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Article

Predictive Biomarkers for Endocrine Therapy: Retrospective Study in Tamoxifen and Exemestane Adjuvant Multinational (TEAM) Trial

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ABSTRACT

Background: Aromatase inhibitors improve disease-free survival compared with tamoxifen in postmenopausal women with hormone-receptor-positive breast cancer. The Tamoxifen and Exemestane Adjuvant Multinational (TEAM) trial compared exemestane monotherapy versus sequential therapy of tamoxifen followed by exemestane. The trial failed to show a statistically significant difference between treatment arms. A robust translational program was established to investigate predictive biomarkers.

Methods: A tissue microarray was retrospectively constructed using a subset of patient tissues (n=4631) from the TEAM trial (n=9766). Immunohistochemistry was performed for biomarkers, classed into three groups: MAPK pathway, NF-kappa B pathway, and ER phosphorylation. Expression was analysed for association with relapse-free survival (RFS) at 2.5 and 10 years and treatment regimen using Kaplan-Meier curves and log-rank analysis. All statistical tests were two-sided.

Results: On univariate analysis, ER^{167} (HR 0.71 95% CI 0.59-0.85, p<0.001), IKKα (HR 0.74 95% CI 0.60-0.92, p=0.005), $Raf\text{-}1^{338}$ (HR 0.64 95% CI 0.52-0.80, p<0.001), and p44/42 $MAPK^{202/204}$ (HR 0.77 95% CI 0.64-0.92, p=0.004) were statistically significantly associated with improved RFS at 10 years in patients receiving sequential therapy. Associations were strengthened when IKKα, $Raf\text{-}1^{338}$ and ER^{167} were combined into a cumulative prognostic score (HR 0.64 95% CI 0.52-0.77, p<0.001). Patients with an all negative IKKα, $Raf\text{-}1^{338}$ and ER^{167}

score, favoured exemestane monotherapy (OR 0.56 95% CI 0.35-0.90). On multivariate analysis, the IKKα, Raf-1^{338} and ER^{167} score (p=0.001) was an independent prognostic factor for RFS at 10 years in patients receiving sequential therapy.

Conclusions: The IKKα, Raf-1^{338} and ER^{167} score is an independent predictive biomarker for lower recurrence on sequential therapy. Negative expression may further offer predictive value for exemestane monotherapy.

INTRODUCTION

Treatment of breast cancer has evolved and tamoxifen is no longer the only adjuvant endocrine therapy available for postmenopausal women with estrogen receptor-positive (ER-positive) (1). Third generation aromatase inhibitors (AIs, anastrozole, exemestane and letrozole), which induce suppression of circulating estrogens increase disease free survival in postmenopausal hormone receptor-positive breast cancer. Treatment with AIs for 5 years improved disease-free survival compared with tamoxifen for 5 years (2, 3). In the Intergroup Exemestane Study (IES), patients who switched to exemestane after 2–3 years of tamoxifen had statistically significantly improved disease-free survival and overall survival compared to those remaining on tamoxifen (4). Therefore AIs alone or in sequence with tamoxifen are now recommended as adjuvant therapy for postmenopausal breast cancer (5).

Although currently there are established recurrence score such as Oncotype DX, Pam50 and the combined endocrine score (6-8), there are no biomarkers available to predict which patients will gain maximum benefit from each treatment strategy. NICE guidelines suggest that decisions should be based on discussions between the patient and oncologist, focussing on benefits and risks of each option, risk of recurrence and previous tamoxifen use (5). Clearly biomarkers are required to aid clinician decision-making. The Tamoxifen and Exemestane Adjuvant Multinational (TEAM) trial compared exemestane monotherapy versus sequential therapy of tamoxifen followed by exemestane for a total of five years (9), providing an ideal cohort to retrospectively investigate biomarkers that predict patients most likely to benefit from either exemestane monotherapy or tamoxifen followed by exemestane. The TEAM trial did not show a specific benefit of one therapeutic regimen over the other, both after 5 and 10 years (9).

The current study investigated potential predictive biomarkers for endocrine therapy,

classed into three groups; MAPK pathway (10), NF-kappaB pathway (11), and phosphorylation of ER (12, 13) that have been previously reported as prognostic in postmenopausal hormone receptor-positive (HRec-positive) breast cancer.

PATIENTS AND METHODS

Patients and study design

The TEAM trial is a multinational, randomized, open-label, phase III trial in postmenopausal women with HRec-positive early breast cancer (Trial registration number NCT00032136) (9). Women randomly received either exemestane (25mg) once daily for five years or tamoxifen (20mg) once daily for 2.5 years followed by exemestane for a total of five years. The study complied with the Declaration of Helsinki, individual ethics committee guidelines, and the International Conference of Harmonization and Good Clinical Practice Guidelines. All patients provided informed consent. Further details are in the **Supplementary Methods**.

From the 9766 patients from the TEAM trial, only five of the nine eligible TEAM countries (n=6210) agreed to provided tumour samples. 4781 patients had available tissue from surgical resections for tissue microarray (TMA) construction with linked clinicopathological data (14). Of these 4631 patient samples were eligible for biomarker staining (**Figure 1**). 86 patients were ER-negative, and 101 had cores missing for all stains and were therefore excluded leaving 4444 eligible patients for analysis.

