Markers of steroid receptor, kinase signalling pathways and Ki-67 expression in relation to tamoxifen sensitivity and resistance

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Background: It remains clinically important to identify ER positive breast cancers likely to respond to tamoxifen (TAM) and so we aimed to select a group of biomarkers able to predict response. We also assessed whether data from different sample types [tumor microarrays (TMAs) and core biopsies] or tumor sites could be combined for biomarker studies.

Methods: A total of 123 endocrine treatment naïve patients with known ER and HER2 status treated with TAM had paraffin-embedded tumor tissue available either as TMAs (n=102) or core biopsies (n=21). TMA cores were collected from three different tumor sites, two central and one peripheral. Ten biomarkers were evaluated by immunohistochemistry, for % positivity and/or H-Score, comprising: ER, HER2, Ki-67, phosphorylated forms of ER (Ser118), IGF1R, PRAS40, Akt & MAPK (ERK1/2), and PTEN & androgen receptor expression (AR). Each tumor was analysed for Akt1 E17K somatic mutation using BEAMing technology. Patient outcome was assessed by clinical benefit (CB) rate & survival analyses [time to progression (TTP) and time to death (TTD)].

Results: There was no significant difference in % positivity or H-Score between central & peripheral tumor sites for all biomarkers examined. After False Discovery Rate (FDR) correction differences ($P<0.05$) were observed between the two central samples only for HER2 & pER118 and pPRAS40. However, differences in biomarker expression were common between core biopsies and TMAs. Only 2/123 (1.6%) tumors had Akt1 E17K mutations. Univariate and multivariate analyses identified that lower levels of PTEN and higher levels of Ki-67 (% positivity) were predictive of poor outcome (TTP & TTD) following TAM. Higher ER. lower Ki-67 and AR/ER ratio <2 predicted increased CB rate.

Conclusions: There were few differences in marker expression between TMAs from different intra-tumoral sites. More marked differences between TMAs and core biopsies suggest caution if combining such datasets. Loss of PTEN, a key regulator of the PI3K/Akt pathway, was the only RTK/kinase signaling biomarker related to poorer clinical outcome. PTEN along with ER & lower Ki-67 proved the most predictive markers for better outcome (TTP & TTD and/or CBR) following TAM treatment.

Keywords: ER+ breast cancer; Akt pathway; tamoxifen

Introduction

Resistance to endocrine therapy (ET) is an ongoing clinical challenge in the treatment of estrogen receptor positive ER+ breast cancer (BC). Between 50–80% of ER+ patients initially show clinical benefit to ETs that target estrogen/ER signaling (1,2). Tamoxifen (TAM), a SERM, is one ET that has been used for nearly 40 years in BC (3) and acts by competitively binding to the ER thus blocking the ability of estrogen to drive tumor growth (4). Many patients now receive aromatase inhibitor (AI) treatment, which acts by causing estrogen deprivation, yet TAM has not been superceded by AIs in every clinical scenario, e.g., remaining an important adjuvant ET in premenopausal patients. In postmenopausal patients, 10 years treatment with an AI has not been established as superior to 5 years. Thus, the best extended ET options in post-menopausal BC are still unclear and may incorporate TAM since all of the following have been shown in RCTs to be better than 5 years of TAM alone, i.e., (I) 10 years of TAM (ATLAS & aTTOM) (5,6)], (II) 5 years of TAM followed by 3–5 years of AI (MA17) (7) or (III) 5 years of AI (ATAC, BIG 1–98) (8). TAM still has a significant role in BC chemoprevention (9-12) where studies up to 20 years (9,11) provide data on its long-term efficacy *vs*. side-effects. It remains clinically-important to identify BCs most likely to be sensitive to TAM.

Steroid hormone receptors, growth factor signaling and resistance to ET

Resistance can either be 'de-novo' (i.e., no evidence of hormone sensitivity—defined in the advanced setting as progression within 6 months of starting ET) or 'acquired' (i.e., in this setting, after a period of response/stable disease for at least 6 months the tumor progresses). In ER+ BC the proliferation marker Ki-67 is a prognostic tool that may be valuable in defining responsiveness to ET (13,14).

It may be possible to define other molecular predictive biomarkers based on ET signalling. ER-alpha (ER) expression is commonly retained in ET resistance (15) and is mostly in its wild type form in BCs refractory to TAM since its mutation is enriched primarily in estrogen deprivation resistant patients where it is believed to promote a constitutively-active receptor (16-18). A second ER, ER beta, has five isoforms and while the data for ERβ is engaging it has not yet been established as a marker with clinical utility in breast cancer. For example, in one study of 177 postmenopausal women who received adjuvant tamoxifen, the recurrence-free interval was found to differ with respect to degree of nuclear ERβ1 expression (19). In another study from our own research group of 880 patients with primary breast cancer, nuclear ERβ2 and ERβ5, but not ERβ1, significantly correlated with OS and ERβ2 additionally with DFS. ERβ2 also predicted response to endocrine therapy and correlated positively with ERα, progesterone receptor and androgen receptor (20). Not only were the tumor and clinical outcome correlations different between these two studies for ERβ1 but the positivity varied from 33% to 99% respectively, despite the fact both used the same mouse monoclonal antibody clone PPG5/10 which was raised against the unique COOH-terminal peptides of ERβ1. In a further study involving 911 patients with breast cancer, ERβ1 expression (again using this antibody) reported positivity in 89% of tumors (21). In this study, high ERβ1 expression was a favourable prognostic marker, especially in chemotherapy treated patients, but not in the endocrine therapy-treated breast cancer patients. Furthermore, in a pre-surgical study comparing the effect of short-term tamoxifen or three doses of fulvestrant (50, 125 and 250 mg) versus placebo we previously reported significant changes in ERα, PR and Ki-67 (22). However, there was no significant difference between any of the endocrine arms and placebo in terms of either ERβ1 or ERβ2 (personal communication J Robertson). Therefore, while the role of ERβ in breast cancer continues to be explored (23) we did not include it as a biomarker in the present study but focussed on ERα.

