer, HER2, HER2-low, Ki67, oestrogen receptor, progesterone receptor

Introduction

Breast cancer is a heterogeneous disease and the management decisions are largely based on the assessment of clinicopathological factors, as well as on the expression status of the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Ki67, a proliferation marker, although extremely valuable, is not routinely performed on all breast cancers since controversies exist regarding its optimum scoring methods and cutoff values.[1] The current gold standard is immunohistochemistry (IHC), a semiquantitative method for assessing ER/PR/HER2 and Ki67 protein expression. IHC limitations, including inter- and intraobserver variability, have been previously highlighted, particularly for Ki67.[2] HER2 status is tested by *in situ* hybridization (ISH) techniques such as fluorescent *in situ* hybridisation (FISH) on the equivocal IHC 2+ scores[3] or upfront on all cases[4] to determine gene amplification for tumours with equivocal IHC HER2 results (IHC 2+ score).[5] The mRNA-based tests have the potential to overcome some of the issues encountered with IHC and ISH.

The MammaTyper (Cerca Biotech, Berlin, Germany) is a conformite Europeenne (CE) marked *in vitro* diagnostic test that quantifies the mRNA expression of the four genes, *ERBB2*, *ESR1*, *PGR*, and *MKI67* using reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR). In an international multicentre prospective validation study, MammaTyper was shown to be highly accurate and reproducible in the quantitative determination of *ERBB2*, *ESR1*, *PGR*, and *MKI67* mRNA in breast cancer.[6] In 2018, the United Kingdom National Institute for Health and Care Excellence (NICE) published a Medtech innovation briefing that concluded that the test is reliable in classifying breast cancer subtypes.[7] Recent data from the OPTIMA prelim trial, which was presented at the San Antonio Breast Cancer Symposium 2023,[8] showed that high PGR expression by MammaTyper was superior to progesterone receptor IHC in predicting a low OncotypeDx score. The positive predictive value of MT-PGR ≥ 36.5 for Oncotype DX RS < 25 equals 93.2%.

However, direct comparison between MammaTyper and IHC in routine diagnostic practice using core needle biopsy has not been performed.

In this prospective study, we aimed to assess the concordance rates of MammaTyper testing of breast cancer core needle biopsies with ER, PR, and Ki67 status by IHC as well as HER2 status, by IHC +/− FISH in a large UK tertiary referral institution. We also aimed to analyse in detail the *ERBB2* mRNA expression in the HER2-Low breast cancer compared with IHC within this cohort.

Materials and Methods

The study was approved by the University Hospitals of Birmingham NHS Trust Clinical Audit Registration and Management System (CARMS NO-14418) as a prospective audit entitled "Prospective audit of incorporating MammaTyper use into routine breast cancer care in an NHS setting."

SAMPLE SELECTION AND PREPARATION OF FFPE TISSUE SECTIONS

Core needle biopsy formalin-fixed paraffin-embedded (FFPE) samples of breast cancer were prospectively collected between 2018 and 2019 at Queen Elizabeth Hospital Birmingham, a large UK tertiary referral hospital. Cases were fully anonymized, for a prospective audit (CARMS Registration No: 14418) and therefore, ethical approval and patient consent were not required. The tissue content of invasive tumour had to be at least 20% as determined by a breast pathologist. Sections of 10-μm thickness were prepared from the FFPE block and transferred into a 1.5 ml tube (RNase-free, safe-lock) using clean forceps. Measures were taken to prevent RNase contamination (e.g. from hands, skin, dust), by cleaning the work area beforehand with RNase decontamination reagents (e.g. RNase Away), wearing clean disposable gloves and using clean or disposable microtome blades and forceps.

IMMUNOHISTOCHEMICAL STAINING AND INTERPRETATION

ER, PR, Ki67, and HER2 status of samples was performed via IHC according to previously standardized protocols for routine diagnostic practice.

ER, PR, and Ki67 staining was performed using DAKO (Agilent, Santa Clara. CA, USA) Omnis automated staining platforms and HER2 IHC performed using the Ventana BenchMark Ultra (Roche, Indianapolis, IN, USA). Ready to Use (RTU) primary antibodies for ER; clone EP1, DAKO, PR; clone PgR 1294, DAKO, Ki67; Clone MIB-1, DAKO and HER2; clone 4B5, Ventana were incubated following the manufacturer's instructions.

An invasive carcinoma was defined as positive if ER and PR staining was seen in ≥1% cells and 1–10% of ER staining was reported as ER low positivity according to the current UK and ASCO/CAP guideline.[9] A PR cutoff of ≥20% was applied to distinguish between 'Luminal A-like' and 'Luminal B-like' subtypes as recommend by the 13th St Gallen International Breast Cancer Conference expert panel.[10] HER2 was scored as 0, 1+, 2+, and 3+ and tumours with 2+ score were further assessed with gene amplification based on the Cep17 and HER2 gene copy number by FISH. On choosing the cutoff value for Ki67 immunohistochemistry, we considered the available guidelines and evidence from the literature. A majority of the St Gallen's Panel voted that a threshold of ≥20% was indicative of 'high' Ki67 status.[10] Meanwhile, it is difficult to get acceptable agreement between pathologists on the Ki67 IHC cutoff between 5% and 30% although ≤5% or ≥30% can be used to estimate prognosis according to the International Ki67 Working Group.[1] Two Ki67 IHC scoring methods (eyeballing and hotspot analysis by digital image analysis) were used in the current study.

KI67 MANUAL AND DIGITAL ANALYSIS

Ki67-immunostained sections were scanned using a Leica Aperio AT2 slide scanner (Leica Biosystems Imaging, Vista, CA, USA) at ×40 magnification. Tumours were scored by a pathologist (N.M.B.) supervised by a specialist breast pathologist (A.M.S.). Both pathologists assessed the digitalized whole-slide images (WSIs) to provide a global eyeball score (pathologist global proliferation index %) and a hotspot score per case.

Using a deep-learning-based application for Ki67 assessment in Breast Cancer, AI APP, for research use only, (Visiopharm, Denmark), selection of the tumour area and different functions were used by a pathologist (N.M.B.) who annotated Ki67-positive and -negative cells. Regions of interest (ROIs) were selected to identify whole tissue, tumour area, and noninvasive areas. Four fields each including at least 200 nuclei were selected and counted. An average (Global score) was calculated for each case. A hotspot percentage count was recorded for each case.

