mRNA expression of specific HER ligands and their association with clinical outcome in patients with metastatic breast cancer treated with trastuzumab

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Abbreviations: ER, oestrogen receptors; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; MBC, metastatic breast cancer; PgR, progesterone receptors; R-MBC, relapsed metastatic breast cancer; RARA, retinoic acid receptor α; THRA, thyroid hormone receptor α; TTP, time to progression

Key words: metastatic breast cancer, trastuzumab, biomarkers, HER2, HER ligands

Abstract. Prognostic and predictive biomarkers are being studied for the diagnosis and treatment of breast cancer. The present study retrospectively assessed the mRNA expression of HER family receptor ligands and of other potential prognostic biomarkers and their association with time to progression (TTP), survival and clinicopathological characteristics in patients with metastatic breast cancer (MBC) treated with trastuzumab. A total of 145 tumour tissue samples were analysed. mRNA expression analysis of the transcripts of interest was performed and the association of these markers with selected clinicopathological parameters was examined. HER2 status was centrally re-evaluated. Only 67.6% of patients were truly HER2-positive according to the central HER2 re-evaluation. Heparin binding epidermal growth factor (EGF)-like growth factor, transforming growth factor β1 (TGFB1) and thyroid hormone receptor α (THRA) mRNA expression was higher in HER2-positive patients (P=0.026, P<0.001 and P<0.001). Insulin-like growth factor binding

protein 4 was correlated with retinoic acid receptor α, TGFB1 and THRA (rho=0.45, rho=0.60 and rho=0.45). In HER2-positive patients, high neuregulin 1 and high betacellulin were unfavourable factors for TTP [hazard ratio (HR) = 1.78, P=0.040 and HR=2.00, P=0.043, respectively]. In patients with *de novo* MBC, high EGF expression was associated with a non-significant prolongation of TTP (HR=0.52, P=0.080) and significantly longer survival (HR=0.40, P=0.020). The present study examined clinical and biological implications of specific genes and it was concluded that their expression has an impact on the outcome of trastuzumab-treated patients with MBC.

Introduction

Breast cancer is a disease characterised by molecular heterogeneity (1,2). Gene expression profiling has identified molecular subtypes of breast cancer with differences in prognosis and therapeutic options. In particular, the HER2-enriched subtype is characterised by upregulation of the HER2 gene (3), while the HER2 gene is amplified in 15-20% of all breast carcinomas (4).

The HER2/neu protein is a component of a four-member family of closely related growth factor receptors, including EGFR or HER1 (ERBB1), HER2 (ERBB2), HER3 (ERBB3) and HER4 (ERBB4). HER receptors exist as monodimers, and they can form homodimers or heterodimers when they bind to a ligand. Numerous ligands are associated with HER1, HER3 and HER4, while HER2 is characterised as an 'orphan' receptor, since there is no known ligand that can promote homodimers of HER2. In terms of the HER ligands, epidermal growth factor (EGF), transforming growth factor α, the heparin binding EGF-like growth factor (HBEGF), betacellulin (BTC), amphiregulin (AREG) and epiregulin (EREG) bind to the HER1 receptor (5). Neuregulin 1 (NRG1) and neuregulin 2 bind to HER3, while HBEGF, BTC, EREG and neuregulins 1, 2, 3 and 4 bind to HER4 (6,7).

The importance of HER2 as a prognostic or predictive marker in invasive breast cancer is well recognised and, therefore, HER2 status should be determined in all cases of invasive (early stage or recurrent) breast cancer (8,9). HER2 testing includes the evaluation of HER2 protein upregulation assessed by immunohistochemistry (IHC) and HER2 gene amplification assessed by *in situ* hybridisation, fluorescence *in situ* hybridisation (FISH), chromogenic *in situ* hybridisation, silver-enhanced *in situ* hybridisation or quantitative PCR (10). Although mRNA expression profiling has been used to classify breast tumours into molecular subtypes, assessment of oestrogen receptors (ER)/progesterone receptors (PgR)/HER2 status via IHC is currently the standard in clinical practice for the selection of patients that are more likely to respond to hormone or anti-HER2 treatments, according to international guidelines (11-13).