Breast cancer models have helped reveal multiple potential mechanisms underpinning resistance (24). Cross talk between ER and deregulated growth factor-driven kinase signaling pathways has been most implicated (25,26), including subverting responsiveness to TAM (27). Such mechanisms have been proposed as a key focus for progress in overcoming endocrine resistance (28). Three membrane receptor tyrosine kinases (RTKs) with reported roles in cell proliferation in particular have been linked to TAM resistance: insulin-like growth factor (IGF) 1 receptor (IGF1R), epidermal growth factor receptor (EGFR), and HER2. Inhibition of such receptors can lead to restoration of the therapeutic effect of TAM (29). IGF1R has been reported in relation to the endocrine responsive phenotype (30). Blockade of the IGFR pathway with the monoclonal antibody Ganitumab showed a negative therapeutic impact (31). Downstream of such RTKs, PI3K signaling through hyper-activation of Akt plays an important role ER+ BC progression, being able to facilitate resistance to ET (32-37). An E17K mutation in AKT1 is also capable of constitutively-activating

this enzyme (38). Loss of the enzyme phosphatase and tensin homolog (PTEN) can furthermore result in Akt hyperactivity (39,40) and its loss has been associated with TAM resistance (30). Phosphorylation on Thr246 of Proline-rich Akt substrate of 40 kDA (PRAS40), an Akt substrate and indicator of PI3K pathway activation status (37,41), might also feasibly relate to worsened prognosis but to date there is a paucity of studies exploring the contribution for pPRAS40 in TAM resistant ER+ BC.

Several routes exist for subsequent cross-talk between deregulated RTK/kinase pathways and ER, including ligand independent activation of ER (24). Nuclear ER phosphorylation of AF-1 residues including serine 118 and 167 (pER118 and pER167 respectively) can be triggered by these kinase pathways. Non-genomic (plasma membrane) ER interacts with RTKs activating downstream kinases that can drive such ligand-independent nuclear ER phosphorylation. ERK1/2 MAP kinase (MAPK) has been linked to induced phosphorylation of ER's serine 118 residue, thereby promoting hormone independent growth in models (42,43). Hyperactive Akt can phosphorylate ER on serine 167, for example when driven by PTEN loss (40). Ligand-independent ER activation in turn may enhance expression of growth factors (44,45). Such signaling can be causal in BC models resistant to ET (28) including TAM (44,45).

An alternative nuclear steroid hormone receptor, the androgen receptor (AR), a relatively new marker in relation to BC response to ET, is potentially important (46). The expression of AR has been related to expression of ER (47) and subsequently in a larger series of BCs associated with multiple phenotypes and biomarkers (48). AR has also been reported as a prognostic marker (49). Thus, AR positive tumors are associated with luminal A disease (50), low grade tumors (50,51), and better outcome in patients with ER+ BC (49,50). In contrast, AR overexpression has been linked with TAM resistance in pre-clinical experiments (52,53); Cochrane *et al*. reported that the risk of TAM failure for patients with a nuclear AR/ER ratio ≥2 was 4 times that of the lower ratio subgroup (54).

To identify if any of these molecular markers implicated in steroid receptor and RTK/kinase pathways (including PI3K/Akt signaling) predicts response or resistance to TAM in patients with ER+ BC 10 biomarkers were immunohistochemically-evaluated. By including biopsy specimens and tumor microarrays (TMAs), as well as from different tumor locations, the study was able consider whether biomarker data derived from different intra-tumoral locations or sample types can be combined for

such studies. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/tbcr-20-31).

Methods

Patients and immunohistochemical

A total of 123 endocrine treatment naïve patients with known ER and HER2 status were treated with TAM and had clinical outcome data: 102 had their tumor samples in a tumor microarray format (TMAs) and 21 as core biopsies (*Figure 1*)]. An additional five patients with TMAs but no outcome data were included only in the analysis of biomarker expression in TMA samples from different intra-tumoral locations (i.e., central and peripheral) (i.e., total TMA samples n=107).

Patients reported here were commenced on TAM between 1983–2002. During the earlier part of this period patients with ER unknown tumors or even on occasion known ER negative indolent disease were given TAM. For this report, in keeping with the ASCO guidelines, ER+ has been defined as ≥1% of tumor cells staining positive (55). 103/123 (84%) patients had ER+ tumors and of these 83/103 patients had surgery as initial treatment of their tumors. For these 83 patients, three 0.6 mm TMA samples were prepared from two central and one peripheral regions from the resected tumor tissue block for each patient: these patients received no adjuvant ET and were treated with TAM when they developed recurrent, advanced BC. The remaining 20 ER+ patients who did not have initial surgery for their tumors had core biopsies only, and were treated with TAM as a primary ET: these were either elderly patients with primary BC and were being treated by primary ET or patients who presented with advanced BC for whom TAM was the initial ET. Survival analyses included only ER+ patients with TAM clinical outcome data (n=103) (*Figure 1*). Categorization by HER2 status is also shown in *Figure 1*. Clinical data included measurements for time to progression (TTP), time to death (TTD), duration of clinical benefit (DoCB), clinical benefit (CB) and objective response (OR).