The label function was used to annotate the cells in the invasive tumour areas where Ki67-positive cells were labelled with a different colour from the nonstained tumour cells. Global Ki67 count and hotspot percentage were calculated by selecting four fields, one of which included the hotspot following the International Ki67 in Breast Cancer Working Group guidelines.[1]

RNA ISOLATION AND RT-QPCR

The MammaTyper kit was developed and validated for use with RNA from FFPE breast cancer tissue samples, which were extracted and purified using the RNXtract RNA Extraction Kit according to the manufacturers' instructions (Cerca Biotech, Ref CC0 01011). MammaTyper testing was used to assess the mRNA expression of the four genes (*ESR1*, *PGR*, *ERBB2*, and *MKI67*) on a CFX96 qPCR cycler (Bio-Rad CFX Manager Software v. 3.1, Hercules, CA, USA) according to the manufacturer's instructions in this study. Calculations of 40-ddCq values were described previously in the latest MammaTyper IFU (Cerca Biotech, Ref CC0 01010).

$$\begin{aligned} 40-\Delta\Delta Cq = ((&\text{Median Cq target}_{\text{sample}} - - \\ &\text{Combined Reference}_{\text{sample}}) - - \\ &(\text{Median Cq target}_{\text{PositiveControl}} - - \\ &\text{Combined Reference}_{\text{PositiveControl}})) \end{aligned}$$

Cutoffs for categories variable (positive/negative) of markers are provided in Table S1 according to the MammaTyper IFU (Cerca Biotech, Ref CC0 1010).

STATISTICAL ANALYSIS

For continuous variables, the median, minimum, and maximum values were calculated. Categorically scaled variables were presented as absolute and relative frequencies (counts and percentages). The agreement between MammaTyper and IHC for biomarkers was assessed using positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) along with their two-sided 95% confidence interval (CI) and Cohen's kappa with its two-sided 95% CI. A kappa (κ) statistic ≤0.2 was regarded as poor agreement, >0.2–0.4 as fair, >0.4–0.6 as moderate, >0.6–0.8 as substantial, and

>0.8 as almost perfect agreement, as previously described.[11]

Additionally, the correlation between different IHC scores was assessed by Pearson correlation coefficient and the linear regression coefficient (R^2). All statistical analyses were performed using the SPSS 20.0 statistical software (IBM, Armonk, NY, USA) and figures were generated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). A two-sided $P \leq 0.05$ was considered statistically significant. Following analysis, discordant tumours were identified and reviewed in detail to identify reasons for discordance. Figure 1 shows a flow chart of the study design.

Results

CLINICOPATHOLOGICAL CHARACTERISTICS

A total of 133 breast cancer core biopsy samples diagnosed between 2018 and 2019 within a single UK institution (Queen Elizabeth Hospital Birmingham) were included in this study (Figure 1). The clinicopathological characteristics are shown in Table 1. The median age of patients was 61 years (range: 26–-92 years) and 60.3% of tumours were of grade 2. Mammatyper testing was performed on all core needle biopsy samples. One tumour containing solid papillary carcinoma was excluded from the immunohistochemical staining pathway.

AGREEMENT BETWEEN MAMMATYPER AND IMMUNOHISTOCHEMISTRY (CATEGORIZED MEASUREMENTS) FOR ER, PR, HER2

ER, PR, Ki67, and HER2 status for cutoffs detected by IHC and MammaTyper testing (RT-qPCR) had been previously categorized (e.g. positive or negative) (Table S1). The OPA (95% CI) was above 90% for all markers; 94.7% (89.5–97.4%) for ER, 90.1% (83.9–94.1%) for PR, and 95.0% (90.0–97.8%) for HER2, Table 2.

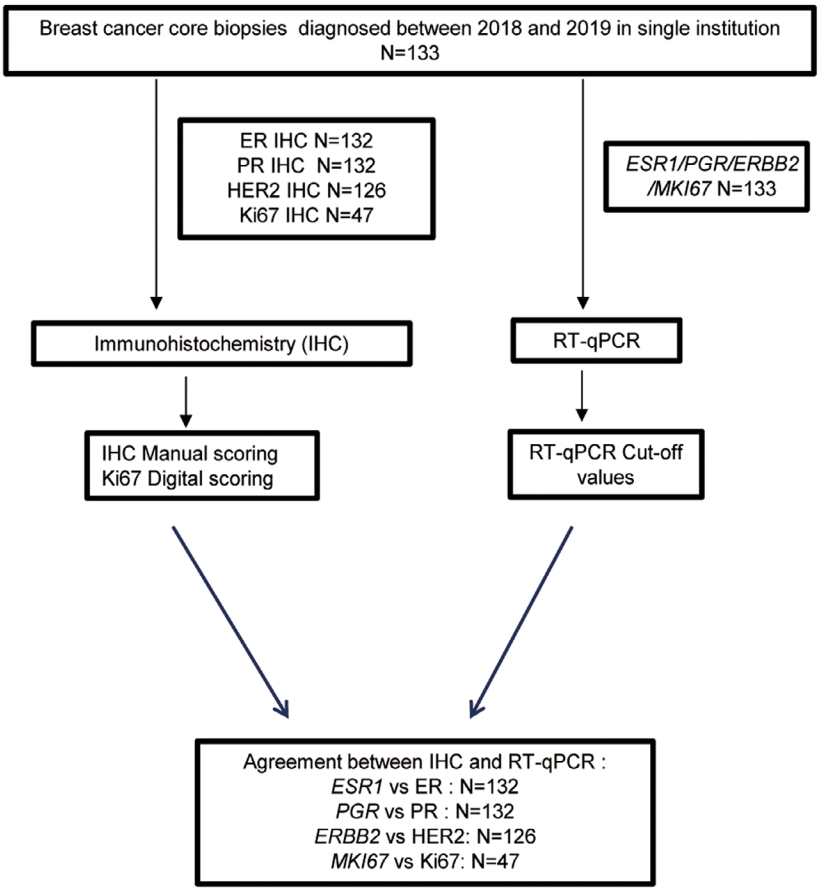


Figure 1. Flow chart of the studydesign. Immunohistochemistry, ER; Estrogen receptor, PR; Progesterone receptor; PGR; Progesterone receptor gene, CI; Confidence interval.

Table 1. Clinicopathological characteristics of the study cohort ($N = 133$)

Parameter	*N* (%)
Age (years) Median (IQR)	61 (50–74.5)
Gender	
Male	2 (1.5)
Female	131 (98.5)
Tumour type	
Ductal/NST	108 (81.2)
Lobular	15 (11.3)
Metaplastic	3 (2.25)
Mucinous	2 (1.5)
Tubular	2 (1.5)
Mixed/rare types	2 (1.5)
Solid papillary carcinoma	1 (0.75)
Tumour grade	
I	12 (9.0)
II	79 (59.4)
III	40 (30.1)
Unknown	2 (1.5)

IQR, interquartile range. NST, no special type

a The OPA between IHC and RT-qPCR using a lower cutoff point for *ESR1* reflecting 1% IHC staining was 94.7%. Similar concordance was seen when a low ER-positive category was included (91.7%).The kappa indicated almost perfect agreement over 0.8 (Table 2). The Pearson correlation coefficient and the linear regression coefficient (R^2) between ER IHC and *ESR1* was 0.933 and 0.871, respectively, with $P < 0.001$ (Figures 2A and alluvial plot Figure S1). RT-qPCR results of *ESR1* had highly concordant rates when ER was classified as negative (below 1%) and high positive (above 10%) excluding the ER low category (1–10%). Eleven ER highly positive IHC tumours were categorized into five negative and six low positive categories by MammaTyper testing, respectively.