Immunohistochemistry

Immunohistochemical expression of IKKα, p65^{536}, N-Ras, Raf-1^{338}, p44/42 MAPK$^{202/204}$, ER^{118}, and ER^{167} was conducted on a TMA using the Benchmark XT (Ventana Medical Systems, Roche, Tucson, Arizona) automated staining platform (14). Stained TMA sections were scanned using a Hamamatsu NanoZoomer at x20 magnification and visualized on Slidepath Digital Image Hub (Leica Biosystems, Newcastle, UK). If cores were missing or contained less than 10% tumour tissue they were excluded from analysis (**Figure 1**). Assessment of cytoplasmic IKKα expression was performed by a single examiner (LB) blinded to clinical data at x20 magnification (total magnification x400) using the weighted histoscore and 10% double scored by JE. The interclass coefficient (ICC) was 0.95. All other proteins were assessed using an automated computer algorithm of the weighted histoscore for nuclear expression, except N-Ras and Raf-1^{338} were cytoplasmic expression was assessed (Leica Biosystems). 10% of tumours were manually scored by a single examiner (JE) blinded to the clinical data. The ICC for each biomarker was ER^{118} 0.79, ER^{167} 0.99, p65^{536} 0.98, N-Ras 0.96, Raf-1^{338} 0.92, and p44/42 MAPK$^{202/204}$ 0.99. Further details are in the **Supplementary Methods**.

Outcomes

The co-primary outcomes were relapse-free survival at 2.5 years (RFS; defined as time to earliest documentation of disease relapse or death due to breast cancer) and RFS at 10 years. Secondary outcomes included biomarker associations with clinicopathological factors and treatment regimen.

Statistical Analysis

The prospectively powered outcome analysis for this study compared high expression (approximately 50%) and low expression (approximately 50%). By using a two-sided α=0.05 analysis and assuming a hazard ratio (HR) of 1.2 and a low expression prevalence of 50%, a sample size of >1000 patients gave >90% power to detect a treatment-biomarker interaction. Therefore the 4646 eligible samples from this trial population would be adequate to identify treatment-biomarker interactions, with at least 90% power.

Histograms were assessed for each protein and IKKα, ER^{167}, Raf-1^{338} and p44/42 MAPK$^{202/204}$ histograms determined that negative and positive expression was the appropriate threshold. p65^{536}, N-Ras, and ER^{118} were analysed using ROC analysis in a discovery cohort and validated using the current cohort, the following thresholds were determined for each protein: 25 for p65^{536}, 100 for N-Ras, and 110 for ER^{118}. SPSS (version 22, IBM, Armonk, New York) was used for statistical analysis unless otherwise stated. Pearson's $\chi 2$ test assessed associations between biomarkers, treatments, and clinicopathological features. Odds ratios compared biomarker associations with treatment regimens using tamoxifen followed by exemestane as the control group and exemestane monotherapy as the experimental group and were displayed as Forrest Plots (showing I^2 for heterogeneity and Z-score for overall effect) with each biomarker as a separate study (RevMan 5.3, Cochrane, London, UK). Kaplan-Meier and log-rank analysis compared RFS at both time points. HRs and CIs were calculated from univariate cox regression survival analysis. Multivariate cox regression survival analysis using a backward conditional elimination model and a statistical significance threshold of $p<0.01$ was performed to identify independent prognostic biomarkers. The study conformed to the REMARK guidelines (15) and statistical significance was set as $p<0.01$. All statistical tests were two-sided.

RESULTS

Study group

Of the 9766 patients from the TEAM trial treated for HRec-positive early breast cancer, 4444 patients were included in this study (**Figure 1**). Patient characteristics are shown in **Table 1**. In brief, 100.0% were ER-positive, 68.3% PR-positive, 39.1% HER2-positive, and 67.7% 60 years or older at surgery. 51.4% had grade II disease and 30.8% had grade III/IV disease. 57.6% were node positive and 8.9% had lymphovascular invasion. 2240 patients received exemestane monotherapy (50.4%) and 2204 patients received tamoxifen followed by exemestane therapy (49.6%). The median follow-up of survivors was 8.8 years (range 1.0-13.9 years) with 1054 recurrences and 1161 deaths. When compared to the full TEAM trial, the characteristics of the patients were similar. However, the patients in the present study appeared to have higher grade, larger tumors and more nodal involvement. Nevertheless, the present cohort was a fair representation of the full TEAM trial.

Associations between biomarkers and relapse-free survival at 2.5 and 10 years.

Univariate analysis of biomarker associations with relapse-free survival (RFS) at 2.5 and 10 years are shown in **Table 2**. ER^{118} phosphorylation was not associated with RFS at either time point. However, ER^{167} phosphorylation statistically significantly associated with improved RFS at both 2.5 years (HR 0.73 95% CI 0.59-0.92, p=0.007) and 10 years (HR 0.82 95% CI 0.71-0.94, p=0.004). For the NF-kappaB pathway, $p65^{536}$ phosphorylation did not associate with RFS at either time point. However, positive IKKα expression statistically significantly associated with improved RFS at 10 years (HR 0.80 95% CI 0.69-0.93, p=0.003). For the MAPK pathway,

N-Ras did not associate with RFS at either time point. However, phosphorylation of Raf-1^{338} (HR 0.62 95% CI 0.48-0.81, p<0.001) and p44/42 MAPK$^{202/204}$ (HR 0.70 95% CI 0.56-0.88, p=0.002) strongly associated with improved RFS at 2.5 years. Both Raf-1^{338} (HR 0.69 95% CI 0.59-0.80, p<0.001) and p44/42 MAPK$^{202/204}$ (HR 0.76 95% CI 0.66-0.86, p<0.001) associations with improved RFS were strengthened at 10 years.