All tissue evaluated was formalin-fixed paraffin-embedded (FFPE) tumor material and specimens and clinical data were provided by the University of Nottingham and City Hospital (now Queens University Hospital), Nottingham. The study was conducted in accordance with the Declaration of Helsinki (revised 2013) and the Harmonized Tripartite Guideline for Good Clinical Practice from the
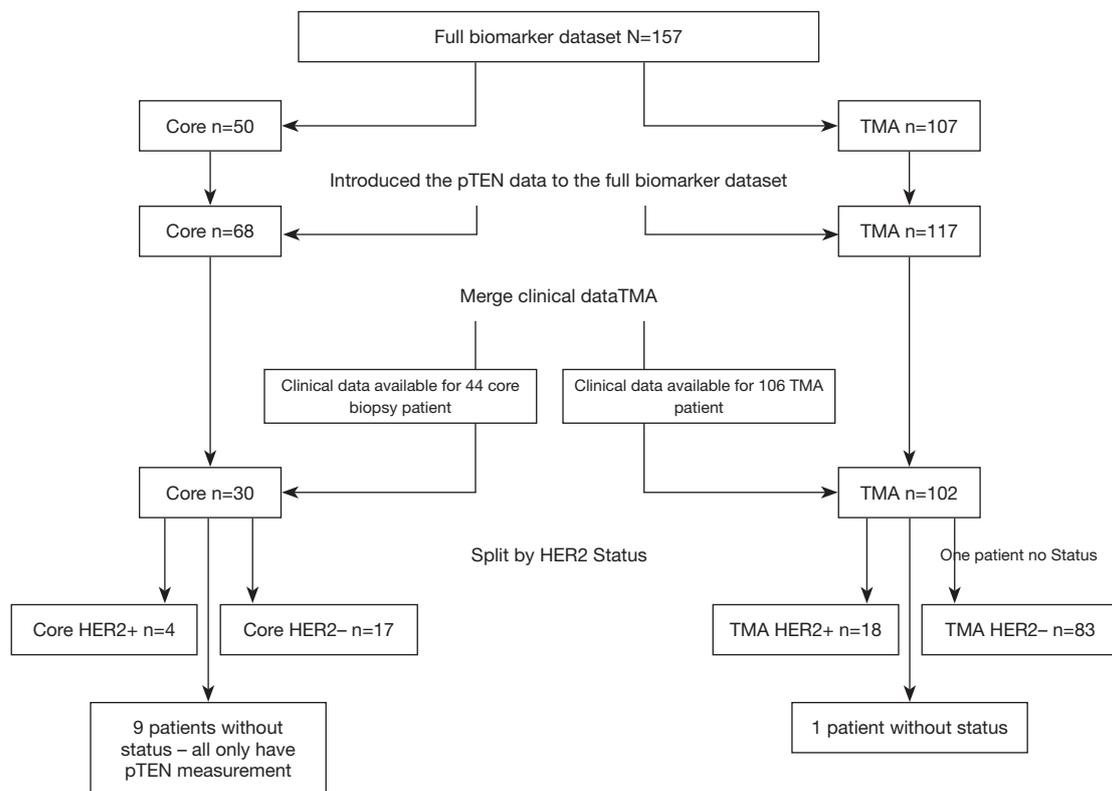
Figure 1 The consort diagram.

International Conference on Harmonization. Informed consent was waived and archival tissues were used with approval of the Ethics Committee and R & D Committee. The research protocol had approved from Nottingham Research Ethics Committee (Ref: 2020313) and R & D approval from the hospital (Ref: 03H101).

Biomarker measurements were performed by the University of Nottingham, the Breast Cancer Molecular Pharmacology Group in Cardiff University, and the Division of Oncology and Pathology, Department of Clinical Sciences in Lund University, Sweden. Ten biomarkers were evaluated by immunohistochemical (IHC) staining (assessing % positivity and/or H-Score), reflecting steroid receptor and RTK-kinase signaling as well as proliferation, comprising: ER-alpha (ER), HER2, Ki-67, phosphorylated (p) forms of ERK1/2 MAPK, Akt, ER (Ser118), PRAS40, and IGF1R, and PTEN & AR expression. All assays were applied to the clinical BC TMAs and core biopsies, except PTEN which was measured on core biopsies and full tumor sections (FTS) and AR which was measured only on TMAs (*Table 1*). Table S1 details the monoclonal antibodies used, phosphorylation site detected and IHC. A minimum of 100

tumor epithelial cells were required for assessment of the IHC marker staining. Eight IHC markers were evaluated in Cardiff University using antibodies for ER expression, pER118, pMAPK, Ki-67, HER2, pIGF1R, pPRAS40 and pAkt followed by assessment by consensus of 2 experienced personnel (JMW Gee, P Finlay) blinded to the clinical data. All assays were performed by running small batches of test slides, together with an in-house internal assay positive breast cancer control to monitor for adequate IHC assay staining performance in each assay and so ensure no marked inter-assay differences. Each TMA was run in separate assays along with a subset of the biopsy samples. PTEN staining was performed in Nottingham. For this, staining was assessed by one observer and an H-score measured. Equivocal or borderline cases were re-examined and a consensus score was reached with a second observer. AR staining was performed and assessed in Lund by one observer. PTEN and AR IHC methodologies are again shown in *Table 1*. *Table 1* shows the cellular location of staining (n = nuclear, c = cytoplasmic, m = plasma membrane) evaluated and the scoring method for all biomarkers. Figure S1 shows examples of higher and weaker staining for the various markers.

Table 1 The biomarkers

Biomarker	Locations	H-score, TMA	H-score, Core	% Pos, TMA	% Pos, Core
RTKs					
HER2	Plasma membrane	Yes	Yes	Yes	Yes
pIGF1R	Plasma membrane	Yes	Yes	Yes	Yes
Intracellular kinase signaling					
pPRAS40	Cytoplasm & nucleus	Yes	Yes	Yes	Yes
pMAPK	Cytoplasm & nucleus	Yes	Yes	Yes	Yes
pTEN*	Cytoplasm	Yes (*FTS)	Yes	Yes (*FTS)	Yes
pAKT	Cytoplasm & nucleus	Yes	Yes	Yes	Yes
Steroid hormone receptor					
pER118	Nucleus	Yes	Yes	Yes	Yes
ER	Nucleus	Yes	Yes	Yes	Yes
AR	Nucleus	Yes	N/A	Yes	N/A
Proliferation					
Ki-67	Nucleus	No	No	Yes	Yes

*, FTS-PTEN was measured on core biopsies and on full tissue sections rather than TMAs and was scored for % cells positive and H-score.

Each tumor was also analysed for Akt1 E17K somatic mutation using BEAMing (Beads, Emulsions, Amplification, and Magnetics) technology by Sysmex Inostics GmbH, Hamburg, Germany (56).

Analysis cohorts

The analysis cohorts consisted of (I) ER+ patients only, (II) ER+ patients sub-grouped by HER2 status (i.e., luminal A: ER+/HER2– & luminal B ER+/HER2+) and iii) grouped according to tissue type (core biopsy or TMA; also TMA combined with core biopsy) (*Figure 1*). HER2m status was categorized into negative "0" ≤30% or positive "1" >30% according to % of 3+ complete membrane staining. For each patient, the clinical TAM outcome & levels of staining for each biomarker comprised the analysis data.