The discordant cases were examined in detail (See Table S2). It is of note that two samples contained a small amount of invasive tumour tissue and were designated as ER low positive by MammTyper and three tumours showed ER weak nuclear staining in a small proportion of nuclei and were ER negative by MammaTyper.

CONCORDANCE RATES OF PR BETWEEN MAMMATYPER AND IHC

The OPAs of PR/*PGR* were over 90% using three cutoffs (1%, 10%, and 20%) (Table 2 and Table S3). The Pearson correlation coefficient and the linear regression coefficient (R^2) between PR IHC and *PGR* was 0.864 and 0.746, respectively, $P < 0.001$ (Figure 2B).

CONCORDANCE RATES OF HER2 BETWEEN MAMMATYPER AND IHC

The OPA was 95.0% with a lower PPA of 84.6% (Table 2). The kappa indicated substantial agreement (0.759). 13/126 (10.3%) cancers were HER2-positive by IHC/FISH, whereas 15/126 (11.9%) were classified as HER2-positive by MammaTyper testing, scatter plots (Figure 2C).

HER2-LOW BREAST CANCER

The HER2-low category was defined as IHC scores of 1+ or 2+ with FISH negative results. Among the 113 HER2 IHC negative tumours, 72 (63.7%) were HER2 0 and 41 (36.3%) were HER2-low. The latter group was classified into 35 HER2-low, three HER2 negative, and three positive categories by MammaTyper testing. In all, 72 IHC HER2 0 tumours were classified into 40 HER2-low, 31 HER2 0, and one positive category by MammaTyper testing (Figure 3). Therefore, 40 out of 72 IHC HER2 0 tumours showed *ERBB2* low expression by MammaTyper. The medians of HER2 mRNA expression (40-ddCq) were 38.5 ($n = 72$, range: 36.5–40.6) for IHC HER2 0 tumours and 39.4 ($n = 41$, range: 38.1–41.0) for IHC HER2-Low tumours. The expression of *ERBB2* in the IHC HER2-Low group was significantly higher than that in IHC HER2 0 group ($P < 0.001$) (Figure 4).

DISTRIBUTION OF ER/PR/HER2 IHC AND MRNA EXPRESSION

The frequencies of expression of ER/PR/HER2 using both techniques are plotted in Figure 5.

ER and PR IHC exhibited two peaks; the highest was for the strongly positive expression and the second for negative (0) staining. However, mRNA exhibited a wider dynamic range of expression. Similarly,

Table 2. Agreement between MammaTyper and immunohistochemistry for ER/*ESR1*, PR/*PGR*, HER2/*ERBB2*

	ER/*ESR1* $N = 132$		PR/*PGR* $N = 132$	HER2/*ERBB2* $N = 126$
IHC cutoff	Binary, ≥1%	Binary, ≥10%	Binary, ≥1%	Binary, negative vs. 3+ or 2+ /FISH+
RT-qPCR cutoff	Binary, ≥37.1	Binary, ≥38.2	Binary, ≥35.0	Binary, ≥40.4
PPA (95% CI)	92.9% (86.1–96.5%)	88.7% (80.8–93.6%)	91.5% (83.4–95.8%)	84.6% (57.7–95.7%)
NPA (95% CI)	100.0% (89.6–100.0%)	100% (90.1–100%)	88.0% (76.2–94.4%)	96.5% (91.3–98.6%)
OPA (95% CI)	94.7% (89.5–97.4%)	91.7% (85.7–95.3%)	90.1% (83.9–94.1%)	95.0% (90.0–97.8%)
Kappa (95% CI)	0.868 (0.768–0.947)	0.806 (0.697–0.911)	0.792 (0.689–0.892)	0.759 (0.553–0.916)

Concordance rates of ER between MammaTyper and IHC. IHC; Immunohistochemistry, ER; Estrogen receptor, PR; Progesterone receptor; PGR; Progesterone receptor gene, CI; Confidence interval; FISH; flourescence insitu hybridisation

the standard HER2 IHC scores (0, 1+, 2+, and 3+) corresponded to a wider range of mRNA levels, suggesting that IHC scores 0, 1+ and 2+ can further be classified using the MammaTyper assay.

CONCORDANCE RATES OF KI67 EXPRESSION BETWEEN MAMMATYPER AND IHC

Ki67 IHC data scored manually and digitally were available on a subset of the total cohort ($n = 47$), since the test is not routinely performed on all breast cancers in the UK.

Comparing *MKI67* by MammaTyper to Ki67 IHC using a cutoff value of 20%, a PPA of 92.3% was achieved with NPA of 61.9% by using standard pathologist eyeballing analysis, but NPA was improved to 80.0% when hotspot analysis (digital image analysis) of Ki67 was used with small compromise of PPA (Table 3). Scatter plots between MammaTyper and IHC are shown in Figure 2D,E. The linear regression coefficient (R^2) of two Ki67 IHC methods was 0.836 (Figure 2F). Analysis of two other Ki67 thresholds, 5% and 30%, was done. The highest concordance rate was obtained at 5% cutoff using hotspot analysis (OPA: 95.7% (85.7–98.8%) (Table 3). The agreement at 20% cutoff value is presented in Table S4.

Discussion

In this prospective study, we show that RT-qPCR-based assessment of the mRNA expression of *ERBB2*, *ESR1*, and *PGR* had a high concordance with IHC. *MKI67* by MammaTyper exhibited a higher concordance with the digital Ki67 image analysis.