A cumulative prognostic score of the NF-kappa B and MAPK pathways was examined (IKKα and Raf-1^{338}). Patients with positive IKKα expression and phosphorylation of Raf-1^{338} were classified as both-positive, patients with positive IKKα expression or Raf-1^{338} phosphorylation as one-positive and patients with negative IKKα and Raf-1^{338} expression as both-negative. A both-positive IKKα and Raf-1^{338} score strengthened the association with improved RFS at 10 years (HR 0.78 95% CI 0.71-0.87, p<0.001) and 2.5 years (HR 0.77 95% CI 0.65-0.91, p=0.005) as shown in **Table 2.** ER^{167} phosphorylation was added to the cumulative prognostic score. Patients with negative IKKα, Raf-1^{338} and ER^{167} were defined as all-negative, patients with positive expression of one or two from IKKα, Raf-1^{338} or ER^{167} as one/two-positive and patients with positive IKKα, Raf-1^{338} and ER^{167} as all-positive. Patients with an all-positive IKKα, Raf-1^{338} and ER^{167} score strengthened associations with improved RFS at 10 years (HR 0.73 95% CI 0.64-0.85, p<0.001).

Associations between biomarkers, treatment regimens, and relapse-free survival at 2.5 and 10 years.

Univariate analysis was performed for treatment by biomarker interaction and its associations with RFS at 2.5 and 10 years as shown in **Table 3**. Patients receiving tamoxifen followed by exemestane therapy had a statistically significant improvement in RFS at 10 years

with phosphorylation of ER^{167} (HR 0.71 95% CI 0.59-0.85, p<0.001). This was not observed in patients receiving exemestane monotherapy (HR 0.96 95% CI 0.79-1.16, p=0.66). Similarly, patients receiving tamoxifen followed by exemestane therapy had a statistically significant improvement in RFS at 10 years with positive IKKα expression (HR 0.74 95% CI 0.60-0.92, p=0.005) (**Figure 2A**). This was not observed in patients receiving exemestane monotherapy (HR 0.86 95% CI 0.69-1.07, p=0.17) (**Figure 2A**). Furthermore, patients receiving tamoxifen followed by exemestane therapy had improved RFS at 2.5 years when Raf-1^{338} (HR 0.58 95% CI 0.41-0.83, p=0.003) or p44/42 MAPK$^{202/202}$ (HR 0.66 95% CI 0.49-0.89, p=0.006) is phosphorylated, which was not observed in patients receiving exemestane monotherapy (Raf-1^{338}: HR 0.68 95% CI 0.47-0.98, p=0.04 or p44/42 MAPK$^{202/204}$: HR 0.77 95% CI 0.56-1.06, p=0.11). Similarly, patients receiving tamoxifen followed by exemestane therapy had a statistically significant improvement in RFS at 10 years for both Raf-1^{338} (HR 0.64 95% CI 0.52-0.80, p<0.001) and p44/42 MAPK$^{202/204}$ expression (HR 0.77 95% CI 0.64-0.92, p=0.004) as shown in **Table 3**. However, the different pattern between treatment regimens for Raf-1^{338} and p44/42 MAPK$^{202/204}$ was lost by 10 years with patients receiving exemestane monotherapy also having a statistically significant improvement in RFS (Raf-1^{338}: HR 0.74 95% CI 0.60-0.91, p=0.004 or p44/42 MAPK$^{202/204}$: HR 0.75 95% CI 0.62-0.90, p=0.002). Furthermore, no biomarker by treatment associations (**Figure 3**) were observed at 10 years for either Raf-1^{338} (OR 0.97 95% CI 0.83-1.12) or p44/42 MAPK$^{202/204}$ (OR 0.96 95% CI 0.83-1.11).

For the IKKα and Raf-1^{338} score, patients with a both-positive score receiving tamoxifen followed by exemestane therapy also showed a statistically significant improvement in RFS at 10 years (HR 0.73 95% CI 0.63-0.84, p<0.001) (**Figure 2B**). However, when assessing biomarker by treatment interactions, patients with negative IKKα expression receiving tamoxifen

followed by exemestane therapy had shorter RFS than patients receiving exemestane monotherapy, although this did not reach statistical significance (HR 1.24 95% CI 0.1.02-1.51, p=0.03) (**Figure 4A**). Furthermore, patients with a both-negative IKKα and Raf-1^{338} score do statistically significantly worse on tamoxifen followed by exemestane compared to exemestane monotherapy (HR 1.35 95% CI 1.08-1.68, p=0.009) (**Figure 4B**). These results were then confirmed with forest plots for biomarker by treatment interactions (**Figure 3**) with both negative IKKα expression (OR 0.79 95% CI 0.63-0.99) and a both-negative IKKα and Raf-1^{338} score (OR 0.72 95% CI 0.55-0.93) favouring exemestane monotherapy.

