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Gene expression of estrogen receptor, progesterone receptor and microtubule-associated protein Tau in high-risk early breast cancer: a quest for molecular predictors of treatment benefit in the context of a Hellenic Cooperative Oncology Group trial

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Abstract *Background* Estrogen receptor (ER) and progesterone receptor (PgR) protein expression carry weak prognostic and moderate predictive utility for the outcome of early breast cancer patients on adjuvant chemohormonotherapy. We sought to study the predictive significance and correlations of transcriptional profiling of the ER, PgR and microtubule-associated protein Tau (MAP-Tau) genes in early breast cancer. *Materials and methods*

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Messenger RNA (mRNA) was extracted from 279 formalin-fixed paraffin-embedded breast carcinomas (T1-3N0-1M0) of patients enrolled in the Hellenic Cooperative Oncology Group (HeCOG) trial HE 10/97, evaluating epirubicin-alkylator based adjuvant chemotherapy with or without paclitaxel (E-T-CMF versus E-CMF). Kinetic reverse transcription polymerase chain reaction (kRT-PCR) was applied for assessment of the expression of estrogen receptor, progesterone receptor and MAP-Tau genes in 274 evaluable patients. Cohort-based cut-offs were defined at the 25th percentile mRNA value for ER and PgR and the median for MAP-Tau. *Results* Two hundred and ten patients (77%) were ER and/or PgR-positive by immunohistochemistry (IHC).

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Positive ER and MAP-Tau mRNA status was significantly associated with administration of hormonal therapy and low grade, while MAP-Tau mRNA status correlated with premenopausal patient status. MAP-Tau strongly correlated with ER and PgR mRNA status (Spearman $r = 0.52$ and 0.64 , $P < 0.001$). The observed chance corrected agreement between determination of hormonal receptor status by kRT-PCR and IHC was moderate (Kappa = 0.41) for ER and fair (Kappa = 0.33) for PgR. At a median follow-up of 8 years, univariate analysis adjusted for treatment showed positive ER mRNA status to be of borderline significance for reduced risk of relapse (HR = 0.65, 95% CI 0.41–1.01, $P = 0.055$) and death (HR = 0.62, 95% CI 0.36–1.05, $P = 0.077$), while positive MAP-Tau mRNA status was significantly associated with reduced risk of relapse (HR = 0.50, 95% CI 0.32–0.78, $P = 0.002$) and death (HR = 0.49, 95% CI 0.29–0.83, $P = 0.008$). In multivariate analysis, only axillary nodal metastases (HR = 2.33, 95% CI 1.05–5.16, $P = 0.04$) and MAP-Tau mRNA status (HR = 0.46, 95% CI 0.25–0.85, $P = 0.01$) independently predicted patient outcome. However, MAP-Tau mRNA levels did not predict enhanced benefit from inclusion of paclitaxel in the adjuvant chemotherapy regimen (test for interaction $P = 0.99$). No correlation was evident between increasing ER and PgR mRNA transcription and increasing benefit from endocrine therapy in 203 ER and/or PgR IHC-positive patients receiving adjuvant hormone therapy (Wald $P = 0.54$ for ER, 0.51 for PR). **Conclusions** ER gene transcription carries weak predictive significance for benefit from endocrine therapy or for outcome, with no apparent dose-response association. The predictive significance is possibly exerted via MAP-Tau gene expression, an ER-inducible tubulin modulator with strong predictive significance for patient outcome. However, MAP-Tau mRNA did not predict benefit from the addition of a taxane to adjuvant chemotherapy. Further study of the biologic function and utility of MAP-Tau for individualising adjuvant therapy is warranted.

Keywords Estrogen receptor · Progesterone receptor · Microtubule · MAP-Tau · Predictive value · Breast cancer

Introduction

Breast adenocarcinoma is the most common malignant tumor in females with 60–70% of affected patients presenting with localised disease. These women become disease-free following surgical resection of the tumor and may be candidates for adjuvant systemic therapy aiming at eradication of micrometastatic deposits [1]. Estrogen receptor (ER) protein expression, studied by means of immunohistochemical (IHC) staining, is the gold standard

for the selection of patients who will be managed with hormonal therapy, though its predictive value for benefit from such treatment is modest (30–60%) [2, 3]. Several clinicopathological parameters, including tumor size, grade, ER status, axillary nodal status, patient age, and Human Epidermal growth factor Receptor-type 2 (HER2) overexpression are taken into consideration in order to identify which patients are most likely to benefit from administration of adjuvant cytotoxic chemotherapy and which drugs should be used [4, 5]. Still, the power of these complex predictive models is at best moderate: patients may be undertreated or overtreated. Ultimately, we are unable to reliably predict the true binary outcome of recurrence versus no recurrence at the level of the individual, as these clinicopathological guidelines do not capture the rich molecular heterogeneity of the malignancy and are by no means able to pinpoint the exact clinical behaviour of breast tumors [6].

Even when conventional IHC studies were used, evidence emerged that incremental ER protein expression was associated with higher probability of benefit from endocrine therapy [7]. However, (IHC) studies show considerable intra- and inter-laboratory variability even when reporting binary results, due to variation in fixation time, antigen retrieval and staining methods, observer subjectivity or image acquisition threshold differences [8–10]. Accordingly, efforts at quantification of ER and progesterone receptor (PgR) protein expression face problems of technical feasibility and reproducibility. The advent of robust, sensitive and reproducible molecular techniques that reliably quantify expression of one or several genes offers promise for more accurate quantification of ER and PgR. Reverse-transcriptase polymerase chain reaction (RT-PCR) techniques analysing messenger RNA (mRNA) extracted from formalin-fixed paraffin-embedded tumors have recently been developed, able to provide normalised gene expression data over a broad dynamic range. Preliminary reports suggest satisfactory correlation of quantitative mRNA results with categories of IHC protein expression [11–13]. Still, the prognostic and predictive value of the continuous range of tumoral ER and PgR gene transcription data for patient survival and benefit from therapy has not been examined to date.

Tau is a microtubule-associated protein (MAP-Tau) that stabilises tubulin polymerisation. Early preclinical and clinical reports suggest that the MAP-Tau gene is induced by ER and that expression of Tau protein modulates sensitivity of malignant cells to taxanes [14]. Low levels of Tau protein expression render mitotic and cytoskeletal microtubules more sensitive to disruption by paclitaxel, affecting mitosis and cell signalling. We retrospectively examined gene expression of ER, PgR and MAP-Tau in high-risk localised breast carcinomas of women enrolled in

a prospective, randomised Hellenic Cooperative Oncology Group (HeCOG) trial of adjuvant chemotherapy in order to study their correlation and concordance with IHC expression of the relevant proteins as well as their utility for predicting clinical benefit from adjuvant hormonal therapy and taxane-containing chemotherapy.