Refining the classification of breast cancer and selection of patients for targeted therapy remains an urgent clinical need. While IHC for ER, PR, HER2, and Ki67 protein expression is currently the gold standard, the technique is semiquantitative with subjective assessment and requires pathologists' expertise both in the histological interpretation and biomarker analysis. The IHC assessment of Ki67, in particular, has been challenging, with various proposed cutoff values including 14%,[12] 18%, 20%, and 30%,[13] thus hindering its use in routine practice. The 20% cutoff value, used in the current study was previously proposed by the St Gallen's consensus and others as optimal in providing the best prognostic information,[10,12] and is used to confirm eligibility for the Food and Drug Administration (FDA) approved adjuvant amicaciclib therapy with endocrine therapy in high-risk ER-positive, HER2-negative, node negative early breast cancer.[14]

We report a high level of concordance between mRNA and protein levels in all markers studied. The rates of concordance are higher than previous reports. In their study of 397 Asian breast cancers, Chen et al[15] reported concordance rates of 81.6% ($\kappa = 0.4075$) for ER, 87.2% ($\kappa = 0.5647$) for PR, and 79.1% ($\kappa = 0.2767$) for HER2. Data from the OPTIMA Prelim study presented at the American Society of Clinical Oncology (ASCO) Annual Meeting 2022 highlighted marked disparity between Ki67 expression by IHC and three widely used genomic tests using standard methods by their manufacturers. Concordance was 62% for Oncotype DX, 69% for Prosigna, and 68% for MammaPrint. Best concordance was achieved when high Ki67 expression was defined as ≥30%.[16] The 2021 St Gallen Consensus[17] endorsed the thresholds of Ki67 5% and 30% for rejecting or recommending, respectively, adjuvant chemotherapy in ER+ early breast cancer, and hence

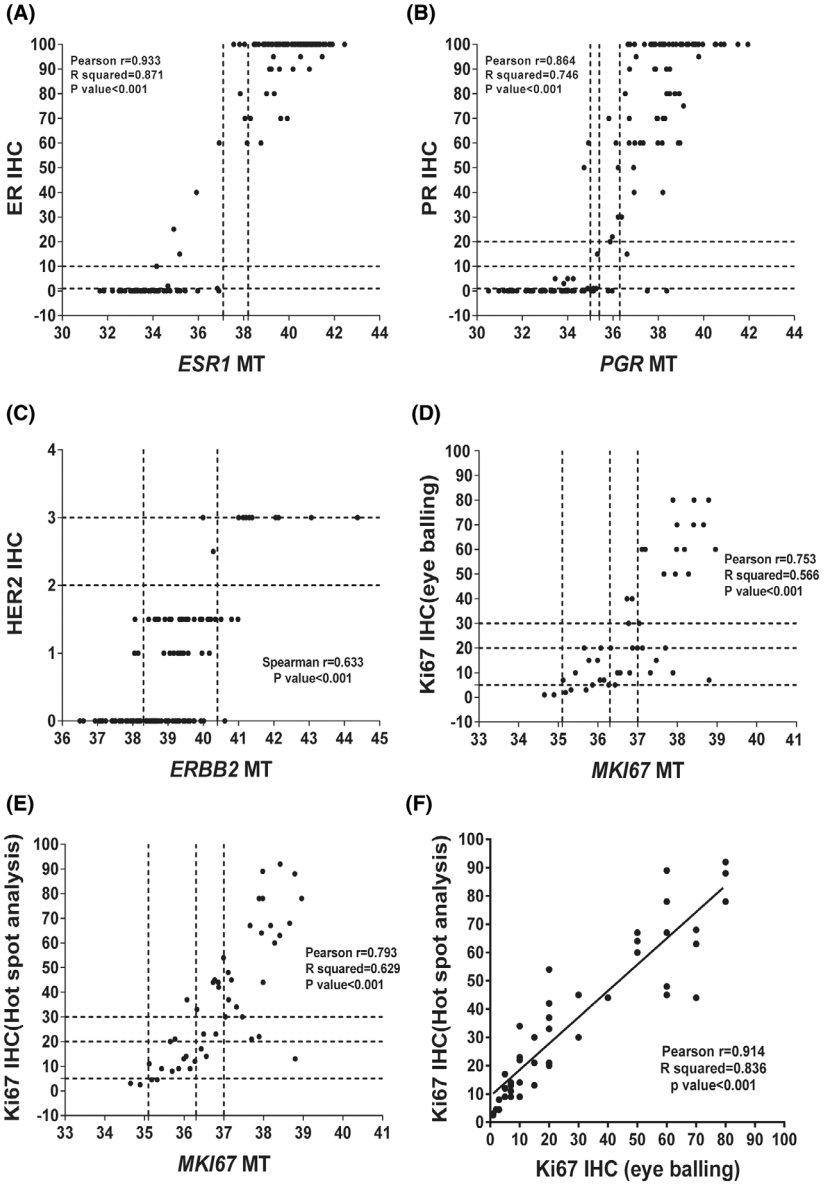
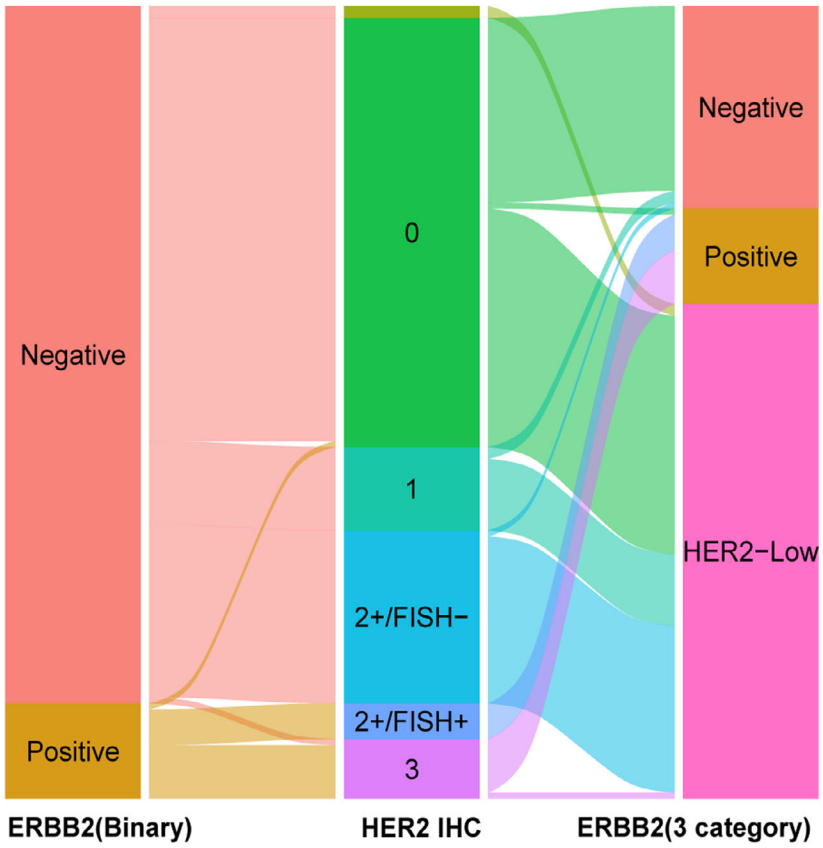