To assess how interactions between all three pathways associate with treatment regimen, the IKKα, Raf-1^{338} and ER^{167} score was assessed (**Table 3**). Patients receiving tamoxifen followed by exemestane therapy with an all-positive IKKα, Raf-1^{338} and ER^{167} score had statistically significantly improved survival at 10 years (HR 0.64 95% CI 0.52-0.77, p<0.001) (**Figure 2C**). This was not observed for exemestane monotherapy (HR 0.75 95% CI 0.70-1.04, p=0.27) (**Figure 2C**). When assessing biomarker by treatment associations, patients with an all-negative IKKα, Raf-1^{338} and ER^{167} score favoured exemestane monotherapy (OR 0.56 95% CI 0.35-0.90, **Figure 3**).

Associations between biomarkers and common clinicopathological characteristics

Since IKKα, Raf-1^{338}, p44/42 MAPK$^{202/204}$, and ER^{167} associated with RFS and patient treatments, associations with common clinicopathological factors were assessed (**Supplementary Table 1**). ER^{167} phosphorylation_statistically significantly associated with lower grade (p=0.001), whereas IKKα statistically significantly associated with lower nodal status (p<0.001) and Ki67 index (p=0.008). Raf-1^{338} statistically significantly associated with

lower grade (p<0.001), lower nodal status (p<0.001), smaller size (p<0.001), increased PR status (p<0.001), and decreased Ki67 index (p=0.004). Whereas p44/42 MAPK$^{202/204}$ statistically significantly associated with lower age (p=0.001), lower grade (p<0.001), lower nodal status (p<0.001), and decreased lymphovascular invasion (p<0.001).

Multivariate analysis of biomarkers, treatment regimen, clinicopathological characteristics and relapse-free survival at 2.5 and 10 years

IKKα, Raf-1^{338}, p44/42 MAPK$^{202/204}$, and ER^{167} were then taken forward into multivariate analysis along with age, grade, nodal status, size, PR status and Ki67 index (**Table 4**). In the full cohort, multivariate survival analysis for RFS at 2.5 years (n=2827) showed age (p<0.001), nodal status (p<0.001), size (p=0.002), and Ki67 index (p<0.001) were independent prognostic factors. Whereas multivariate survival analysis for RFS at 10 years (n=1963) demonstrated that age (p<0.001), size (p<0.001), HER2 status (p=0.001), and Ki67 index (p=0.006) were independent prognostic factors. Patients were then split by treatment regimen and multivariate analysis performed for each cohort (**Table 4**). For patients receiving exemestane monotherapy, RFS at 2.5 years (n=1429) showed age (p=0.004), nodal status (p=0.001), size (p=0.008), and Ki67 index (p=0.001) were independently prognostic. Similarly, RFS at 10 years (n=980) showed age (p<0.001), size (p<0.001), and PR status (p=0.001) were independent prognostic factors. Whereas for patients receiving tamoxifen followed by exemestane therapy, RFS at 2.5 years (n=1398) showed age (p<0.001), nodal status (p=0.008), and Ki67 index (p=0.001) were independently prognostic. However, RFS at 10 years (n=983) showed age (p=0.001), size (p=0.001), ER^{167} phosphorylation (p=0.002), the IKKα and Raf-1^{338} score

(p=0.008), and the IKKα, Raf-1^{338} and ER^{167} score (p=0.001) were independent prognostic factors.

DISCUSSION

The current study demonstrated that an all-positive IKKα, Raf-1^{338} and ER^{167} score is an independent predictor of response to sequential therapy of tamoxifen followed by exemestane. Furthermore, an all-negative IKKα, Raf-1^{338} and ER^{167} score is predictive for response to exemestane monotherapy. Utilizing IKKα, Raf-1^{338} and ER^{167} could ensure the most effective endocrine therapy regimen is administered to patients.

Bennett *et. al.* reported that high IKKα expression was associated with shorter RFS in ER-positive tamoxifen-treated patients (11). In contrast, the present study reports that IKKα expression is associated with improved RFS in ER-positive patients receiving tamoxifen followed by exemestane. This discordance may be explained by differences in the thresholds employed; Bennett et al. (11) employed the median whereas the present study used positive and negative, making direct comparisons difficult. Nevertheless, the data suggests that the addition of exemestane changes the prognostic power of IKKα. During long-term tamoxifen treatment (5 years), blockade of ER lead to higher free estrogen levels promoting the formation of an ER/IKKα complex to enhance transcriptional activity, which results in reduced RFS after 2 years as observed by Bennett *et al.* (16). However, if exemestane is administered following short-term tamoxifen treatment (2.5 years), estrogen levels fall and the ER/IKKα complex is released to phosphorylate ER in a ligand-independent manner resulting in improved RFS as observed in the

current study (16). This is not observed in the exemestane monotherapy patients, as the initial formation of the ER/IKKα complex is required to improve RFS.