Given the size of the dataset it was necessary to combine the IHC scores from the TMA and core biopsies for analysis versus outcome for each marker. Previous publications, including from our own unit, have reported that core biopsies and surgical excision specimens show good correlation for ER status (57-59) with the H-score only slightly lower (between 10–20 on a 0–300 scale) in the surgical specimen compared to the core biopsy (57,58). A meta-analysis of 21 published studies involving almost 2,500 patients also reported good overall concordance between core and surgical excision of 92.8 % for ER and 85.2 % for PR (60). Fewer prior publications exist regarding biomarkers of RTK-kinase pathways (61), in particular phospho-specific markers compared according to type of sample, although one publication has reported some differences (62).

In exploring biomarker relationship to TAM outcome it was thus first important to be aware of any impact of combining steroid receptor, RTK/kinase or proliferation data from the different types of sample in the present study. This was achieved by evaluating differences in staining for individual biomarkers between TMAs and core biopsies (which in this study was an inter-patient, inter-tumor comparison). To more robustly achieve this, the number of core biopsies for staining was further increased by including further diagnostic pre-treatment core biopsies that were available without clinical outcome data (thus giving a total number of cores for this analysis n=50). The results were evaluated by non-parametric testing (Wilcoxon test applies throughout) before false discovery rate (FDR) correction in an exploratory analysis of sample positivity rate and staining level within the TMAs versus core biopsies for the biomarkers. Exploratory correlations were evaluated between levels of the signaling biomarkers to gauge whether the biological observations noted were also rational for the

Table 2 Samples analysed for each biomarker by type of biopsy (TMA or Core), with [n (%)] of samples positive

Biomarker	TMA: % Pos	TMA: H-score	Core: % Pos	Core: H-score
ERn	103 [88 (85%)]	107 [88 (82%)]	50 [46 (92%)]	50 [46 (92%)]
HER2m	106 [97 (92%)]	107 [97 (91%)]	50 [48 (96%)]	50 [48 (96%)]
Ki-67	106 [95 (90%)]	N/C	50 [48 (96%)]	N/C
pAKTc	106 [78 (74%)]	107 [78 (73%)]	50 [39 (78%)]	50 [39 (78%)]
pAKTn	106 [73 (69%)]	107 [73 (68%)]	50 [41 (82%)]	50 [41 (82%)]
pER118n	102 [72 (71%)]	107 [72 (67%)]	50 [47 (94%)]	50 [47 (94%)]
pIGFR1131	100 [28 (28%)]	107 [28 (26%)]	50 [46 (92%)]	50 [46 (92%)]
pMAPkc	102 [54 (53%)]	107 [54 (50%)]	50 [44 (88%)]	50 [44 (88%)]
pMAPkn	102 [83 (81%)]	107 [83 (78%)]	50 [48 (96%)]	50 [48 (96%)]
pPRAS40c	101 [88 (87%)]	107 [88 (82%)]	50 [50 (100%)]	50 [50 (100%)]
pPRAS40n	101 [52 (51%)]	107 [52 (49%)]	50 [15 (30%)]	50 [15 (30%)]
PTEN	117 [98]*	117 [98]*	20 [19]	20 [19]
ARn	86 [72 (84%)]	86 [72 (84%)]	N/C	N/C

*, FTS-PTEN was measured on core biopsies and on full tissue sections rather than TMAs and was scored for % cells positive and H-score. N/C, no comparison possible.

combined TMA plus core biopsy sample set.

Biomarkers in TMAs (n=107) obtained from the two central and the peripheral regions of the tumors were similarly statistically-examined, this time to evaluate any biomarker differences according to sample location within a tumor (i.e., intra-patient, intra-tumoral comparison) and so gauge if sample location might influence biomarker findings. The two central and one peripheral value were subsequently combined to get a single TMA value for each patient by taking the mean of all three values (central a, central b, peripheral) for analysis versus outcome.

Statistical analysis of IHC biological (steroid receptor, RTK-kinase and proliferation) endpoints versus patient outcome

The ability of the IHC biomarkers to predict response to TAM as measured by CB, TTP, and TTD was examined in the ER+ patient cohort and according to HER2 status or sample type. In addition to analyzing the full set of IHC markers, 3 further analyses were performed examining a combination of ER & Ki-67, pAkt and pPRAS40 (as an indicator of active Akt signaling) and also analysis of AR/ER ratio.

The potential role of the markers in prediction of response to TAM was explored through logistic and survival models of clinical outcomes. Univariate and multivariate methods were

used to model the markers individually and in combination. Logistic regression and Cox proportional hazards models were used to model clinical benefit and survival endpoints respectively. FDR correction was applied to results from univariate analyses, and variable selection methods including regression trees were used to avoid over fitting the multivariate model. Bootstrap methods (resampling from the original data) and model selection based on AIC were used to validate variable selection for logistic models and the final model for survival analyses. Interquartile range (IQR) odds ratios and IQR hazard ratios are presented along with confidence intervals, illustrating difference between high and low (3rd and 1st quartiles) measures for each of the markers.

In the absence of any predefined cut points, the biomarkers were considered continuous predictors. Significance was set at one sided with P<0.05.