Figure 2. Comparison between IHC and RT-qPCR (MammaTyper) for ER, PR, HER2, and Ki67 expression. Scatter plot with IHC scores on the y-axis and MT values on the x-axis. Cutoff values of IHC sores and RT-PCR values are shown by black dotted lines. The linear regression coefficient (R^2) and the Pearson correlation coefficient values are shown in the graph. (A) Scatter plot of ER IHC scores and *ESR1* MT values, cutoff values at 1% and 10% for ER IHC and 37.1 and 38.2 for *ESR1* MT. (B) Scatter plot of PR IHC scores and *PGR* MT values, Cutoff values at 1%, 10%, and 20% for PR IHC and 35.0, 35.4, and 36.3 for *PGR* MT. (C) Scatter plot with HER2 IHC scores and *ERBB2* MT values, cutoff values at 1 and 2+/FISH+ for HER2 IHC and 38.3 (unpublished) and 40.4 for *ERBB2* MT. (D) Comparison of *MKI67* MT values and Ki67 IHC score by eyeballing. (E) Comparison of *MKI67* MT values and Ki67 IHC score by hotspot analysis using Visiopharm digital platform. (F) Correlation of Ki67 IHC eyeballing method and Ki67 IHC hotspot analysis.

those thresholds were analysed in this study. We herein also report an excellent correlation on hotspot analysis at 5%. Other studies previously highlighted the discrepancy in HER2 assessment by IHC and Oncotype DX with a percent positive agreement of only 50%.[18]

While there were no technical failures in this cohort, few tumours exhibited discrepant results between IHC and mRNA results. We examined the reasons of ER and HER2 discordance between the two techniques. Those included the presence of a small amount of invasive carcinoma (small tumour



		HER2 IHC					
		0	1	2+/FISH-	2+/FISH+	3	Total
ERBB2 (Binary)	Negative	71	14	24	1	1	111
	Positive	1	0	3	2	9	15
Total		72	14	27	3	10	126

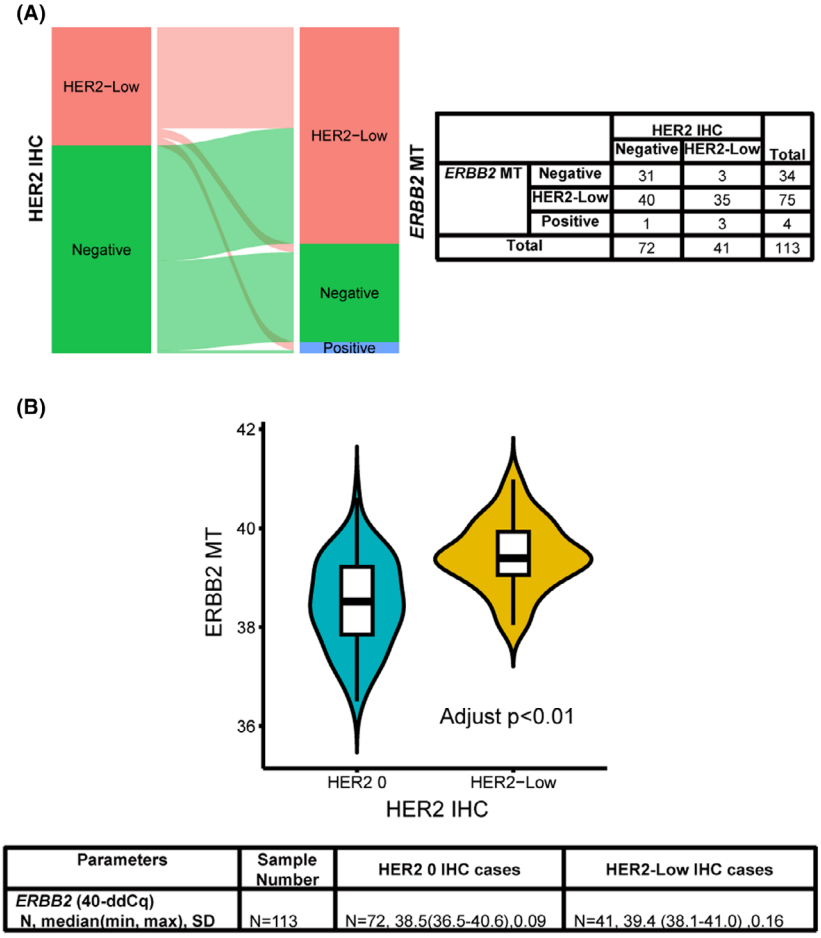
		HER2 IHC					
		0	1	2+/FISH-	2+/FISH+	3	Total
ERBB2 (3 category)	HER2 0	31	2	1	0	0	34
	HER2-Low	40	12	23	1	1	77
	Positive	1	0	3	2	9	15
Total		72	14	27	3	10	126

Figure 3. Alluvial plot of HER2 IHC and *ERBB2* mRNA categories. Alluvial plot showing re-categorisation of HER2 IHC scores by RT-qPCR (MammaTyper). IHC; Immunohistochemistry, FISH; flourescence insitu hybridisation.

content), large amount of DCIS, and tumours with weak ER expression. Care should be taken in those scenarios and testing other samples with good invasive tumour content should be considered.

Similar figures to our study were, however, achieved in the ABCSG-6 biomarker cohort comparing central IHC testing with mRNA expression assessed by CE-Marked test (STRAT4) of surgical excisions. The results, published in an abstract form, showed concordance rates of 98.6% for ER, 92.6% for PR, 98.4% for HER2, and 88.7% for Ki67.[19]

One of the strengths in our study is that the histological assessment and molecular testing were performed at a central specialist tertiary referral unit



		HER2 IHC		
		Negative	HER2-Low	Total
ERBB2 MT	Negative	31	3	34
	HER2-Low	40	35	75
	Positive	1	3	4
Total		72	41	113

Parameters	Sample Number	HER2 0 IHC cases	HER2-Low IHC cases
ERBB2 (40-ddCq) N, median(min, max), SD	N=113	N=72, 38.5(36.5-40.6),0.09	N=41, 39.4 (38.1-41.0) ,0.16

Figure 4. HER2-low analysis: (A) Alluvial plot of HER2 IHC and *ERBB2*. More tumours are classified as HER2-Low by RT-qPCR testing. (B) Violin plot of HER2 mRNA expression by HER2 IHC status. A significant difference in mRNA levels is found between IHC 0 and HER2-Low cancers. IHC; Immunohistochemistry.

and a UK Local Genomics Laboratory with validated protocols that is a referral unit for HER2 IHC and FISH testing. The study was prospective, included representation from all breast cancer molecular subtypes, and was performed on core biopsies that are used in routine practice for receptor assessment. Another strength is the analysis, in detail, of the HER2-Low breast cancer group and the detailed assessment of Ki67 IHC staining both by eyeballing and by digital image analysis and using different previously proposed cutoff values.