Of interest, McGlynn *et al.* reported that high Raf-1^{338} was associated with shorter RFS on tamoxifen (10). However, in the present study, patients that received tamoxifen for 2.5 years showed an increase in RFS, which was also observed for both treatments at 10 years. Again, this discordance may be due to differences in the thresholds used for the two studies; McGlynn et al. used the upper quartile whereas the present study assessed negative or positive, making direct comparisons difficult. Therefore it would appear that depending on the threshold employed slightly different results may be obtained. Of note, these studies are retrospective and may be subject to selection biases. However, other studies observed that when estrogen production is ablated, p44/42 MAPK phosphorylates ER^{167} to induce transcription of alternative ER-dependent genes (17). When tamoxifen is administered long-term, the partial agonist activity of tamoxifen causes activation of ER, which in turn activates Raf-1 causing increased tumor growth as observed by McGlynn *et al.* However, when estrogen production is ablated by exemestane alone or following short-term tamoxifen treatment, ER-independent phosphorylation of Raf-1^{338} can promote p44/42 MAPK to phosphorylate ER^{167} resulting in up-regulation of alternative gene transcription, conveying good prognosis to the patients as observed in the present study (18).

The cumulative prognostic score demonstrated that patients with both-positive IKKα and Raf-1^{338} expression had a better prognosis on tamoxifen followed by exemestane therapy compared to patients with negative expression of one of the biomarkers, suggesting it could be used as a predictive biomarker for patients receiving sequential therapy. Previous studies have demonstrated that IKKα can phosphorylate p44/42 MAPK suggesting IKK alpha may work with Raf-1 to enhance ER^{167} phosphorylation (19). To assess this ER^{167} phosphorylation was added to

the cumulative prognostic score. Associations with improved RFS were enhanced in patients receiving tamoxifen followed by exemestane therapy, suggesting IKKα and Raf-1 independently phosphorylate ER^{167}. This was confirmed in exemestane monotherapy patients as no improvement in RFS was observed suggesting the addition of the primed ER/IKK alpha complex enhances the beneficial effects of ER^{167}. This data suggest that an all-positive IKKα, $Raf\text{-}1^{338}$ and ER^{167} score could be utilized as a predictive biomarker for lower recurrence in patients receiving tamoxifen followed by exemestane therapy.

Conversely, patients with an all-negative IKKα, $Raf\text{-}1^{338}$ and ER^{167} score favored exemestane monotherapy with statistically significantly improved RFS. This suggests that when IKKα and Raf-1 are not present, the build-up of free estrogens during tamoxifen treatment promote tumour progression by competing for ER and exemestane is unable to stop this detrimental effect without IKKα and Raf-1. However, in the exemestane monotherapy patients, estrogens never activate ER, inhibiting tumour recurrence. These results suggest that an all-negative IKKα, $Raf\text{-}1^{338}$ and ER^{167} score might be utilized to select patients for exemestane monotherapy.

The present study also assesses HER2 status and uses 10-year follow-up, which was not available when the trial was originally reported. The main limitation of this study is its retrospective nature and that of the 9766 patients included in the TEAM trial, only 4444 patients had tissue available for use in this study. Furthermore, of the 4444 patient in the present cohort, only 2827 were included in the multivariate analysis due to missing data. However, even with these limitations the IKKα, $Raf\text{-}1^{338}$ and ER^{167} score was demonstrated to predict recurrence in postmenopausal breast cancer patients treated with endocrine therapy. Patients with an all-positive score should be treated with sequential therapy of tamoxifen followed by exemestane,

whereas patients with an all-negative score should be treated with exemestane monotherapy. As the results describe predictive biomarkers for response to different endocrine therapy regimens and not prognostic biomarkers of general recurrence, it is not clear how the present work may be incorporated into existing recurrence models.

In conclusion, although validation is warranted in other translational studies of comparative clinical trial patients, utilizing the IKKα, Raf-1^{338} and ER^{167} score could ensure the most effective endocrine therapy regimen is administered to patients most likely to gain maximum benefit. Furthermore, this cumulative score is readily translatable to the clinical scenario as it utilizes techniques already in daily use and is scored as negative or positive which is easily automated within this setting. These observations may only apply to a minority of patients, however applying the correct treatment at the correct time is a key goal in personalised medicine and is likely to improve the outcome of patients with breast cancer.

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Notes

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FIGURE LEGENDS

Figure 1. Flow diagram showing criteria for exclusion of patients from study. Tissue Micro Array = TMA, Estrogen receptor = ER, Inhibitory Kappa Kinase = IKK, Mitogen Activated Protein Kinase = MAPK

Figure 2. IKKα, Raf-1^{338} and ER^{167} score as a prognostic biomarker for recurrence in patients receiving tamoxifen followed by exemestane therapy. (A) Kaplan Meier showing RFS at 10 years for IKKα in patients receiving either exemestane monotherapy or tamoxifen followed by exemestane therapy. (B) Kaplan Meier showing RFS at 10 years for the IKKα and Raf-1^{338} score in patients receiving either exemestane monotherapy or tamoxifen followed by exemestane therapy (C) Kaplan Meier showing RFS at 10 years for the IKKα, Raf-1^{338} and ER^{167} score in patients receiving either exemestane monotherapy or tamoxifen followed by exemestane therapy. All statistical tests were two-sided. HR=hazard ration; CI=confidence interval.

Figure 3. Biomarker associations with treatment regimen. Forest plot for RFS at 10 years, showing whether a biomarker favours exemestane monotherapy (experimental group) or tamoxifen followed by exemestane therapy (control group), in postmenopausal early breast cancer patients. All statistical tests were two-sided and error bars represent 95% CI. CI=confidence interval; df=degrees of freedom; I^2=heterogeneity score; Z=z-score; ■=individual odds ratio ◆=combined odds ratio.