Results

Exploratory analyses of biological (steroid receptor, RTK/kinase & proliferation) IHC endpoints

Analysis of the IHC biomarker positivity rate in core biopsy *vs.* TMA samples

Table 2 includes the number (and %) of biopsy samples (core or TMA)—showing any degree of immuno-positivity for

Table 3 Tissue type and tissue location (FDR corrected)

Biomarker	TMA vs. Core Biopsy (FDR P value)		Central vs. peripheral (FDR P value)		Central a vs. central b (FDR P value)	
	% Positive	H Score	% Positive	H Score	% Positive	H Score
ERn	0.29	<0.01	1	1	1	1
HER2m	<0.01	<0.01	1	1	<0.05	0.23
Ki-67	1	N/C	1	N/C	0.24	N/C
pAKTc	0.27	0.04	1	1	0.81	0.53
pAKTn	1	0.18	0.66	0.40	1	1
pER118n	<0.01	<0.01	0.10	0.35	<0.05	0.19
pIGFR1131m	<0.01	<0.01	1	1	1	1
pMAPkc	<0.01	<0.01	1	1	1	1
pMAPkn	1	0.34	1	1	0.81	1
pPRAS40c	<0.01	<0.01	1	1	0.19	<0.05
pPRAS40n	0.07	0.12	1	1	1	1
ARn	N/C	N/C	1	1	0.81	1
PTEN*	1	0.08	N/C	N/C	N/C	N/C

*, PTEN was measured on core biopsies and on full tissue sections rather than TMAs and was scored for % cells positive and H-score. N/C, no comparison possible.

each marker (considering n, m or c staining localization). As stated above, 50 core biopsies and up to 107 TMAs (with or without clinical outcome data) were included in this exploratory analysis. pIGFRm had substantially more positive core biopsies than positive TMAs and for pAKTn, pER118n, pMAPKc, pMAPKn and pPRAS40c somewhat increased positivity rate was also seen in core biopsies. No marked discordance was noted for the % tumors showing any immunopositivity for total nuclear ER (ERn), HER2m, pAktc or Ki-67 between core biopsy vs. TMA sample types. Only pPRAS40n had a reduced % positivity rate in the core biopsies compared with TMA samples (*Table 2*). PTEN was measured on either FTS or core biopsies and therefore was not included in this comparison of TMAs versus core biopsies. AR was not measured in cores, but showed a substantial % nuclear positivity rate in the TMAs (*Table 2*).

Analysis of IHC biomarker staining level in core biopsy vs. TMA samples and inter-marker correlations in the combined dataset

The positive staining level (H-Score or %) in the TMAs compared with core biopsies was different for some of the biomarkers; when FDR correction was applied to the resultant individual Wilcoxon P values the hypothesis that % positivity or H-scores cannot be assumed to be the same was verified (*Table 3*). Staining values were generally lower in TMAs, and after FDR correction significant differences remained for pER118n, pIGF1Rm, pMAPKc, HER2m, pAktc and pPRAS40c, and for ER H-Score only, but there were no significant differences for pAktn, pMAPKn or pPRAS40n, or for ER or Ki-67 when measured by percentage positivity, between core biopsy and TMA samples.

Exploratory analysis revealed several biologically-expected significant correlations between markers in the combined biopsy cores and TMAs (Figure S2), notably between total and pER118n; for pMAPKn, pAktn and pPRAS40c with pER118n; between pAktc and pPRAS40c, and several for pIGF1Rm (including with HER2m, PRAS40c and pER118n). An inverse correlation was noted between ER expression and Ki-67 (Figure S2).

Comparing the IHC biomarkers in TMAs obtained from central & peripheral regions of tumors

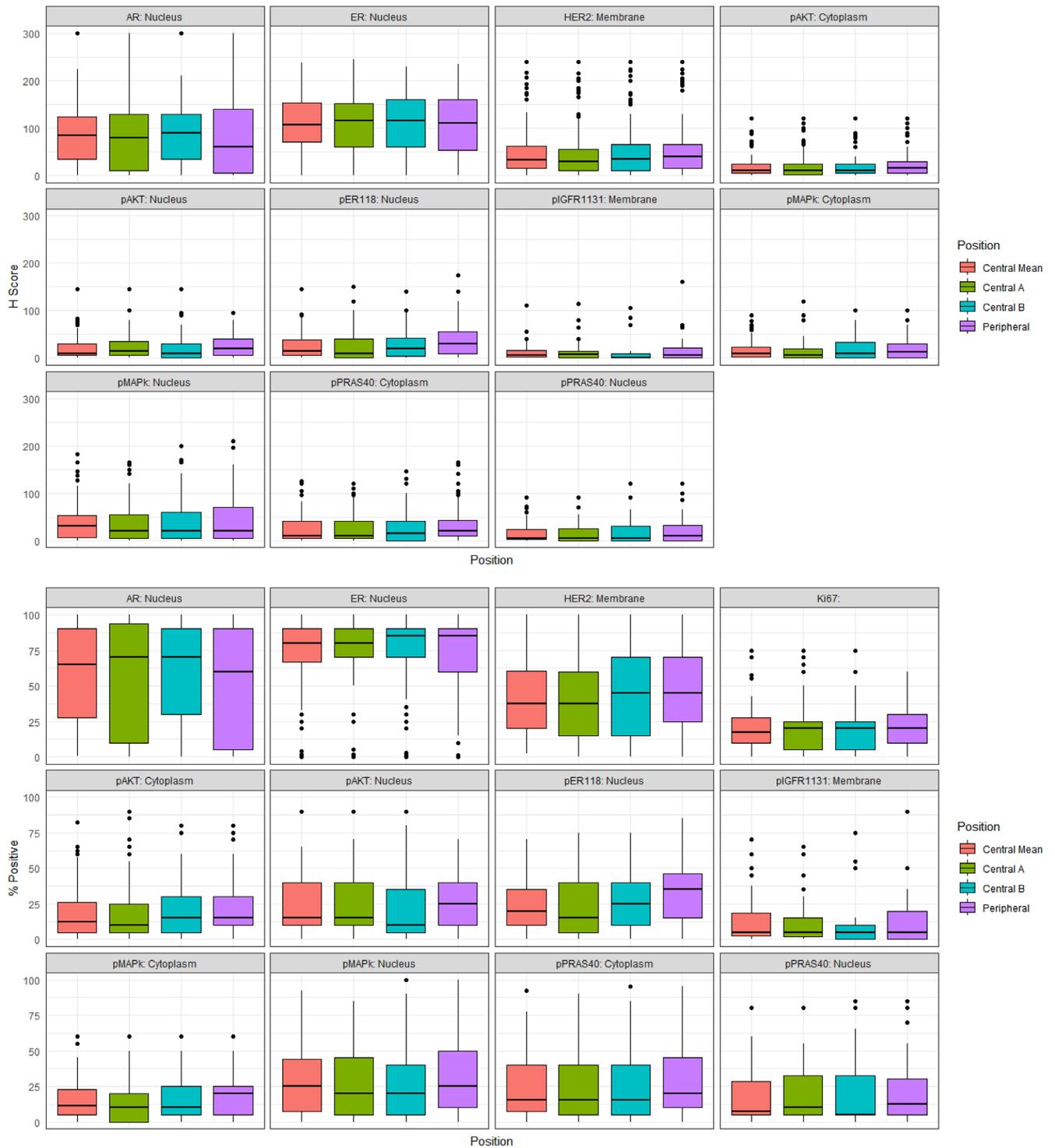*Figure 2* show the box plots of % positivity and H-score

Figure 2 Box plots of % positivity and H-score.