Materials and methods

Clinical trial

The HeCOG prospective trial HE10/97 randomised a total of 595 high-risk (T1-3N1M0 or T3N0M0) breast cancer patients in the period 1997–2000, to receive either four cycles of epirubicin followed by four cycles of intensified cyclophosphamide, methotrexate and 5-fluorouracil combination chemotherapy (E-CMF) or three cycles of epirubicin followed by three cycles of paclitaxel and three cycles of intensified CMF (E-T-CMF) [15]. Chemotherapy cycles were administered every 2 weeks and patients received granulocyte-colony stimulating factor (GCSF) support. The clinical protocol and translational research studies were approved by the HeCOG Protocol Review Committee, by the Institutional Review Board of Kyanous Stavros Hospital and the AHEPA University Hospital. All patients provided written informed consent prior to enrolment.

Molecular and immunohistochemical studies

This was a retrospective translational research study among patients who had been enrolled in a prospective clinical trial. Accordingly, collection of formalin-fixed paraffin-embedded (FFPE) tumor tissue samples was possible in 279 patients only, due to logistical/organisational barriers. An experimental method based on proprietary magnetic beads from Siemens Healthcare Diagnostics (Cologne, Germany) resulted in mRNA isolation from 274 FFPE blocks. For all tumor samples included in the analysis the number of malignant cells represented at least 75% of all nucleated cells per section as verified by hematoxylin-eosin staining. Kinetic reverse transcription polymerase chain reaction (kRT-PCR) was applied for assessment of the expression of estrogen receptor (ESR1), progesterone receptor and MAP-Tau genes using gene specific Taq-ManTM based Primer/Probe sets. Forty cycles of nucleic acid amplification were applied and the cycle threshold (CT) values of the target genes were identified. CT values were normalized by subtracting the CT value of the housekeeping gene RPL37A from the CT value of the target gene (Δ CT). RNA results were then reported as 40- Δ CT values, which would correlate proportionally to the mRNA expression level of the target gene.

In short, each FFPE slide (5 μ m thick) was deparaffinized in xylol and ethanol, the pellet was washed with ethanol and dried at 55°C for 10 min. The pellet was then lysed and proteinised overnight at 55°C with shaking. After adding a binding buffer and the magnetic particles (Siemens Healthcare Diagnostics, Cologne, Germany) nucleic acids were bound to the particles within 15 min at room temperature. On a magnetic stand, the supernatant was aspirated and the beads were washed several times with a washing buffer. After adding elution buffer and incubating for 10 min at 70°C the supernatant was aspirated on a magnetic stand without touching the beads. After normal DNase I treatment for 30 min at 37°C and inactivation of DNase I the solution was used for RT-PCR. The Primer/Probe sets used for amplification of the target genes were the following:

ER Probe ATGCCCTTTTGCCGATGCA

Forward Primer GCCAAATTGTGTTTGATGGAT TAA

Reverse Primer GACAAAACCGAGTCACATCAG TAATAG

PgR Probe TTGATAGAAACGCTGTGAGCTCGA

Forward Primer AGCTCATCAAGGCAATTGGTTT

Reverse Primer ACAAGATCATGCAAGTTATCAA GAAGTT

MAP-Tau Probe AGACTATTTGCACACTGCCGCCT

Forward Primer GTGGCTCAAAGGATAATATC AAACAC

Reverse Primer ACCTTGCTCAGGTCAACTGGTT

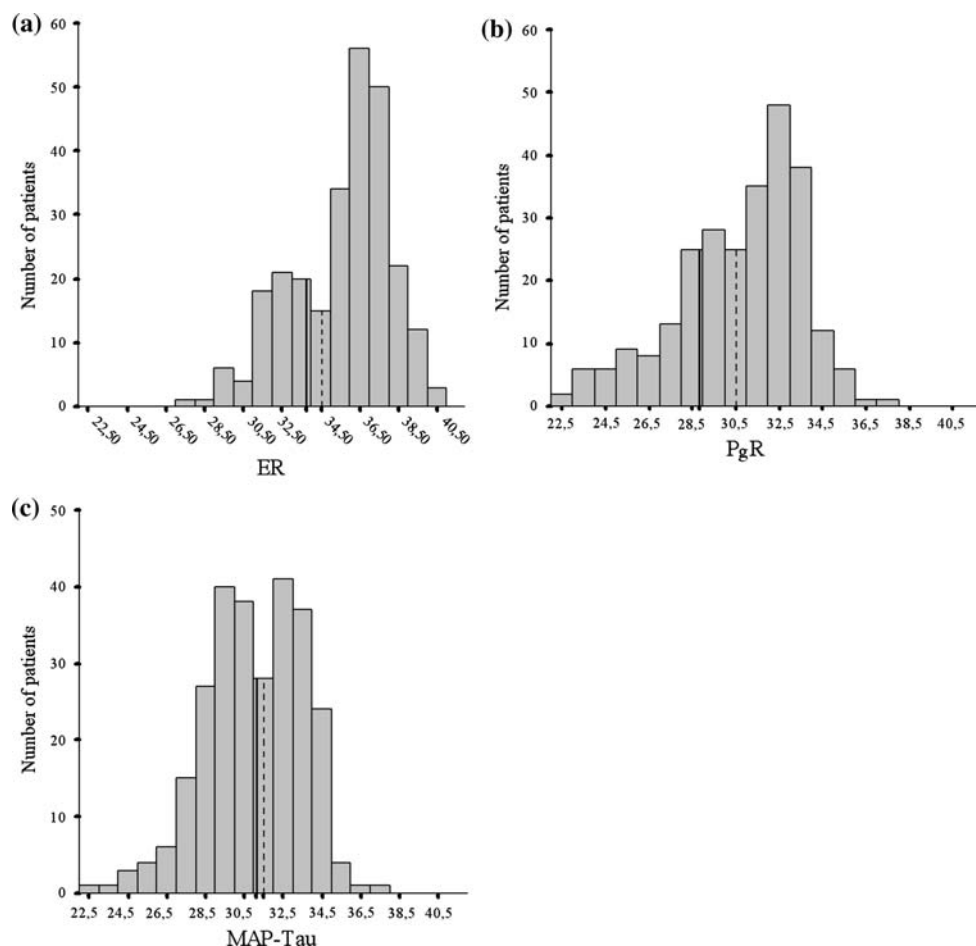
The quality and quantity of RNA was checked by measuring absorbance at 260 and 280 nm. Pure RNA has an A260/A280 ratio of 1.9–2.0. Human reference total RNA pooled from ten human cell lines (Stratagene, La Jolla, California, USA) was used as a positive control. RNA-free DNA extracted from tumor tissues was used as a negative control.

ER and PgR protein expression was evaluated immunohistochemically in the pathology department of the different centers involved in the study, by means of streptavidin–biotin complex immunoperoxidase assay on 5 μ m sections from FFPE tumor tissue. Tumor samples were categorised as ER or PgR-positive if at least 1% of malignant cells stained for ER or PgR protein, respectively.