Figure 4. Both negative IKKα and Raf-1^{338} score as a predictive biomarker for exemestane monotherapy. (A) Kaplan Meier showing RFS at 10 years for treatment regimen in patients with either negative or positive expression of IKKα. (B) Kaplan Meier showing RFS at 10 years

for treatment regimen in patients with either a both negative or both positive IKKα and Raf-1[338] score. All statistical tests were two-sided. HR=hazard ration; CI=confidence interval.

Table 1. Baseline characteristics of patients

Characteristics	TEAM study (n=9766)	Pathology substudy (n=6120)	Biomarker substudy (n=4444)					
				Univariate Analysis				
				RFS at 2.5yrs			RFS at 10yrs	
	No. of Patients (%)	No. of Patients (%)	No. of Patients (%)	No. of Events	HR (95% CI)	p value*	No. of Events	HR (95% CI)	p value*
Age					1.31 (1.16-1.49)	<0.001		1.42 (1.32-1.53)	<0.001
<50	331 (3.2)	211 (3.4)	115 (2.6)	7			22		
50-59	3017 (30.9)	1874 (30.6)	1322 (29.7)	88			240		
60-69	3731 (38.4)	2373 (38.8)	1703 (38.3)	129			357		
>70	2687 (27.5)	1662 (27.2)	1304 (29.4)	146			435		
Grade					1.27 (1.11-1.45)	0.001		1.15 (1.07-1.24)	<0.001
1	1677 (17.2)	616 (10.1)	520 (11.7)	33			91		
2	4795 (49.1)	3166 (51.8)	2284 (51.4)	168			529		
3-4	2438 (25.0)	1835 (30.0)	1369 (30.8)	150			371		
Unknown	856 (8.7)	503 (8.1)	271 (6.1)	19			63		
Nodal Status					1.62 (1.30-2.02)	<0.001		1.52 (1.34-1.73)	<0.001
absent	5113 (52.4)	2604 (42.5)	1884 (42.4)	117			353		
present	4585 (46.9)	3494 (57.1)	2560 (57.6)	253			701		
unknown	68 (0.7)	22 (0.4)	0 (0)	-			-		
Size					1.95 (1.56-2.42)	<0.001		1.97 (1.74-2.24)	<0.001
<20mm	5697 (58.3)	3028 (49.4)	2139 (48.1)	124			363		
>20mm	4047 (41.4)	3027 (49.4)	2172 (48.9)	237			662		
Unknown	22 (0.3)	65 (1.2)	133 (3.0)	-			-		
PR status					1.14 (0.99-1.31)	<0.001		1.12 (1.03-1.21)	<0.001
absent	1724 (17.7)	1070 (17.5)	826 (18.6)	101			261		
present	7301 (74.8)	4378 (71.5)	3034 (68.3)	224			658		
not performed	741 (7.5)	672 (11.0)	584 (13.1)	45			135		
HER2 Status					1.21 (0.96-1.52)	0.11		1.22 (1.06-1.40)	0.005
absent	-	2796 (45.7)	1776 (39.7)	134			377		
present	-	1908 (31.2)	1738 (39.1)	159			469		
not performed	-	1416 (23.1)	940 (21.2)	-			-		
Ki67 status					1.87 (1.48-2.36)	<0.001		1.48 (1.29-1.71)	<0.001
low (<15)	-	2308 (37.7)	2277 (51.2)	156			489		
high (>15)	-	1064 (17.4)	1027 (23.1)	127			305		
unknown	-	2748 (44.9)	1140 (25.7)	-			-		

Treatment					1.22 (1.00-1.50)	0.05		1.05 (0.93-1.18)	0.45
exemestane	4898 (50.2)	3075 (50.2)	2240 (50.4)	1169			523		
tamoxifen then exemestane	4868 (49.8)	3045 (49.8)	2204 (49.6)	1201			531		
Lymphovascular Invasion					0.78 (0.56-1.08)	0.13		0.90 (0.73-1.10)	0.31
no	-	3845 (62.8)	3727 (83.9)	299			881		
yes	-	403 (6.6)	394 (8.9)	40			101		
unknown	-	1872 (30.6)	323 (7.2)	-			-		

RFS = relapse-free survival; HR = Hazard Ratio; CI = Confidence interval; -- = data not available [*]Kaplan-Meier and log-rank analysis compared RFS at both time points. HRs and CIs were calculated from univariate cox regression survival analysis. All statistical tests were two-sided.

Table 2. Univariate analysis of associations between biomarkers and RFS at 2.5 and 10 years in postmenopausal HRec-positive early breast cancer patients.