Table 4 % tumor cells positive univariate results (CBR, TTP & TTD)

Biomarker	Cohort	IQR OR/HR (95% CI)
CBR		
ER+, TMA + Core	ERn	OR 2.63 (1.43, 4.83)*
ER+, TMA + Core	Ki-67	OR 0.45 (0.27, 0.78)*
ER+, HER2–	Ki-67	OR 0.40 (0.21, 0.74)*
ER+, TMA only	ERn	OR 3.20 (1.57, 6.54)*
TTP		
ER+, TMA + Core	pTEN	HR 0.83 (0.73, 0.94)*
ER+, TMA + Core	Ki-67	HR 1.70 (1.24, 2.32)*
ER+, HER2–	Ki-67	HR 1.76 (1.24, 2.49)*
ER+, TMA only	pTEN	HR 0.83 (0.73, 0.94)*
TTD		
ER+, TMA + Core	pTEN	HR 0.84 (0.74, 0.94)*
ER+, TMA + Core	Ki-67	HR 1.64 (1.20, 2.23)*
ER+, HER2–	Ki-67	HR 1.75 (1.21, 2.54)*
ER+, TMA only	pTEN	HR 0.84 (0.75, 0.95)*

*, FDR P value <0.05. pTEN was measured on core biopsies and on full tumor sections rather than TMAs. Its staining was included in the analyses with all the other markers measure on TMAs.

respectively comparing each of the different sample locations within each tumor for the biomarkers. After FDR correction there was no significant difference between the peripheral sample and the mean of the two central TMA samples for % positivity or H-Score of any of the markers (*Table 3*). Between the two central samples, there was a modest significant difference (P<0.05) detected for % positivity only for HER2m and pER118n (*Table 3*) while for H-score there was a modest significant difference (P<0.05) detected for pPRAS40c (*Table 3*). The lack of marked differences support feasibility of combining staining data for these biomarkers (including Ki-67) at the different tumor sites (as a mean) for analysis versus outcome

Analyses of Akt1 E17K somatic mutation

Only 2/123 (1.6%) tumors had Akt1 E17K mutations.

Relationships between IHC biomarkers and tamoxifen outcome

While the IHC biomarkers were scored by % positive

staining and H-Score, the majority of the significant results versus outcome pertained to the % positive results (*Table 4*). There were also too few patients in the HER2+ cohort to obtain conclusive data, so the following results refer only to the full ER+ combined TMA + Core biopsy cohort, the ER+ TMA only cohort, and the luminal A (ER+/HER2–, TMA + Core biopsy) cohort.

Clinical benefit on tamoxifen

In the ER+ combined TMA + Core biopsy sample cohort, univariate models suggested there was an increased odds of clinical benefit (CB) associated with decreased levels of Ki-67 and increased levels of ERn expression, as measured by % positivity (*Table 4*). Given the differences in some biomarkers (other than Ki-67) between core biopsy and TMAs, studies were also performed within the TMA cohort alone, where the observation with ERn was retained. The significant association with Ki-67 was also seen in the luminal A (ER+/HER2–) cohort using the combined (ER+ TMA+ core biopsy) cohort. After adjustment for FDR no other significances for any of the RTK-kinase signaling molecules were obtained versus CB (*Table 4*). To avoid overfitting in the multivariate model, variable selection methods (regression trees of 1,000 bootstrapped samples) were used; the only markers included in the multivariate model were ERn and Ki-67, and also pPRAS40c and pAkt (as an indicator of active Akt signaling). Only Ki-67 and ER % positivity were retained as significant for CBR in the final multivariate model for the full sample cohort (*Table 5*). In the Luminal A cohort only the Ki-67 association remained when analyzing the full ER+ sample set (*Table 4*).

While AR alone was not predictive, the AR/ER staining ratio revealed 61 had a ratio <2, and 10 had a ratio ≥2. Fishers exact test showed that the odds of achieving CB was enriched in patients with an AR/ER ratio <2 [P<0.05, odds ratio 5.1 (1.01, 33.4)].

Survival endpoints following tamoxifen treatment

Univariate models were also fitted for TTP and TTD. After FDR correction a significant increased hazard of disease progression (TTP) and death (TTD) was seen in the full ER+ and also the Luminal A cohorts for those with higher Ki-67. Within the full ER+ cohort (FTS+ core biopsy or FTS only, but not for the luminal A cohort) a significantly increased risk of disease progression and death was found for those with lower PTEN % positivity levels (*Table 4*). No other associations were seen for the survival endpoints.