Statistical methodology

In the absence of a reliable gold standard and following distributional studies, we used the 25th percentile of observed hormonal receptor mRNA expression levels and the median of observed MAP-Tau mRNA levels as thresholds for categorization of tumors to positive or negative cases. The chosen cut-offs were found to be

Fig. 1 Distribution of ER (a), PgR (b) and MAP-Tau (c) mRNA levels in the studied population. Solid vertical lines on the graphs correspond to the chosen cut-offs (25th percentile for ER and PgR and the median for MAP-Tau) and dashed lines to the natural cut-offs as determined by the corresponding distributions



close to the natural cut-offs in distributional studies (Fig. 1a–c). Moreover, cut-offs on the quartiles offer easily interpretable, reproducible and objective results. Fisher's exact test was applied to compare standard clinical and pathological factors between ER, PgR and MAP-Tau mRNA levels. Spearman's rank correlation was used as a measure of association between the three biomarkers. The Mann–Whitney test was used to compare MAP-Tau levels with ER and PgR expression levels.

Overall percentages of agreement, uncorrected for agreement by chance, were calculated and given along with the 95% confidence intervals. The degree of agreement corrected by chance was estimated using the Kappa coefficient. Kappa estimations were interpreted following the guidelines described by Landis and Koch [16]: $\kappa \leq 0$, poor agreement; $\kappa > 0$ but ≤ 0.20 , slight agreement; $\kappa > 0.20$ but ≤ 0.40 , fair agreement; $\kappa > 0.40$ but ≤ 0.60 , moderate agreement; $\kappa > 0.60$ but ≤ 0.80 , substantial agreement; and $\kappa > 0.80$ –1.00, almost perfect agreement.

Disease-free survival (DFS) was defined as the interval from study entry to first locoregional recurrence, first

distant metastasis, contralateral breast cancer, secondary neoplasm, death from the disease, or death from any cause non-related to breast cancer, whichever occurred first [17]. Overall survival (OS) was measured from study entry until death from any cause. Surviving patients were censored at the date of last contact. Estimation of disease-free and overall survival was performed using the Kaplan–Meier product-limit method and differences between survival curves were assessed with the log-rank test. The Cox proportional hazards model was used to assess the strength of the association of OS and DFS with various clinical and histological variables in the presence of treatment group, ER, PgR, and MAP-Tau mRNA expression levels. A backward selection procedure with removal criterion $P > 0.10$, identified a subclass of significant variables among the following: menopausal status (pre versus post), tumor grade (I–II versus III–Undifferentiated), tumor size (≤ 2 cm vs. 2–5 cm vs. > 5 cm), and number of positive lymph nodes (0–3 vs. ≥ 4). Multivariate P values were based on Wald statistics. Results of this study were presented according to reporting recommendations for tumor marker prognostic studies [18].

Results

Patient and tumor characteristics

Patients included in the present study were comparable in terms of basic characteristics (age, menopausal status, ER status, PgR status, proportion of patients receiving hormonal treatment, number of positive nodes and tumor size) with patients from the HE10/97 trial that were not included. Two-hundred and seventy-four patients (median age 51) harboured breast carcinomas, most commonly hormone-receptor positive by IHC (77% of patients), T2-3 (70%) of moderate to poor differentiation (96%), from which neoplastic FFPE material underwent molecular analysis for gene expression of ER, PgR and MAP-Tau. After modified radical mastectomy or breast conserving surgery and nodal dissection, malignant metastases in axillary lymph nodes were present in all but four cases, with presence of four or more involved nodes in 77% of patients. Slightly more patients were managed with adjuvant E-CMF chemotherapy than E-T-CMF. With the exception of grade, basic clinicopathological characteristics were well balanced when the 274 analysed patients were stratified by adjuvant chemotherapy arm (Table 1). Following the completion of adjuvant chemotherapy, 92% of the 274 women received some form of hormonal therapy, mostly tamoxifen. Among women with IHC ER-negative tumors who took tamoxifen, a proportion had PgR-unknown or PgR-positive tumors, while some received hormonal therapy despite having both ER and PgR-negative tumors, a practice not followed anymore in HeCOG participating centres.

Correlation of molecular and clinicopathological parameters

Although mRNA isolation was successful in 274 out of 279 tumor blocks, the study of several other genes in companion projects resulted in availability of high-quality mRNA in only 271 (MAP-Tau) and 263 (ER, PgR) blocks respectively. The correlations of ER, PgR and MAP-Tau mRNA status (positive or negative) with standard clinical and pathologic factors are shown in Table 2. The associations of ER mRNA and MAP-Tau mRNA status with administration of adjuvant hormonal therapy and histological grade were statistically significant. Histological grade was also significantly associated to PgR mRNA status. Moreover, MAP-Tau mRNA status was significantly associated with patient menopausal status. Specifically, patients with negative ER, PgR or MAP-Tau mRNA levels had significantly higher grade (ER negative: 78% vs. positive: 41%, $P < 0.001$; PgR negative: 67% vs. positive: 45%, $P = 0.003$; MAP-Tau negative: 64% vs. positive: 36%, $P < 0.001$). Significantly more patients with

positive ER and MAP-Tau mRNA levels received adjuvant hormonal therapy (ER negative: 85% vs. positive: 95%, $P = 0.012$; MAP-Tau negative: 85% vs. positive: 98%, $P < 0.001$). Premenopausal patients were significantly more numerous in patients with positive MAP-Tau mRNA levels (MAP-Tau negative: 43% vs. positive: 58.5%, $P = 0.015$). On the contrary, mRNA status of any of ER, PgR and MAP-Tau did not exhibit significant correlations with patient age, adjuvant chemotherapy arm (E-CMF versus E-T-CMF), administration of adjuvant radiotherapy, tumor size, and number of involved axillary lymph nodes.

Tumoral MAP-Tau mRNA status was significantly associated with both ER (Spearman rank correlation $r = 0.52$, $P < 0.001$) and PgR mRNA status (Spearman rank correlation $r = 0.64$, $P < 0.001$). In fact, positive MAP-Tau mRNA expression was seen in 67% of cases with positive ER mRNA expression versus only 3% of cases with negative ER mRNA expression (Fisher exact test, $P < 0.001$). Similarly, positive MAP-Tau mRNA expression was observed in 61% of cases with positive PgR mRNA expression versus 20% of cases with negative PgR mRNA status (Fisher exact test, $P < 0.001$). The distribution of tumoral MAP-Tau mRNA levels in accordance to ER mRNA status is shown in Fig. 2.

Concordance between immunohistochemical and molecular hormonal receptor studies

Two hundred and ten patients (77%) were ER and/or PgR-positive by IHC. The observed overall agreement between the determination of hormonal receptor status by kRT-PCR (mRNA) and IHC was 77% (95% confidence interval (CI): 71.5%–82%) for ER and 71% (95% CI: 65%–79%) for PgR. Among ER-mRNA positive tumors, 82.5% stained positively for the ER protein whereas 17.5% did not. Among ER-mRNA negative tumors, 61% were IHC negative but 39% stained positively for the ER protein. These rates of concordance and disagreement between the RT-PCR and IHC methodologies were quite similar for the PgR parameter as well: among PgR-mRNA positive tumors, 26% exhibited negative protein staining, while among PgR-mRNA negative tumors 37.5% stained positively for the PgR protein.