Biomarkers	Number of Patients	RFS 2.5 events	RFS 10 events	Relapse-free survival at 2.5yrs		Relapse-free survival at 10yrs	
				HR (95% CI)	p value*	HR (95% CI)	p value*
phospho ER^{118} (n=4218)				0.96 (0.75-1.22)	0.73	0.83 (0.71-0.96)	0.01
low expression (<110 WHS units)	3157	266	777				
high expression (>110 WHS units)	1061	86	218				
phospho ER^{167} (n=4133)				0.73 (0.59-0.92)	0.007	0.82 (0.71-0.94)	0.004
negative expression (0 WHS units)	1150	115	304				
positive expression (>0 WHS units)	2983	222	670				
IKK alpha (n=3024)				0.86 (0.68-1.11)	0.24	0.80 (0.69-0.93)	0.003
negative expression (0 WHS units)	1579	144	400				
positive expression (>0 WHS units)	1445	114	298				
phospho p65^{536} (n=4053)				1.03 (0.79-1.34)	0.81	0.89 (0.77-1.04)	0.15
low expression (<25 WHS units)	857	70	219				
high expression (>25 WHS units)	3196	271	739				
N-Ras (n=4039)				0.66 (0.28-1.61)	0.36	1.36 (0.94-1.96)	0.11
low expression (<100 WHS units)	3952	337	929				
high expression (>100 WHS units)	87	5	28				
Raf-1^{338} (n=4030)				0.62 (0.48-0.81)	<0.001	0.69 (0.59-0.80)	<0.001
negative expression (0 WHS units)	2797	266	728				
positive expression (>0 WHS units)	1233	74	224				
p44/42 MAPK$^{202/204}$ (n=4055)				0.70 (0.56-0.88)	0.002	0.76 (0.66-0.86)	<0.001
negative expression (0 WHS units)	2249	217	590				
positive expression (>0 WHS units)	1806	125	369				
IKK alpha and Raf-1^{338} (n=2980)				0.77 (0.65-0.91)	0.005	0.78 (0.71-0.87)	<0.001
both negative	1154	115	316				
one positive	1158	102	254				
both positive	668	37	116				
IKK alpha, Raf-1^{338} and ER^{167} (n=2904)				0.71 (0.56-0.89)	0.01	0.73 (0.64-0.85)	<0.001
	339	37	104				
all negative	2037	178	471				
one or two positive	528	29	93				
all positive							

RFS = relapse-free survival; HR = Hazard Ratio; CI = Confidence interval; WHS = weighted histoscore . *Kaplan-Meier and log-rank analysis compared RFS at both time points. HRs and CIs were calculated from univariate cox regression survival analysis. All statistical tests were two-sided..

Table 3. Univariate analysis of associations between biomarkers, treatment regimen, and RFS at 2.5 and 10 years in postmenopausal HRec positive early breast cancer patients

Biomarkers	Exemestane							Tamoxifen then exemestane						
	Number of Patients	RFS 2.5 events	RFS 10 events	Relapse-free survival at 2.5yrs		Relapse-free survival at 10yrs		Number of Patients	RFS 2.5 events	RFS 10 events	Relapse-free survival at 2.5yrs		Relapse-free survival at 10yrs	
				HR (95% CI)	p value*	HR (95% CI)	p value*				HR (95% CI)	p value*	HR (95% CI)	p value*
ER[118]				1.22 (0.87-1.71)	0.26	0.87 (0.71-1.07)	0.19				0.77 (0.54-1.09)	0.14	0.78 (0.63-0.97)	0.03
low expression	1587	112	380					1570	154	397				
high expression	550	47	115					511	39	103				
ER[167]				0.68 (0.49-0.95)	0.02	0.96 (0.79-1.16)	0.66				0.77 (0.57-1.05)	0.10	0.71 (0.59-0.85)	<0.001
negative expression	594	55	141					556	60	163				
positive expression	1491	96	342					1492	126	342				
IKK alpha				0.80 (0.55-1.16)	0.23	0.86 (0.69-1.07)	0.17				0.93 (0.67-1.29)	0.66	0.74 (0.60-0.92)	0.005
negative expression	791	65	183					788	79	217				
positive expression	744	49	150					701	65	148				
p65[536]				1.19 (0.79-1.79)	0.41	0.83 (0.67-1.02)	0.08				0.93 (0.69-1.32)	0.69	0.97 (0.78-1.20)	0.76
low expression	422	28	112					453	42	107				
high expression	1623	127	362					1573	144	377				
N-Ras				0.26 (0.04-1.87)	0.15	1.22 (0.72-2.08)	0.46				1.10 (0.41-2.96)	0.85	1.55 (0.91-2.64)	0.10
low expression	1991	154	459					1961	183	470				
high expression	48	1	14					39	4	14				
Raf-1[338]				0.68 (0.47-0.98)	0.04	0.74 (0.60-0.91)	0.004				0.58 (0.41-0.83)	0.003	0.64 (0.52-0.80)	<0.001
negative expression	1390	117	352					1407	149	376				
positive expression	641	37	120					592	37	104				
p44/42 MAPK[202/204]				0.77 (0.56-1.06)	0.11	0.75 (0.62-0.90)	0.002				0.66 (0.49-0.89)	0.006	0.77 (0.64-0.92)	0.004
negative expression	1103	93	286					1146	124	304				
positive expression	942	62	188					864	63	181				
IKK alpha and Raf-1[338]				0.81 (0.63-1.04)	0.18	0.85 (0.73-0.98)	0.05				0.74 (0.59-0.93)	0.03	0.73 (0.63-0.84)	<0.001
both negative	570	48	137					584	67	179				
one positive	590	46	132					568	56	122				
both positive	346	18	58					322	19	58				
IKK alpha, Raf-1[338] and ER[167]				0.73 (0.51-1.03)	0.17	0.75 (0.70-1.04)	0.27				0.70 (0.51-0.95)	0.08	0.64 (0.52-0.77)	<0.001
all negative	167	15	41					172	22	63				
one or two positive	1027	80	229					1010	98	242				
all positive	271	13	49					257	16	44				

RFS = relapse-free survival; HR = Hazard Ratio; CI = Confidence interval. *Kaplan-Meier and log-rank analysis compared RFS at both time points. HRs and CIs were calculated from univariate cox regression survival analysis. All statistical tests were two-sided.