Multivariate models analyses were performed using

Table 5 Tumor cells positive multivariate results (CBR, TTP & TTD)

Cohort	IQR OR/HR (95% CI)	IQR OR/HR (95% CI)
CBR		
ER+, TMA + Core	Ki-67: OR 0.5 (0.28, 0.9)*	ERn: OR 2.25 (1.19, 4.27)*
TTP		
ER+, TMA + Core	Ki-67: HR 1.48 (1.06, 2.07)*	pTEN: HR 0.83 (0.73, 0.94)*
ER+, HER2−	Ki-67: HR 1.57 (1.09, 2.26)*	pTEN: HR 0.81 (0.69, 0.94)*
ER+, TMA only	Ki-67: HR 1.39 (1.02, 1.89)*	pTEN: HR 0.83 (0.74, 0.95)*
ER+, TMA only HER2	Ki-67: HR 1.53 (1.05, 2.24)*	pTEN: HR 0.79 (0.67, 0.94)*
TTD		
ER+, TMA + Core	Ki-67: HR 1.45 (1.04, 2.01)*	pTEN: HR 0.85 (0.75, 0.96)*
ER+, HER2−	Ki-67: HR 1.60 (1.08, 2.37)*	pTEN: HR 0.79 (0.65, 0.96)*
ER+, TMA only	Ki-67: HR 1.42 (1.02, 1.98)*	pTEN: HR 0.85 (0.76, 0.96)*
ER+, TMA only HER2−	Ki-67: HR 1.57 (1.06, 2.35)*	pTEN: HR 0.79 (0.65, 0.96)*

*, P value <0.05. pTEN was measured on core biopsies and on full tumor sections rather than TMAs. Its staining was included in the analyses with all the other markers measure on TMAs.

the full ER+ cohort, considering the Core biopsy + TMA combined or TMA alone samples, although as noted above pTEN was measured in FTS and core biopsies: in both analyses increased Ki-67 and decreased PTEN were predictive of TTP and TTD (*Table 5*). Only Ki-67 proved significant versus these endpoints in the Luminal A cohort. In contrast to the CB data, no significant results were seen for TTP and TTD in relation to AR/ER ratio. These results were validated by bootstrapping and model selection based on AIC.

Discussion

This study adds to the literature on the impact of type of clinical sample and of intra-tumoral site of biopsy when assessing biomarkers as predictors of clinical utility in breast cancer. Even after FDR correction significant differences in staining level remained for the majority of markers according to sample type (pER118n, pIGF1Rm, pMAPKc, HER2m, pAktc and pPRAS40c and for ER H-Score only). There were no significant differences for pAktn, pMAPKn, pPRAS40n, Ki-67 or ER when assessed by percentage positivity or H-score between core biopsy and TMAs. This is an important and reassuring finding since ER and Ki-67 are two of the markers identified as significantly associated with clinical outcome in this study and associations with outcome have also been noted for Ki-

67 in sequential sample studies (13). Differences commonly noted in signaling marker staining for immunopositivity rate and level between core biopsies and TMAs (often staining higher in the former) may be related to differences in fixation/preparation of each type of sample influencing antigenicity and thus the subsequent IHC assay data. This has previously been suggested by other groups (61,62), who reported somewhat higher levels of selected AKT signaling elements and phospho-MAPK in core biopsies. It should however be appreciated that in the present study the core biopsy versus excision specimens were not a direct comparison from the same tumor and so differences reported here may, at least in part, also be due to differences in phenotype of tumors which had core biopsy (primary ET patients) versus TMAs constructed from surgically resected tumors. Importantly, however, there was no significant difference in Ki-67 between TMA and core samples.

For all the biomarkers reported in the present study the findings suggest that the site biopsied within a tumor is not a critical issue: our analysis of multiple biomarkers was a direct comparison within the same tumor. This is important in interpreting the results of studies using percutaneous core biopsy material where differences in biomarkers between groups have been suggested may be due, at least in part, to the sampling of different sites within the tumors. Equally studies assessing the effect of drug therapies using sequential core biopsies in an

individual (22,63) have reported differences in expression/ activity of the various steroid receptor and RTK/kinases. Our study would indicate that such differences are not due to sequential biopsies being taken from different sites within the tumor. These findings are in keeping with the study by Meric-Bernstam and colleagues who reported no significant difference in 7 biomarkers measured by IHC according to tumor location (62): Ki-67, PTEN & p4EBP1 T70 showed strong correlations between central and peripheral expression, pAktS473, pPRAS40 T246 and pS6 S240/244 showed moderate correlations, and only pS6 S235/236 showed a low correlation. This study also measured biomarkers by RPPA and reported that of 154 markers measured, 132 (86%) were not statistically different between the tumor centre and periphery. Tramm and colleagues recently reported on 27 patients assessing five biomarkers in central and peripheral regions, each within two separate tumor blocks (64): there was almost perfect concordance for BCL2, E-cadherin and EGFR between the central and peripheral regions and moderate/substantial agreement for EMMPRIN and Ki-67. Nevertheless, it should be noted that the finding of similar biomarker expression in peripheral and central sites in a tumor remains a matter of debate. Douglas-Jones and colleagues reported differences in ER expression across the width of tumors (58). Likewise, Aleskandarany and colleagues reported heterogeneity of Ki-67 expression within a tumor (although this was between 'full face' sections from different primary tumor blocks rather than from central or peripheral TMA cores within the tumor) (65). There may also be some loss of antigen signal between core biopsies and surgical resected specimens due to technical factors, particularly for phosphorylated proteins (62): where antigen signal has been diminished this might provide some explanation for similarity in signal between different areas of the same resected tumor specimen.

The above highlight the importance of describing the study methodology and technical aspects of biomarker assessment when trying to correlate biomarker expression with clinical utility. We found using univariate and multivariate analysis that measures of clinical response to TAM can be predicted by increased staining for ER, the PI3K/Akt pathway regulator PTEN, or lower Ki-67, either in combination or individually. This was seen in the full ER+ cohort. For the ER+ TMA + Core combined group PTEN, Ki-67 and ERn as measured by % positive staining were all predictive: ERn & Ki-67 predicted CBR whereas Ki-67 and PTEN predicted TTP and TTD. It should be

noted that ER and Ki-67 were also two markers for which there was no evidence of difference between TMA and core biopsy samples (in terms of % positivity) or between central or peripheral sites of the tumor, indicating these may be a robust combination of predictive markers for TAM outcome irrespective of sample type. PTEN was measured on FTS and core biopsies, so there was no central versus peripheral TMA comparison for PTEN in our study. However, Meric-Bernstam and colleagues (62) reported "strong correlation" between PTEN expression of central and peripheral samples, suggesting this may also be a reliable signaling marker. Within the luminal A cohort only decreased Ki-67 was seen to be predictive of increased CB, TTP, and TTD while increased pTEN was predictive of an increased TTP & TTD.