Despite the good observed agreement between the two methods, the Kappa statistic (chance-corrected measure of agreement) between kRT-PCR and IHC was 0.41 (95% CI: 0.29–0.54) for ER, a marker of moderate agreement, and 0.33 (95% CI: 0.20–0.45) for PgR, a marker of fair agreement between methodologies. Using the 25th percentile of the ER and PgR mRNA values as the threshold and considering the more widely used IHC protein expression as the benchmark, the relatively new methodology of kRT-PCR had satisfactory sensitivity (85.5–86.5%) and positive

Table 1 Patient and tumor characteristics

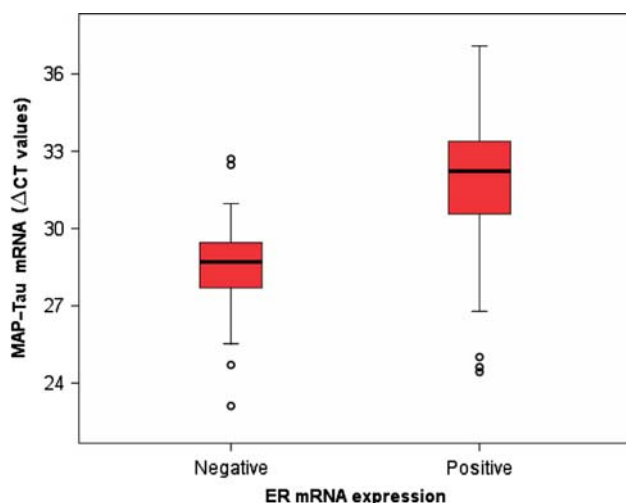
	<i>N</i> (%)			<i>P</i>
	<i>N</i> = 274	E-T-CMF <i>N</i> = 121	E-CMF <i>N</i> = 153	
Age (range)	51 (22.5–76)	52 (28–76)	51 (22–76)	0.873
Menopausal status				0.274
Premenopausal	140 (51)	57 (47)	83 (54)	
Postmenopausal	134 (49)	64 (53)	70 (46)	
Type of operation				0.758
MRM	221 (81)	99 (82)	122 (80)	
Breast conserving surgery	53 (19)	22 (18)	31 (20)	
Interval from operation (weeks)				0.116
<2	43 (16)	17 (14)	26 (17)	
2–4	128 (47)	65 (54)	63 (41)	
>4	103 (38)	39 (32)	64 (42)	
Adjuvant HT				0.387
No	23 (8)	8 (7)	15 (10)	
Yes	251 (92)	113 (93)	138 (90)	
Tamoxifen	218 (87)	100 (88.5)	118 (86)	0.575
LH-RH	107 (43)	49 (43)	58 (42)	0.898
Aromatase inhibitors	11 (4)	5 (4)	8 (6)	0.777
Other	3 (1)	1 (1)	2 (1)	0.999
Unknown	3 (1)	2 (2)	1 (1)	0.590
Adjuvant RT				0.765
No	57 (21)	24 (20)	33 (22)	
Yes	214 (78)	96 (79)	118 (77)	
Unknown	3 (1)	1 (1)	2 (1)	
Estrogen receptor status (IHC)				0.589
Negative	78 (28.5)	32 (26)	46 (30)	
Positive	191 (70)	87 (72)	104 (68)	
Unknown	5 (2)	2 (2)	3 (2)	
Progesterone receptor status (IHC)				0.999
Negative	95 (36)	42 (35)	53 (35)	
Positive	171 (62)	75 (62)	96 (63)	
Unknown	8 (3)	4 (3)	4 (3)	
Tumor size (cm)				0.83
≤2	83 (30)	35 (29)	48 (31)	
2–5	137 (50)	63 (52)	74 (48)	
>5	54 (20)	23 (19)	31 (20)	
Grade ^a				0.001
I	11 (4)	8 (7)	3 (2)	
II	125 (46)	41 (34)	84 (55)	
III	135 (49)	69 (57)	66 (43)	
Undifferentiated	2 (1)	2 (2)	0 (0)	
Missing	1 (0.4)	1 (1)	0 (0)	
Nodes removed				
Median (range)	20 (4–59)	20 (5–59)	20 (4–53)	0.950
Positive nodes				
Median (range)	6 (0–54)	7 (0–54)	6 (0–49)	0.463
0–3	63 (23)	24 (20)	39 (25.5)	0.312
4 or more	211 (77)	97 (80)	114 (74.5)	

^a Distribution of grade differs significantly ($P < 0.001$). This result is identical to the corresponding result in the clinical paper

Table 2 Relationship between mRNA ER, PgR and MAP-Tau levels and standard clinical and pathological factors

	ER mRNA level (<i>N</i> = 263)			PgR mRNA level (<i>N</i> = 263)			MAP-Tau mRNA level (<i>N</i> = 271)		
	Negative	Positive	<i>P</i> -value	Negative	Positive	<i>P</i> -value	Negative	Positive	<i>P</i> -value
Age									
Median	50	52	0.31	51	51	0.90	52	50	0.27
Range	27.5–74	22.5–76		22.5–74	27–76		22.5–74.5	27–76	
	<i>N</i> (%)	<i>N</i> (%)	<i>P</i> -value	<i>N</i> (%)	<i>N</i> (%)	<i>P</i> -value	<i>N</i> (%)	<i>N</i> (%)	<i>P</i> -value
Menopausal status									
Premenopausal	34 (52)	101 (51)	0.89	31 (47)	104 (53)	0.48	59 (43)	79 (58.5)	0.015
Postmenopausal	31 (48)	97 (49)		35 (53)	93 (47)		77 (57)	56 (41.5)	
Treatment group									
E-T-CMF	32 (49)	89 (45)	0.57	26 (39)	95 (48)	0.25	59 (43)	59 (44)	1.0
E-CMF	33 (51)	109 (55)		40 (61)	102 (52)		77 (57)	76 (56)	
Adjuvant HT									
No	10 (15)	10 (5)	0.012	8 (12)	12 (6)	0.11	20 (15)	3 (2)	<0.001
Yes	55 (85)	188 (95)		58 (88)	185 (94)		116 (85)	132 (98)	
Adjuvant RT									
No	17 (27)	37 (19)	0.21	17 (27)	37 (19)	0.21	33 (25)	24 (18)	0.18
Yes	46 (73)	160 (81)		47 (73)	159 (81)		100 (75)	111 (82)	

Italic values denote relationships statistically significant at $P < 0.05$

**Fig. 2** MAP-Tau mRNA levels in ER mRNA positive and negative cases (Mann–Whitney test, $P < 0.001$)

predictive value (74–82.5%) but modest specificity (45–53%) and negative predictive value (61–62.5%). Metrics of concordance between the two methods for ER and PgR determination are summarised in Table 3.