Table 4. Multivariate analysis of associations between biomarkers, clinicopathological characteristics, and RFS at 2.5 and 10 years in postmenopausal HRec-positive early breast cancer patients by treatment

Characteristics And Biomarkers	All Patients (n=4444)				Exemestane Only (n=2240)				Tamoxifen followed by Exemestane (n=2204)			
	Relapse-free survival at 2.5yrs (n=2827)		Relapse-free survival at 10yrs (n=1963)		Relapse-free survival at 2.5yrs (n=1429)		Relapse-free survival at 10yrs (n=980)		Relapse-free survival at 2.5yrs (n=1398)		Relapse-free survival at 10yrs (n=983)	
	HR (95% CI)	p value*	HR (95% CI)	p value*	HR (95% CI)	p value*	HR (95% CI)	p value*	HR (95% CI)	p value*	HR (95% CI)	p value*
Age <50/50-59/60-69/>70	1.43 (1.23-1.67)	<0.001	1.34 (1 20-1.50)	<0.001	1.41 (1.11-1.78)	0.004	1.38 (1.18-1.62)	<0.001	1.48 (1.20-1.83)	<0.001	1.30 (1.11-1.52)	0.001
Grade 1/2/3/4	1.24 (1.04-1.48)	0.02	1.14 (1.01-1.29)	0.04	1.32 (1.01-1.75)	0.04	1.10 (0.92-1.31)	0.28	1.12 (0.90-1.40)	0.32	1.16 (0.97-1.37)	0.10
Nodal Status absent/present	1.78 (1.34-2.36)	<0.001	1.39 (1.08-1.79)	0.01	2.02 (1.31-3.11)	0.001	1.36 (0.93-1.98)	0 12	1.65 (1.14-2.38)	0.008	1.26 (0.87-1.83)	0.22
Size <20mm/>20mm	1.54 (1.18-2.02)	0.002	1.70 (1.42-2.06)	<0.001	1.75 (1.16-2.64)	0.008	1.82 (1.37-2.40)	<0.001	1.44 (1.01-2.06)	0.04	1.61 (1.23-2.11)	0.001
PR status absent/present	1.13 (0.94-1.36)	0.20	1.17 (1.00-1.38)	0.05	1.33 (1.02-1.72)	0.04	1.46 (1.16-1.85)	0.001	0.96 (0.74-1.25)	0.78	0.98 (0.79-1.22)	0.86
HER2 status absent/present	-	-	1.39 (1.15-1.62)	0.001	-	-	1.35 (1.04-1.79)	0.02	-	-	1.40 (1.06-1.85)	0.02
Ki67 status Low/High	1.87 (1.44-2.42)	<0.001	1.33 (1.09-1.62)	0.006	1.96 (1.32-2.92)	0.001	1.37 (1.04-1.79)	0.03	1.78 (1.27-2.50)	0.001	1.32 (1.00-1.73)	0.05
phospho ER^{167} negative/positive	0.88 (0.67-1.16)	0.35	0.79 (0.65-0.96)	0.02	0.78 (0.52-1.17)	0.23	0.99 (0.74-1.32)	0.92	1.00 (0.69-1.45)	0.99	0.65 (0.50-0.85)	0.002
IKK alpha absent/present	-	-	0.91 (0.76-1.14)	0.34	-	-	1.09 (0.83-1.42)	0.55	-	-	0.81 (0.63-1.06)	0.11
Raf-1^{338} (n=4030) negative/positive	0.71 (0.52-0.97)	0.03	0.85 (0.65-1.12)	0.26	0.72 (0.46-1.14)	0.16	1.01 (0.68-1.49)	0.98	0.81 (0.51-1.29)	0.37	0.66 (0.50-0.98)	0.04
p44/42 $MAPK^{202/204}$ negative/positive	0.92 (0.70-1.20)	0.53	0.92 (0.76-1.13)	0.45	1.21 (0.81-1.81)	0.36	0.90 (0.70-1.19)	0.46	0.70 (0.49-1.01)	0.06	0.98 (0.74-1.30)	0.90
IKK alpha and Raf-1^{338} both negative/one positive/both positive	0.82 (0.68-0.99)	0.38	0.89 (0.77-1.04)	0.13	0.79 (0.60-1.05)	0.11	1.03 (0.83-1.27)	0.80	0.81 (0.63-1.05)	0.11	0.76 (0.62-0.93)	0.008
IKK alpha, Raf-1^{338} and ER^{167} all negative/one or two positive/all positive	-	-	0.81 (0.68-0.96)	0.01	-	-	0.97 (0.76-1.24)	0.83	-	-	0.67 (0.54-0.85)	0.001

HR = Hazard Ratio; CI = Confidence interval; -- = not included in analysis. *Multivariate cox regression survival analysis using a backward conditional elimination model and a statistical significance threshold of $p<0.01$ was performed to identify independent prognostic biomarkers. All statistical tests were two-sided.