Our ER, PTEN and Ki-67 predictive biomarker findings were not influenced by AKT mutation status, since such mutation was very low in this BC (1.6%), a finding reflective of the literature since a positivity rate for AktE17K mutation frequencies of 6.3% has been reported (66), and where previous published literature ranges from 1.4–8.2 %, with a mean mutation frequency of 3.8% (67).

With regards to AR/ER ratio, the patient numbers were small and so the relationship detected between the <2 ratio and clinical benefit of TAM should be viewed with caution. Nevertheless, since they are in agreement with studies from Cochrane and colleagues where AR/ER ratio correlates with clinical outcomes (54), our findings suggest further research would be worthwhile to confirm or refute whether there is a meaningful relationship between AR/ER ratio and TAM clinical outcome.

Several biologically-plausible correlations were preserved between the signaling steroid receptor and RTK-kinase biomarkers in this TMA plus core biopsy series. Of note, the correlations between total ER and pER118n, and between pMAPKn and pER118n, are in keeping with ER signaling and kinase/ER cross talk via ER activation (42,43). Correlations were also seen between pAkt and pPRAS40, elements both lying in the PI3K/Akt signaling pathway. Furthermore, the inverse correlation noted between ER and Ki-67 has been previously observed (68). However, despite these reassuring biological correlations and the wealth of supportive preclinical data from ET resistant BC models, unfortunately other than PTEN we found none of the examined markers reflective of RTK-kinase signaling and its cross-talk with ER were related to TAM outcome clinically. On one hand this might be surprising since inhibition of the PI3KCA/Akt/mTOR pathway has been reported to

result in significant increases in TTP/PFS (69,70). In these other clinical studies, most of the patients had been heavily pre-treated. Equally there are studies where inhibitors of these pathways have shown no significant improvement in TTP/PFS (71,72). The fact that the present study included hormone naïve patients and also used primary tumor tissue, where the PI3KCA/Akt/mTOR pathway may not be as disrupted as in metastases (73-75), may explain why no further markers reflective of the Akt pathway were intrinsically associated with TAM resistance. This therefore is one limitation of this study and it again highlights the importance of obtaining tumor tissue immediately prior to a new treatment especially within future studies. Likewise, studies comparing the molecular phenotype of primary and metastases within the same patient are important to elucidate the issues related to using primary tumors to predict response at a much later time point. A recent meta-analysis of 5,000 patients with HR+ advanced BC showed that liver visceral metastases have a worse outcome than other sites of metastases (76,77) suggesting it may also be important to biopsy metastases from different organ sites to best predict clinical outcome to targeted therapies.

It would be difficult to biopsy all sites of metastases in routine clinical practice but from a perspective of trying to understand mechanism of resistance this could be viewed as another potential limitation. Furthermore, biopsies taken prior to starting any treatment, whether from the original primary tumor or metastases, may allow prediction of response or 'de-novo' progression. However, to more fully understand the mechanisms of acquired resistance, which can occur over months to many years, multiple sequential tumor biopsies on treatment and at relapse must be studied (78-81). The present study therefore was not able to look for markers which would highlight potential mechanisms of acquired resistance.

From a mechanistic perspective, the observation that EGFR/HER2/IGF1R signalling and its potential cross-talk via kinases with ER phosphorylation were not biomarkers of tamoxifen resistance in our study might also be explained by the abundance of further resistance mechanisms that have emerged from laboratory studies. For example, along with many changes in ER signalling (including changes in ER's potential dimer partners, ER variants and ER coactivators such as AIB1 (82,83), the de-regulation of alternative kinase signalling pathways and transcription factors [e.g., FGFR (84,85)], changes in non-coding RNAs (86,87), cell cycle regulators and apoptotic machinery (88) are just some of the mechanisms that have been proposed

to contribute to endocrine resistance. The establishment and use of breast cancer cell lines that are tamoxifen resistant *in vitro* has been valuable towards the discovery and verification of many resistance mechanisms, with some of these models also having substantial proliferation in the presence of tamoxifen (89) and in some instances reduced ER (90) and PTEN loss (29,91) as observed in the present clinical study. Indeed, several of the deregulated pathways, following promising pre-clinical studies, have progressed to being evaluated and (in the case of CDK4/6 inhibition (92) approval as therapeutic targets alongside endocrine agents. Studies of patient tumor DNA samples and more complex *in vivo* model systems are also linking endocrine resistance to tumor heterogeneity, underpinned by further mechanisms involving genetic/epigenetic events (93), tumor microenvironment and cancer stem cells (94,95). Some of these mechanisms are likely to be important contributors to the control of proliferative activity and might also influence ER level, limiting effectiveness of agents such as tamoxifen.

Conclusions

In conclusion, while we acknowledge the limitation of this analysis due to the available sample size our results in this study support previous work that high ER and low Ki-67 are predictive of clinical utility in hormone naïve patients receiving TAM and also adds PTEN loss. Significantly, considering the findings in this study together with other studies of biopsy type and site (62), all three markers appear independent of sample type and tumor region, adding to the robustness of these markers in relation to TAM outcome. In contrast, for the remaining 7 markers, it is feasible that some of these markers might have had some value if measured within a more advanced phenotype, since type of sample and the disease setting may be critical in relation to prediction of ET response/ progression. It may be more informative for biomarkers to be assessed on biopsies taken just prior to a particular therapy, with analytical variables minimized, in order to best gauge prediction of response in that setting. This should be our aim in prospective randomized trials of new targeted treatments in advanced BC. Furthermore, it remains important that we continue to uncover potential new targets and relevant markers from the most promising of the mechanisms of resistance emerging in experimental studies. In the future, these should continue to be prioritized for evaluation alongside ER, Ki-67 and PTEN as potential biomarkers to aid in selecting patients most likely to derive durable tamoxifen benefit.

Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (revised 2013) and the Harmonized Tripartite Guideline for Good Clinical Practice from the International Conference on Harmonization. Informed consent was waived and archival tissues were used with approval of the Ethics Committee and R & D Committee. The research protocol had approved from Nottingham Research Ethics Committee (Ref: 2020313) and R & D approval from the hospital (Ref: 03H101).

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