Clinicopathological/molecular variables and patient outcome

Survival status of the 274 patients was updated in October 2007. At the time of analysis (February 2008), the median

Table 3 Agreement between kRT-PCR and IHC status of ER, PgR and ER/PgR

	ER (%)	PgR (%)	ER/PgR (%)
Observed agreement ^a	77	71	82
Agreement expected by chance	61	57.5	69
Kappa coefficient ^b	41	33	41.5
kRT-PCR			
Sensitivity	86.5	85.5	91
Specificity	53	45	47
PPV	82.5	74	86.5
NPV	61	62.5	59

^a Proportion of agreement observed between IHC and kRT-PCR methods

^b Kappa coefficients are expressed as percentages

NPV, negative predictive value; PPV, positive predictive value

The third column ER/PgR represents hormone receptor status (any of the two receptors ER, PgR positive versus both ER and PgR negative)

follow up time was 8 years. During this time, 90 patients (33%) had developed a relapse and 62 patients (23%) had died. Malignant relapses occurred in 43% and deaths in 31% of the ER-mRNA negative cases versus 30% relapsed and 21% dead patients with ER-mRNA positive tumors. Similarly, malignant relapses were seen in 41% and deaths in 29% of patients harbouring MAP-Tau negative tumors

versus 23% relapses and 16% deaths among those with MAP-Tau positive carcinomas. In contrast, 33% of PgR-mRNA negative cases suffered a relapse and 21% died versus 33.5% relapsed and 24% dead patients with PgR-mRNA positive tumors.

Univariate Cox regression analysis, adjusted for treatment, showed a non-significant trend for decreased risk of death in patients with ER-mRNA positive tumors (hazard ratio (HR) = 0.62, 95% CI: 0.36–1.05, Wald- P = 0.077), as well as a trend towards a decreased risk of malignant relapse (HR = 0.65, 95% CI: 0.41–1.01, Wald- P = 0.06), in comparison to those affected by ER-mRNA negative breast carcinomas. In contrast, the status of tumoral PgR mRNA did not carry any predictive significance for either risk of death or malignant relapse. Patients with MAP-Tau mRNA positive tumors had a significantly decreased risk of death compared with those with MAP-Tau mRNA negative tumors (HR = 0.49, 95% CI: 0.29–0.83, Wald- P = 0.008) and a significantly decreased risk of disease relapse (HR = 0.50, 95% CI: 0.32–0.78, Wald- P = 0.002). Kaplan–Meier survival curves representing the probability of survival and disease-free survival as a function of MAP-Tau mRNA status are presented in Fig. 3 (log-rank test for DFS P = 0.002, for OS P = 0.006). However, neither MAP-Tau mRNA nor ER mRNA expression status were predictive for benefit from adjuvant treatment with paclitaxel (E-T-CMF arm) for either disease-free survival or overall survival (DFS and OS tests for interaction Wald P = 0.65 and P = 0.73 for MAP-Tau, P = 0.32 and P = 0.99 for ER, respectively). Restricting analysis to ER positive patients, MAP-Tau mRNA expression status remained non-predictive for benefit from adjuvant treatment with paclitaxel (DFS and OS tests for interaction Wald P = 0.50 and P = 0.88 for MAP-Tau among ER positive patients).

The impact of paclitaxel on OS and DFS in MAP-Tau negative and MAP-Tau positive patients are shown in Fig. 4a and b, respectively. Same analysis among ER positive patients is presented in Figs. 5a and 5b, respectively.

Multivariate Cox regression analysis (Table 4), including menopausal status, tumor grade, size, number of positive lymph nodes, treatment group, ER, PgR, and MAP-Tau mRNA expression levels, revealed that only MAP-Tau and number of involved axillary nodes remained significant independent predictors of OS, while MAP-Tau, number of positive nodes and tumor grade independently predicted DFS. More specifically, positive MAP-Tau was associated with a decreased risk of death (HR = 0.46, 95% CI: 0.25–0.85, Wald- P = 0.01). As expected, four or more positive nodes were associated with an increased risk of death (HR = 2.33, 95% CI: 1.05–5.16, Wald- P = 0.04). Regarding DFS, positive MAP-Tau mRNA status was associated with a decreased risk of relapse (HR = 0.53,