In the current study, few discordant tumours were identified and were examined in detail. The reasons for discordance included tumour heterogeneity (particularly with PR), very low levels of expression, and the inclusion of normal mammary tissue. Compared with ER, PR expression is known to be more heterogeneous in distribution. Allott *et al.* reported PR heterogeneity in 16% of tumours compared with 8% for ER,[20] and this may be prognostic. Recent data from the STO-5 trial showed a survival disadvantage for patients whose tumours showed high PR heterogeneity.[21] A total of six tumours were discordant for HER2 mRNA and protein expression; four of which were of the equivocal 2+ IHC category. There were 13 out of 126 HER2-positive tumours determined by IHC/FISH compared to 15 out of 126 tumours that were HER2-positive determined by MammaTyper. This means that two additional patients out of 126 (1.6%) would be treated with anti-HER2 agents if MammaTyper was used to select these patients for anti-HER2 therapies.

The recent development of various target therapies to HER2 low breast cancer has also highlighted the need for accurate categorisation of this not

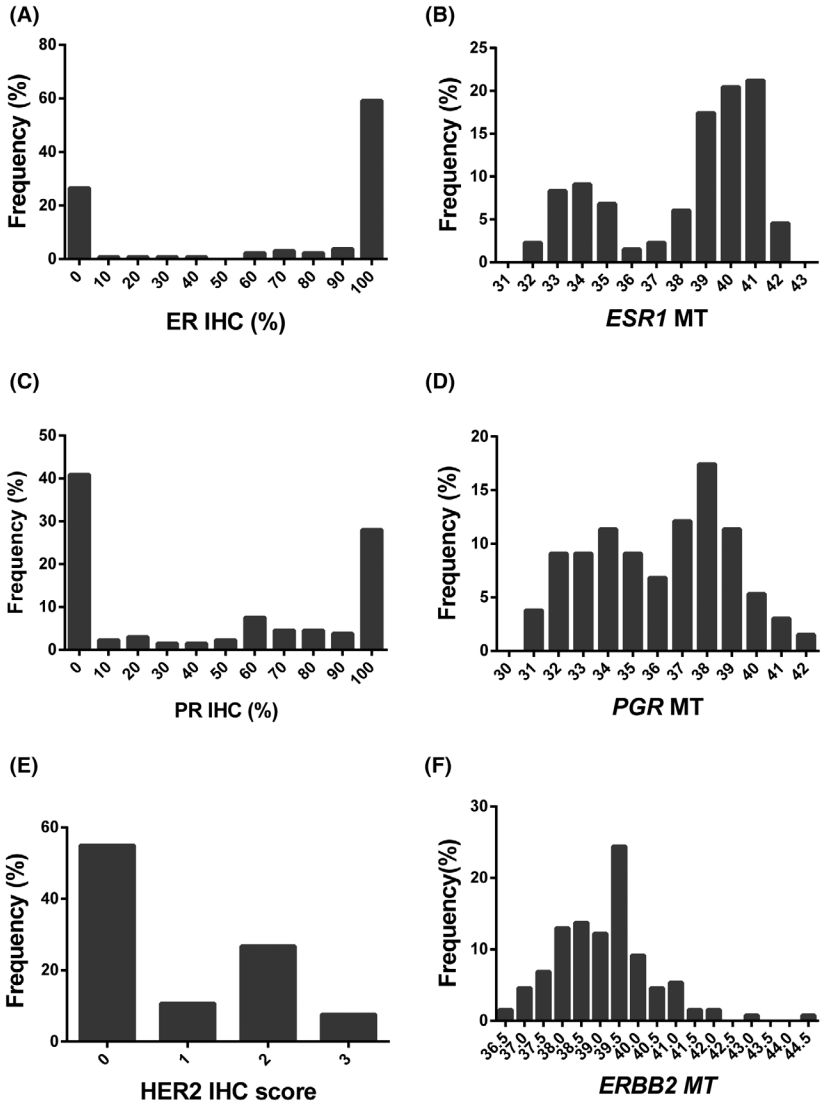


Figure 5. The distribution of ER (A,B), PR (C,D), and HER2 (E,F) scores by IHC and MammaTyper. IHC; Immunohistochemistry, ER; Estrogen receptor, PR; Progesterone receptor; PGR; Progesterone receptor gene.

uncommon group.[22,23] A recent study of 12 expert pathologists concordance in HER2 IHC scoring showed low reproducibility, with 10% of tumours remaining challenging to categcategorise as HER2 IHC score 0 (negative) versus 1+ (Her2-Low).[11] The development of mRNA-based techniques to refine and accurately classify those lesions may be the way forward. In the current study, we show that 55.6% (40 out of 72) of IHC HER2 0 tumours had *HER2* low expression by MammaTyper. This will have therapeutic implications in view of the recent DESTINY-Breast04 study results, which showed that trastuzumab deruxtecan significantly prolonged progression-free survival (9.9 versus 5.1 months) and overall survival (23.4 versus 16.8 months) compared to the physician's choice of chemotherapy in patients with HER2-low metastatic breast cancer.[24] The question remains whether these patients with IHC HER2 0 tumours but detectable HER2 low expression by MammaTyper would respond to trastuzumab deruxtecan or other anti-HER2 targeting agents. The ongoing DESTINY-Breast06 study that included IHC HER2 0 tumours would help to answer these questions. In addition, further studies that include HER2 0 tumours but detectable HER2 low expression in tumours by MammaTyper would confirm whether MammaTyper would be a more reliable test to select patients with HER2 low expression for trastuzumab,

Table 3. Agreement between MammaTyper and immunohistochemistry of Ki67/MKI67

	Ki67 IHC (eyeballing)/*MKI67 N* = 47	Ki67 (hotspot analysis)/*MKI67 N* = 47
IHC cutoff	Binary, ≥20%	
RT-qPCR cutoff	Binary, ≥36.3	
PPA (95% CI)	92.3% (75.9–87.9%)	90.6% (75.8–96.8%)
NPA (95% CI)	61.9% (40.9–79.3%)	80.0% (54.8–95.9%)
OPA (95% CI)	78.7% (65.1–88.0%)	87.2% (74.8–94.0%)
Kappa (95% CI)	0.557 (0.297–0.775)	0.706 (0.470–0.902)

	Ki67 (hotspot analysis)		
	≦5	>5%	Total
MKI67			
≦35.1	2	0	2
>35.1	2	43	45
Total	4	43	47

	Ki67 (eyeballing)		
	≦5	>5%	Total
MKI67			
≦35.1	2	0	2
>35.1	6	39	45
Total	8	39	47