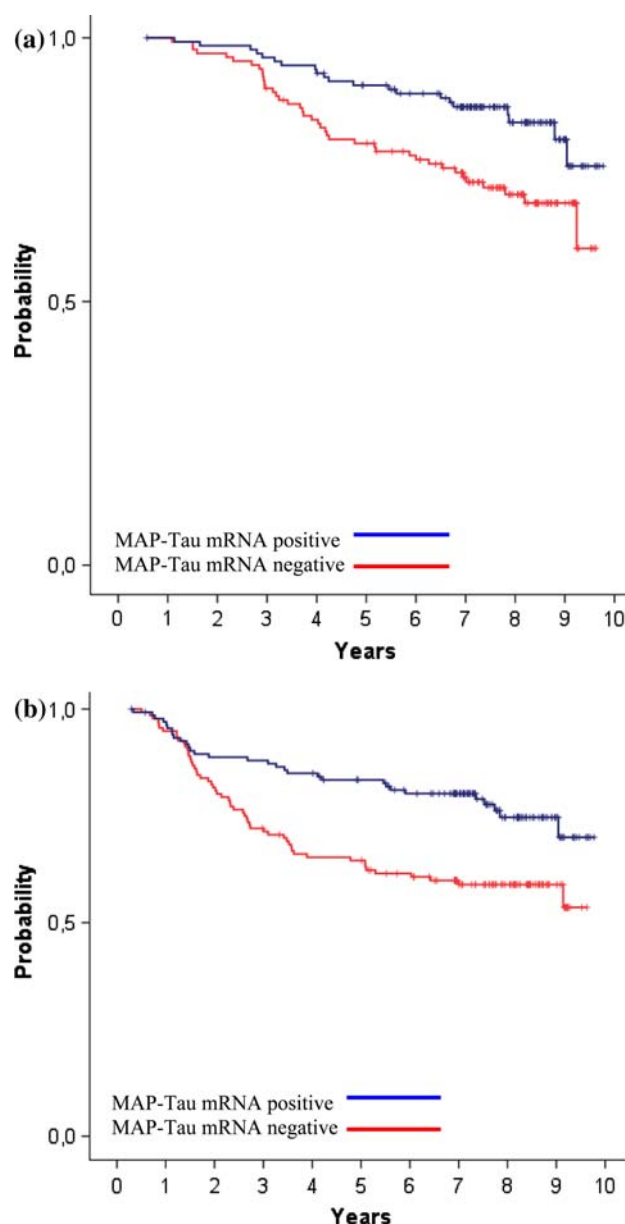
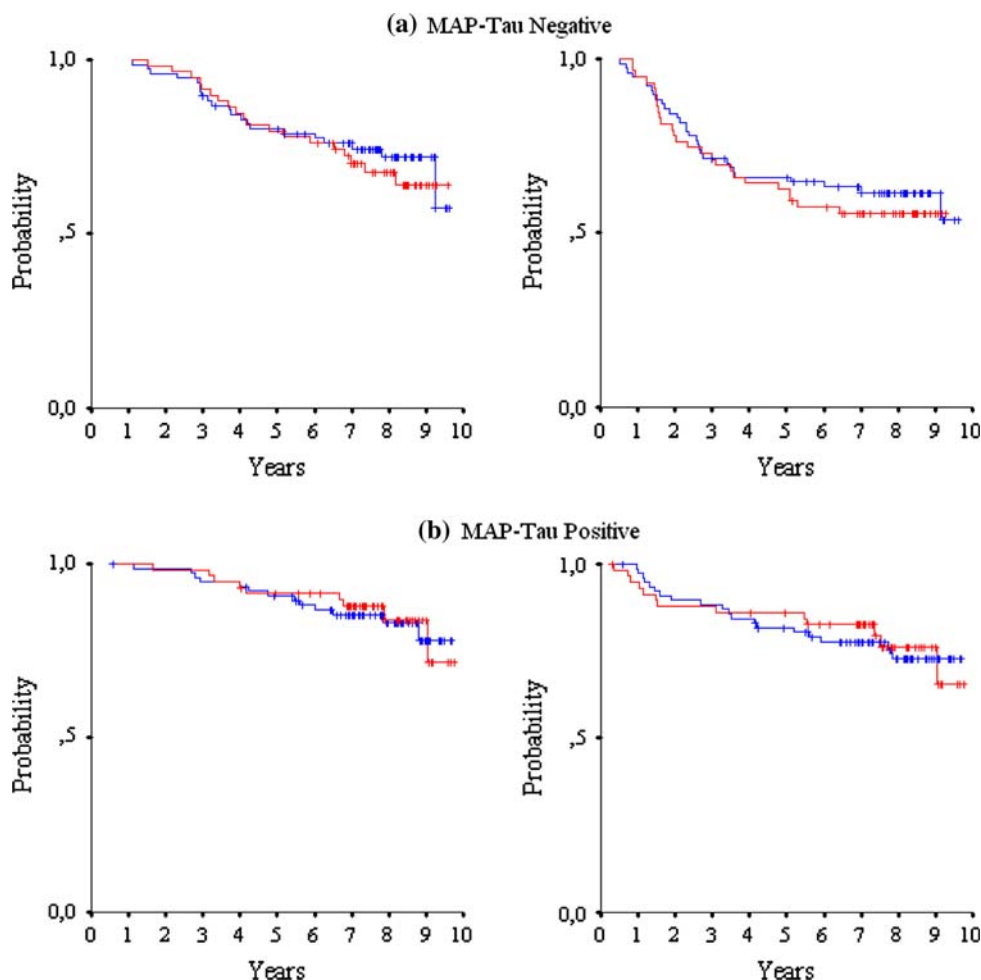


Fig. 3 Overall survival (OS) (a) (log rank P = 0.006) and DFS (b) (log rank P = 0.002) by MAP-Tau mRNA expression

95% CI: 0.32–0.89, Wald- P = 0.02), while four or more positive nodes (HR = 2.30, 95% CI: 1.21–4.38, Wald- P = 0.01) and higher tumor grade (HR = 1.56, 95% CI: 0.98–2.50, Wald- P = 0.06) significantly increased the risk of malignant recurrence. In both multivariate models described above, MAP-Tau mRNA status was not predictive for the benefit of adjuvant treatment with paclitaxel either for OS or for DFS (test for interaction: Wald- P = 0.99 and Wald- P = 0.92, respectively).

As one of the advantages of quantitative measurement of hormonal receptor mRNA expression is a continuous dynamic range of output, we sought to determine whether increasing levels of hormone receptor mRNA correlated

Fig. 4 OS (left) and DFS (right) for E-T-CMF (red line) and E-CMF (blue line) in MAP-Tau negative and MAP-Tau positive patients



with increasing benefit from adjuvant hormonal therapy in a “dose-dependent” manner. We tested this hypothesis in 203 patients harbouring breast carcinomas immunohistochemically positive for ER and/or PgR, who received adjuvant hormonal manipulations. Data were split into four equal groups, using the quartiles as cut off points.

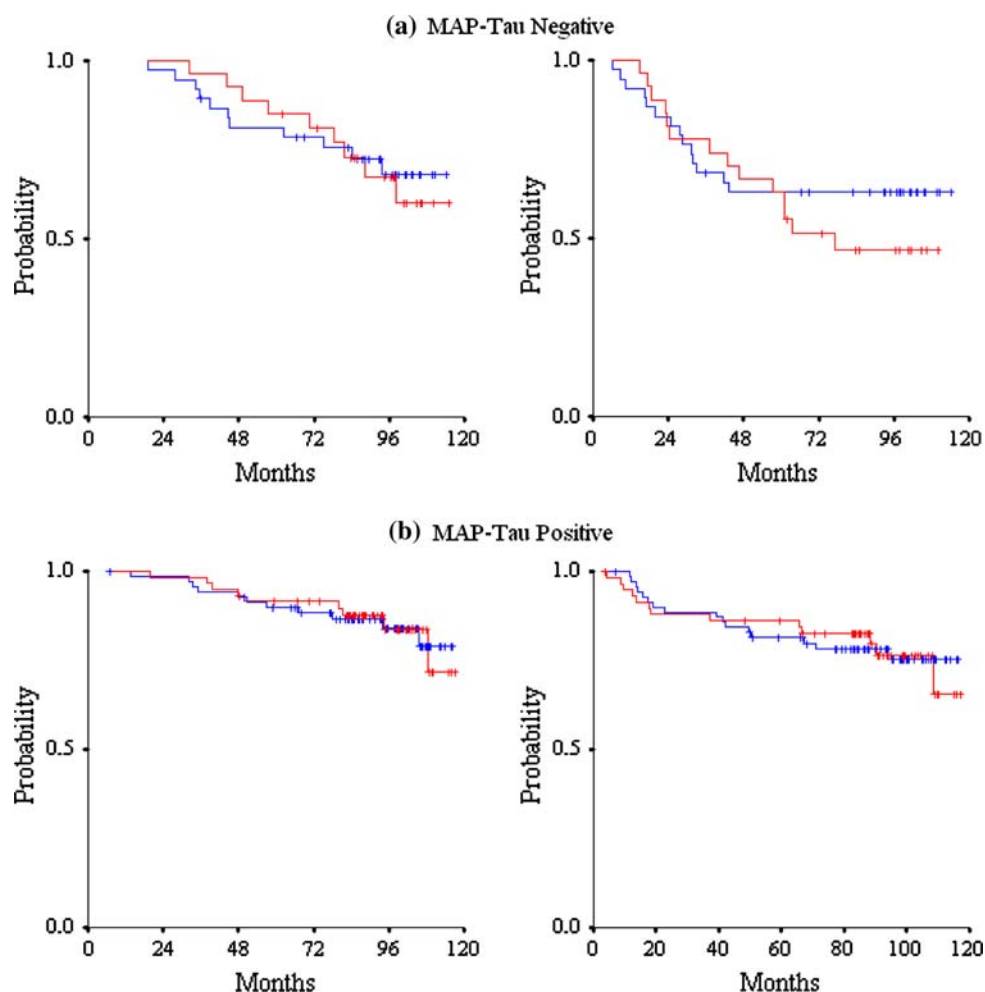
Using Cox regression analysis, we examined the association of very low (ER, PgR mRNA expression \leq first quartile), low (first quartile < ER, PgR mRNA expression \leq second quartile), moderate (second quartile < ER, PgR mRNA expression \leq third quartile), and high (ER, PgR mRNA expression > third quartile) mRNA levels of ER and PgR with disease-free survival. There was no evidence of association between increasing ER or PgR mRNA expression and benefit from hormonal therapy (Wald P values adjusted for treatment group: $P = 0.54$ for ER mRNA and $P = 0.505$ for PgR mRNA). In comparison to tumors with very low ER mRNA expression, the hazard ratios for malignant recurrence in tumors with low, moderate and high expression were 1.08 (95% CI: 0.51–2.31, Wald- $P = 0.835$), 1.61 (95% CI: 0.79–3.29, Wald- $P = 0.19$) and 1.12 (95% CI: 0.52–2.39, Wald- $P = 0.77$),

respectively. Similarly, in comparison to tumors with very low PgR mRNA expression, the hazard ratios for relapse in patients with low, moderate and high mRNA-expressing tumors were 1.06 (95% CI: 0.54–2.10, Wald- $P = 0.86$), 0.61 (95% CI: 0.28–1.32, Wald- $P = 0.21$) and 0.97 (95% CI: 0.48–1.97, Wald- $P = 0.93$), respectively.

Discussion

In our patient cohort, the concordance between immunohistochemical and kRT-PCR determination of ER and PgR status was good (77% for ER, 71% for PgR) but clearly inferior to rates of agreement reported by Badve et al. (88–91%), Bianchini et al. (94%) and Gong et al. (88–90%) [11–13]. After excluding chance agreement, as estimated by the Kappa coefficient, only fair to moderate agreement was found between IHC and RT-PCR determination of ER and PgR. However, in most studies the investigators actually intended to identify those kRT-PCR mRNA cut-off values that would provide the highest concordance with the IHC status of the target protein.

Fig. 5 OS (left) and DFS (right) for E-T-CMF (red line) and E-CMF (blue line) in MAP-Tau negative and MAP-Tau positive patients restricted to ER positive patients



On the contrary, we regarded IHC ER and PgR protein expression determination as the most widely used analytical assay, but not the gold standard, in view of its moderate predictive value for benefit from endocrine therapies, semi-quantitative nature of results and inter- or intra-laboratory reporting variability [2, 3, 8–10]. Using distributional studies of mRNA values and taking into account hormonal receptor protein staining as a gross template, we sought to study the predictive value and correlation of a more conservative threshold, the 25th percentile of mRNA values, in order not to miss an effect of low, but potentially biologically significant level of messenger RNA.

With the use of this threshold, among patients with hormone receptor mRNA-positive tumors, 17–25% lacked immunohistochemical protein staining. This observation could be the result of mRNA failing to translate to protein (alternate splicing, aberrant polyadenylation, microRNA-mediated or other form of translational silencing) or rapid catabolism of the hormone receptor protein. Protein degradation in the tumor block by inadequate fixation, problematic antigen retrieval, protein staining underestimation in old archival material, or finally, RNA

amplification of normal tissue along with the tumor could also be the culprits for absence of protein staining [9, 19–21]. Conversely, when tumoral hormone receptor mRNA was categorised as negative, immunohistochemical protein staining was evident in at least 1% of tumor cells in 37–39% of cases. This discrepancy could reflect the difficulty capturing short-lived, sensitive to degradation mRNA from FFPE tumor blocks, as well as the different fixation and turnover times of ribonucleic acids in relation to proteins [22, 23]. Accumulation of the protein due to aberrant catabolism may also be a factor.

Despite the moderate at best agreement between IHC and kRT-PCR, the ER mRNA status, according to the objective threshold values we set, did show a trend for predictive significance. Estrogen receptor protein expression was shown to be a weak prognostic factor for survival and a moderate predictive factor for benefit from hormonal manipulations in patients with early breast cancer [1]. Its prognostic information may be carried by a multi-gene signature, for which the ER gene itself is either a key regulator or simply the best-known surrogate marker. Pusztai et al. recently used DNA microarrays in order to

Table 4 Estimated hazard ratios (HRs) and 95% confidence intervals (CIs) for OS and DFS—multivariate analysis

	HR	95% CI	Wald <i>P</i> -value
Overall survival			
<i>Treatment group</i>			
E-T-CMF	1		
E-CMF	1.09	0.65–1.82	0.74
<i>Positive nodes</i>			
0–3	1		
≥4	2.33	1.05–5.16	0.04
<i>MAP-Tau mRNA levels</i>			
Negative	1		
Positive	0.46	0.25–0.85	0.01
<i>ER mRNA levels</i>			
Negative	1		
Positive	0.67	0.33–1.36	0.26
<i>PgR mRNA levels</i>			
Negative	1		
Positive	1.78	0.86–3.67	0.12
Disease-free survival			
<i>Treatment group</i>			
E-T-CMF	1		
E-CMF	1.15	0.74–1.78	0.53
<i>Positive nodes</i>			
0–3	1		
≥4	2.30	1.21–4.38	0.01
<i>Tumor grade</i>			
I–II	1		
III–Undifferentiated	1.56	0.98–2.50	0.06
<i>MAP-Tau mRNA levels</i>			
Negative	1		
Positive	0.53	0.32–0.89	0.02
<i>ER mRNA levels</i>			
Negative	1		
Positive	0.87	0.46–1.63	0.66
<i>PgR mRNA levels</i>			
Negative	1		
Positive	1.33	0.72–2.46	0.36

profile mRNA transcripts from snap-frozen breast carcinomas of 38 patients [24]. They reported that ER positive tumors, as assessed by IHC were strongly associated with transcriptional activity of 105 genes involved in regulation of transcription, cellular proliferation, differentiation, intracellular trafficking of proteins, and signal transduction, in contrast to ER negative tumors. Most of these genes are not known to be regulated by ER, suggesting that differences between IHC ER positive and negative tumors are not attributable to the presence or absence of ER function, but rather reflect different molecular phenotypes. These findings were supported by identification of other gene

signatures associated with the presence of ER (Paik et al., 22 genes and Symmans et al., 200 genes) [25, 26]. However, the validity of the above findings was challenged by the low number of tumor samples, the high number of assessed genes and technical variances implicating statistical problems (i.e. over-fitting).