	Ki67 IHC (eyeballing)/*MKI67 N* = 47	Ki67 (hotspot analysis)/*MKI67 N* = 47
IHC cutoff	Binary, >5%	
RT-qPCR cutoff	Binary, >35.1	
PPA (95% CI)	100% (91.0–100%)	100% (91.8–100%)
NPA (95% CI)	25.0% (3.2–65.1%)	50.0% (15.0–85.0%)
OPA (95% CI)	87.2% (74.2–95.2%)	95.7% (85.7–98.8%)
Kappa (95% CI)	0.356 (0.000–0.728)	0.647 (0.198–1.000)

	Ki67 (eyeballing)		
	<30%	≥30%	Total
MKI67			
<37.0	22	3	25
≥37.0	6	16	22
Total	28	19	47

Table 3. (*Continued*)

	Ki67 (hotspot analysis)		
	<30%	≥30%	Total
	Ki67 (hotspot analysis)		
	<30%	≥30%	Total
MKI67			
<37.0	18	7	25
≥37.0	3	19	22
Total	21	26	47

	Ki67 IHC (eyeballing)/*MKI67* *N* = 47	Ki67 (hotspot analysis)/*MKI67* *N* = 47
IHC cutoff	Binary, ≥30%	
RT-qPCR cutoff	Binary, ≥37.0	
PPA (95% CI)	84.2% (62.4–94.5%)	73.1% (53.9–86.3%)
NPA (95% CI)	78.6% (60.5–89.8%)	85.7% (65.4–95.0%)
OPA (95% CI)	80.8% (67.5–89.6%)	78.7% (65.1–88.0%)
Kappa (95% CI)	0.612 (0.385–0.840)	0.577 (0.345–0.810)

deruxtecan, or other anti-HER2 targeting agents. Similarly, out of 11 ER strongly positive tumours, six were classified as *ESR1* low-positive and five as negative. It was also interesting to note that the distribution of ER and PR scoring by IHC was bimodal, while *ERS1* and *PGR* mRNA expression was of a wide and more dynamic range. Those interesting observations require further investigation as to whether mRNA-based tests may refine our current molecular classification of breast cancer and refine selection for targeted therapy.

The prognostic significance of Ki67 expression is proven particularly in ER-positive breast cancer and following neoadjuvant endocrine therapy.[25,26] Its expression correlates well with the mitotic activity[27] and is used to subclassify ER-positive breast cancer into luminal A and B.[28]

A recent study presented at the ASCO conference 2022 has shown marked disparity between Ki67 IHC assessment and gene assay data by Oncotype Dx and Prosigna.[29]

In this current study, we followed the International Ki67 in Breast Cancer Working Group recommendations for Ki67 scoring manually and digitally and the latest St Gallen recommendations of using the 5% and 30% Ki67 thresholds to avoid and recommend adjuvant chemotherapy, respectively.[17] We showed a high level of concordance that was best on using digital and hotspot assessment in comparison with average score and the eyeballing Ki67 estimates. Automated average Ki67 scoring had the potential to improve consistency of Ki67 IHC scoring.[30] The authors, however, reported lower concordance when the maximum Ki67 results were used. We have previously shown that manual Ki67 scoring by eyeballing may overestimate the average Ki67 count, as the human eye gets naturally drawn to the areas of high expression, giving a false impression of a high score. The digital AI method was, faster, quantitative, and accurate.[31]

In conclusion, our data suggest that MammaTyper test on core needle biopsies represents a reliable, efficient, and reproducible alternative for breast cancer 4-marker IHC analysis and molecular subtyping. Technically, none of the tumours failed testing and the tumour content was optimal for all core biopsies tested. This is reassuring and indicates that the test is applicable on small core biopsy samples and surgical excisions. There are discordant results between IHC and the MammaTyper test, especially with regard to HER2-positive and HER2 low status, which may have therapeutic implications for patients. Further validation is ongoing to further assess the predictive values

of MammaTyper test in comparison to ICH/FISH in selecting patients for anti-HER2 treatments.

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Author contributions

NB: data collection and curation, MZ: data collection and curation, QZ: formal analysis, visualization, DK: Resources including sectioning and immunohistochemistry, AK: conceptualization, methodology, formal analysis, review, and editing, AMS: conceptualisation, methodology data collection, formal analysis, supervision, presentation, funding acquisition, wrote the original draft, review, and editing. All authors approved the final article.

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Conflict of interest

MammaTyper RT-Qpcr testing was funded by Biontech Diagnostic GmbH. AK received fees for consulting, advisory, speaker's roles, and/or research funding from PUMA BioTechnology, AstraZeneca, Merck, MSD, Bristol-Myers Squibb, Affini-T Therapeutics. AMS received fees for advisory board member/speaker roles for Exact Sciences, Veracyte, Daiitchi Sankyo, Ventana Roche, and AstraZeneca. None of the above has an impact on the study presented.

Ethics approval statement

N/A. The test is NICE-approved and Ce-Marked. The study was performed as a registered prospective audit (CARMS-14418) entitled: Prospective audit of incorporating MammaTyper use into routine breast cancer care in an NHS setting.

Data availability statement

The authors are happy to share original data with other investigators upon reasonable request.

References

1. Nielsen TO, Leung SCY, Rimm DL *et al*. Assessment of Ki67 in breast cancer: updated recommendations from the international Ki67 in breast cancer working group. *J. Natl. Cancer Inst.* 2021; **113**; 808–819.
2. Hammond ME, Hayes DF, Dowsett M *et al*. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J. Clin. Oncol.* 2010; **28**; 2784–2795.
3. Rakha EA, Tan PH, Quinn C *et al*. UK recommendations for HER2 assessment in breast cancer: an update. *J. Clin. Pathol.* 2023; **76**; 217–227.
4. Wolff AC, Somerfield MR, Dowsett M *et al*. Human epidermal growth factor receptor 2 testing in breast cancer: Asco-College of American Pathologists guideline update. *Clin. Oncol.* 2023; **41**; 3867–3872.
5. McLemore LE, Albarracin CT, Gruschkus SK *et al*. HER2 testing in breast cancers: comparison of assays and interpretation using ASCO/CAP 2013 and 2018 guidelines. *Breast Cancer Res. Treat.* 2021; **187**; 95–104.
6. Varga Z, Lebeau A, Bu H *et al*. An international reproducibility study validating quantitative determination of ERBB2, ESR1, PGR, and MKI67 mRNA in breast cancer using MammaTyper. *Breast Cancer Res.* 2017; **19**; 55.
7. National Institute for Health and Care Excellence. Mammatyper *in vitro* diagnostic test for determining breast cancer subtypes. Medtech innovation briefing [mib135]. 2018.
8. Stein R, Wirtz R, Marshall A et al. Can high progesterone receptor (pgr) expression identify tumours with low-risk tumour gene expression scores? SABCS abstract PO4-16-02. 2023.
9. Allison KH, Hammond MEH, Dowsett M *et al*. Estrogen and progesterone receptor testing in breast cancer: ASCO/CAP guideline update. *J. Clin. Oncol.* 2020; **38**; 1346–1366.
10. Goldhirsch A, Winer EP, Coates AS *et al*. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2013. *Ann. Oncol.* 2013; **24**; 2206–2223.
11. Zaakouk M, Quinn C, Provenzano E *et al*. Concordance of HER2-low scoring in breast carcinoma among expert pathologists in the United Kingdom and the Republic of Ireland -on behalf of the UK National Coordinating Committee for breast pathology. *Breast* 2023; **70**; 82–91.
12. Muftah AA, Aleskandarany MA, Al-Kaabi MM *et al*. Ki67 expression in invasive breast cancer: the use of tissue microarrays compared with whole tissue sections. *Breast Cancer Res. Treat.* 2017; **164**; 341–348.
13. Zurrida S, Bagnardi V, Curigliano G *et al*. High Ki67 predicts unfavourable outcomes in early breast cancer patients with a clinically clear axilla who do not receive axillary dissection or axillary radiotherapy. *Eur. J. Cancer* 2013; **49**; 3083–3092.
14. Food and Drug Administration (FDA). FDA expands early breast cancer indication for abemaciclib with endocrine therapy. 2023.
15. Chen CJ, Chen TH, Lei J *et al*. Correlation of ER, PR, and HER2 at the protein and mRNA levels in Asian patients with operable breast cancer. *Biosci. Rep.* 2022; **42**; BSR20211706.
16. Stein RC, Marshall A, Bayani J *et al*. Disparity between Ki67 measurements and tumor gene expression tests in patients with hormone-sensitive early breast cancer from the optima preliminary trial. *J. Clin. Oncol.* 2022; **40**; 567.

17. Burstein HJ, Curigliano G, Thurlimann B *et al.* Customizing local and systemic therapies for women with early breast cancer: the St. Gallen International Consensus guidelines for treatment of early breast cancer 2021. *Ann. Oncol.* 2021; **32**; 1216–1235.
18. Dvorak L, Dolan M, Fink J, Varghese L, Henriksen J, Gulbahce HE. Correlation between HER2 determined by fluorescence in situ hybridization and reverse transcription-polymerase chain reaction of the oncotype DX test. *Appl. Immunohistochem. Mol. Morphol.* 2013; **21**; 196–199.
19. Filipits M, Rudas M, Singer C *et al.* mRNA expression of ER, PR, HER2 and Ki67 are concordant to central IHC and predict clinical outcome: a validation study from the ABCSG-6 biomarker cohort. *Ann. Oncol.* 2018; **29**; VIII480.
20. Allott EH, Geradts J, Sun X *et al.* Intratumoral heterogeneity as a source of discordance in breast cancer biomarker classification. *Breast Cancer Res.* 2016; **18**; 68.
21. Danielsson O, Dar O, Perez-Tenorio G *et al.* Long-term survival and intra-tumor heterogeneity of progesterone receptor expression in estrogen receptor-positive/progesterone receptor-positive premenopausal women with breast cancer. *Cancer Res.* 2023; **83**; 5; 3-05-03
22. Tarantino P, Viale G, Press MF *et al.* Esmo expert consensus statements (ECS) on the definition, diagnosis, and management of HER2-low breast cancer. *Ann. Oncol.* 2023; **34**; 645–659.
23. Schnitt SJ, Tarantino P, Collins LC. The American Society of Clinical Oncology-College of American Pathologists guideline update for human epidermal growth factor receptor 2 testing in breast cancer. *Arch. Pathol. Lab Med.* 2023; **147**; 991–992.
24. Modi S, Jacot W, Yamashita T *et al.* Trastuzumab deruxtecan (T-Dxd) versus treatment of physician's choice (TPC) in patients (pts) with HER2-low unresectable and/or metastatic breast cancer (mBC): results of destiny-breast04, a randomized, phase 3 study. *J. Clin. Oncol.* 2022; **40**; LBA3.
25. Ellis MJ, Suman VJ, Hoog J *et al.* Ki67 proliferation index as a tool for chemotherapy decisions during and after neoadjuvant aromatase inhibitor treatment of breast cancer: results from the American College of Surgeons Oncology Group Z1031 trial (alliance). *J. Clin. Oncol.* 2017; **35**; 1061–1069.
26. Smith I, Robertson J, Kilburn L *et al.* Long-term outcome and prognostic value of Ki67 after perioperative endocrine therapy in postmenopausal women with hormone-sensitive early breast cancer (poetic): an open-label, multicentre, parallel-group, randomised, phase 3 trial. *Lancet Oncol.* 2020; **21**; 1443–1454.
27. Trihia H, Murray S, Price K *et al.* Ki-67 expression in breast carcinoma: its association with grading systems, clinical parameters, and other prognostic factors--a surrogate marker? *Cancer* 2003; **97**; 1321–1331.
28. Inwald EC, Koller M, Klinkhammer-Schalke M *et al.* 4-IHC classification of breast cancer subtypes in a large cohort of a clinical cancer registry: use in clinical routine for therapeutic decisions and its effect on survival. *Breast Cancer Res. Treat.* 2015; **153**; 647–658.
29. Stein RC, Marshall A, Bayani J *et al.* Disparity between Ki67 measurements and tumor gene expression tests in hormone-sensitive early breast cancer patients from the OPTIMA preliminary trial. *J. Clin. Oncol.* 2022; **40**; 567.
30. Rimm DL, Leung SCY, McShane LM *et al.* An international multicenter study to evaluate reproducibility of automated scoring for assessment of Ki67 in breast cancer. *Mod. Pathol.* 2019; **32**; 59–69.
31. Badr NM, Ramsing TW, Overgaard A *et al.* Concordance between manual pathologist scoring and an artificial intelligence deep learning-based algorithm for Ki-67 immunohistochemical scoring in breast cancer. *Eur. J. Cancer* 2022; **175** (suppl 1); S59.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cutoffs for MammaTyper single marker results for CFX96 (Bio-Rad CFX Manager Software Version 3.1).

Table S2. Agreement between MammaTyper and immunohistochemistry of ER and PR.

Table S3. Clinicopathological features of ER discordant cases.

Table S4. Agreement between MammaTyper and immunohistochemistry of Ki67/MKI67 using immunohistochemistry threshold of 20%.

Figure S1. Alluvial plot of ER IHC and ESR1 RT-qPCR categories [Negative, low positive and Positive].