In our study of patients with high-risk, predominantly node-positive early breast carcinomas, ER mRNA positive status had a favourable prognostic significance for decreased risk of relapse and death, which was of borderline statistical significance. Ninety two percent of the patients received some form of adjuvant hormonal therapy, including as many as 69% of ER mRNA negative patients. Moreover, all did receive chemotherapy. Accordingly, it is unclear whether the transcriptional activity of ER carries prognostic significance for outcome irrespective of the therapy administered, in which case it would reflect inherent biologic characteristics of the tumor, or whether it possesses predictive utility for benefit from antineoplastic or hormonal therapy.

We sought to exploit the availability of a dynamic range of continuous mRNA data to look for an association of increasing hormone receptor gene transcription with enhanced benefit from anti-estrogen therapy but found none. It is unlikely that sample size precluded identification of a modest “dose-response” effect, in view of the absence of any trend for association. Presence of hormone receptor mRNA levels above a certain biological threshold may well be irrelevant, if low levels ensure presence of an amount of protein adequate for biologic effects [27]. Alternatively, increasing concentrations of mRNA may not linearly translate to protein synthesis, since it is modulated by several other regulatory mechanisms [28]. Ultimately, ER and PgR may not be the sole or determining genes that define response or resistance to hormonal therapy.

We found that ER, PgR and MAP-Tau mRNA positive status correlated to low histological grade. In view of recent evidence suggesting that high-grade tumors are driven by a distinct genetic programme, rather than simply lacking some markers of differentiation, tumors with active ER, PgR or MAP-Tau gene function may represent a group with indolent characteristics in terms of proliferation, anaplasia, invasion, and cell cycle control [29]. The association of tumoral MAP-Tau gene transcription with premenopausal patient status, a marker of more aggressive tumor biology, may be a random one in a relatively small sample or may suggest that a subgroup of premenopausal women harbour breast carcinomas of more benign biology. Alternatively, this could be a result of high plasma estrogen levels in premenopausal patients, with increased expression of MAP-Tau in ER positive tumors of comparatively benign nature, compared to the more aggressive ER negative, potentially BRCA1 or BRCA2-positive, premenopausal breast cancer

tumors. In line with this, MAP-Tau gene transcription was strongly associated with ER and PgR gene transcription, an observation that confirms prior evidence that MAP-Tau is an ER-inducible gene. Tau contains an imperfect estrogen response element upstream of its promoter and is an estrogen-inducible protein in cultured neurons and in the MCF7, ZR75 and T47D breast cancer cell lines [30, 31]. Interestingly, MAP-Tau is the target of multiple signalling pathways and thereby may represent an important checkpoint for microtubule and cellular functions beyond its interplay with hormonal activities.

Microtubules are intrinsically dynamic polymers composed of $\alpha\beta$ -tubulin heterodimers and constitute essential components of the mitotic spindle and cytoskeleton. Microtubule dynamics control mitosis and cellular motility. Moreover, microtubules serve as scaffolds for signalling molecules, gene transcription and cell cycle activity [14, 32]. Several proteins bind to microtubules and either stabilize or destabilize their polymerisation. Microtubule-associated protein Tau, one of the most extensively studied, is found in neurons, breast epithelium and breast cancer. Preclinical experiments have shown that the phosphorylated form of MAP-Tau binds to tubulin, resulting in promotion and stabilisation of microtubules. MAP-Tau can compete with paclitaxel for binding to the inner surface pocket of microtubules, a finding that suggests that microtubules assembled in the presence of Tau may be less susceptible to taxane binding and pharmacological hyperpolymerisation [33]. Indeed, Rouzier et al. reported that MAP-Tau gene expression was inversely associated with pathologic complete response (pCR) of breast carcinomas to neoadjuvant paclitaxel-based chemotherapy. Among 82 patients treated with T-FAC induction regimen, pCR occurred in 44% of MAP-Tau mRNA-negative tumors, but only in 17% of MAP-Tau mRNA-positive ones ($P = 0.04$) [34].

We report here for the first time in the literature that positive MAP-Tau mRNA expression strongly predicts decreased risk of relapse and death in patients with high-risk early breast cancer, suggesting that it carries either prognostic information for patient outcome or predictive information for benefit from antineoplastic and/or hormonal therapy. In fact, the observed weak predictive utility of ER mRNA expression was lost in multivariate analysis, suggesting that it may be exerted via induction of MAP-Tau gene transcription. The critical regulatory properties of MAP-Tau for microtubule dynamics make it a potential key protein in controlling mitosis, migration and cell cycle activity, possibly resulting in its strong predictive significance for benefit from antineoplastic and/or hormonal therapy.

In contrast to the findings reported by Rouzier et al., MAP-Tau mRNA status did not predict clinical benefit from taxanes in our cohort, as it had the same predictive

utility in both E-CMF treated and E-T-CMF treated patients. Selection of a different cut-off was unlikely to change this finding and our exploratory analyses using different threshold values confirmed this. Andre et al. found high MAP-Tau gene transcriptional activity to be a bifunctional predictor of endocrine sensitivity and chemotherapy resistance [35]. As the majority of the E-T-CMF patients in our cohort received endocrine therapy after chemotherapy, it is possible that any predictive significance of low MAP-Tau mRNA expression for taxane benefit was cancelled out by resistance to hormonal therapy, resulting in no detectable differences in outcome. Moreover, in the entire HE10/97 trial, the addition of taxanes reduced the hazard of death only in ER-negative patients [15]. Given the correlation of ER and MAP-Tau gene activity, it is probable that ER negative patients who benefit from taxanes are mostly MAP-Tau negative. However, this effect may have been lost in our cohort, as the number of patients evaluated for MAP-Tau mRNA expression in the present study was reduced to less than half, due to limitations in tissue availability. Finally, it is always possible that MAP-Tau has no true predictive value for response to taxanes, or that other molecular characteristics abrogate it: tubulin mutations, variable expression of tubulin isoforms, expression of multidrug resistance proteins, or BCL2 may contribute to taxane resistance in tumors with low MAP-Tau expression. Indeed, Rody et al. performed gene expression profiling in breast carcinomas from 50 patients treated with taxane-based induction chemotherapy and failed to find any predictive significance of MAP-Tau gene expression for pCR [36].

To conclude, we showed the feasibility of obtaining quantitative mRNA expression data of ER, PgR and MAP-Tau from widely available FFPE breast tumor tissue, though the choice of biologically relevant threshold values needs insights from the fields of molecular and systems biology. The use of a dynamic range of continuous hormone receptor mRNA data has yet no established value in quantifying the benefit from endocrine manipulations, as mRNA levels are modulated by several other parameters before exerting biological effects. MAP-Tau is an ER-associated gene that carries a strong predictive significance for patient outcome, though its effects on sensitivity to chemotherapy and hormonal therapy are still unclear. Study of larger and more homogeneous populations of breast cancer patients is warranted in order to unravel these issues and exploit their potential predictive utility for devising tailored therapeutic strategies.

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