Previous work has demonstrated that ERBB/HER ligands, including AREG, BTC, EREG, EGF, HBEGF, NRG1 and transforming growth factor α (TGFA), are co-expressed at the mRNA level in breast cancer in various combinations alongside their receptors, whereas associations have also been established among the mRNA levels of the aforementioned ligands and the four HER receptors (14,15). Furthermore, the mRNA expression patterns of EGF, AREG, TGFA and HBEGF have been linked to clinicopathological parameters, including tumour size and histoprognostic grading (14). However, in the case of EGF, an association with improved prognosis has also been observed for overall survival and relapse-free survival, at least in univariate analyses (14). Specific ERBB/HER ligands, have been linked to treatment response with anti-HER2 therapeutic agents, including trastuzumab, as shown by preclinical or clinical studies (16-18).

Although most HER2-positive patients derive benefit from trastuzumab and other approved anti-HER2 targeted therapies, resistance will eventually develop and cause disease progression (19). Furthermore, there is a subset of HER2-positive patients that will not respond to trastuzumab due to primary resistance (18). Therefore, it is crucial to identify biomarkers that will allow for the categorisation of patients most likely to either respond to or develop primary and secondary resistance to trastuzumab.

Apart from the HER ligands, the mRNA levels of other molecules, including insulin-like growth factor binding protein 4 (IGFBP4), a member of the family of proteins binding to insulin-like growth factors, transforming growth factor β1 (TGFB1), a TGFβ signalling component, the thyroid hormone receptor α (THRA), and the retinoic acid receptor α (RARA), have been studied in breast cancer for their prognostic significance (20-25). The present study retrospectively examined the mRNA expression of several HER ligands and other potential prognostic biomarkers of interest, including IGFBP4, TGFB1, THRA and RARA, in patients with metastatic breast cancer (MBC) who were treated with trastuzumab. Their relationship with other tumour and pathological characteristics, as well as ER/PgR/HER2 status, assessed centrally by IHC, was evaluated and their prognostic role in terms of progression and survival in this subset of patients was explored.

Materials and methods

Patients and tissue processing. The present study was conducted on a previously reported group of patients (26) that was enriched with additional cases to achieve a total study population of 145 cases (all female). The eligibility criteria were as follows: i) Treatment with trastuzumab for histologically confirmed MBC; ii) adequate clinical data on patient history, demographics, tumour characteristics, treatment details (i.e., drug dosages, schedule of administration and serious toxicities) and clinical outcome; and iii) adequate tumour tissue available for biological marker evaluation.

Formalin-fixed paraffin-embedded tumour tissue samples were retrospectively collected from patients treated with trastuzumab-based regimens in the metastatic setting, as previously described in detail (26-28). All tumours were characterised by local pathologists as HER2-positive based on American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria current at that time (29). Consequently, all patients received trastuzumab as part of their treatment. All tumour samples were centrally re-evaluated by IHC for oestrogen receptors (ER), progesterone receptors (PgR), HER2 and the expression of the proliferation marker Ki67. Additionally, HER2 amplification status was assessed using the FISH method, as later described.

The translational research protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (Protocol #4283; January 14, 2008; Thessaloniki, Greece) under the general title 'Investigation of major mechanisms of resistance to treatment with trastuzumab in patients with metastatic breast cancer'. All patients included in the study in 2005 and later provided written informed consent for the provision of biological material for future research studies before receiving any treatment. A waiver of consent was obtained from the Bioethics Committee for patients included in the study before 2005.

Tissue microarrays (TMAs). Representative haematoxylin-eosin-stained 2-μm sections from the tissue blocks were reviewed by a pathologist. A total of 17 TMA blocks were constructed from the 145 eligible cases using a manual tissue microarrayer (Beecher Instruments), as previously described (26). For the construction of the TMA blocks, two core samples (1.5 mm in diameter) were obtained from representative regions of each tumour in the donor blocks. All IHC and FISH markers were assessed on the TMA sections. The cases not represented, which had damaged or inadequate cores on the TMA sections, were re-cut from the original blocks if still available. These sections were used for protein and gene analyses as previously described (26-28,30). Further method details are provided in Data S1.

Immunohistochemistry (IHC)-tumor infiltrating lymphocytes (TILs). Serial 2.5-μm-thick TMA sections or whole tissue sections were stained for ER (clone 6F11; cat. no. NCL-L-ER-6F11; Leica Microsystems, Ltd.), PgR (clone 1A6; cat. no. NCL-L-PGR; Leica Microsystems, Ltd.), HER2 (polyclonal Ab; cat. no. A0485; Dako; Agilent Technologies, Inc.), Ki67 (clone MIB-1; cat. no. M7240; Dako; Agilent Technologies, Inc.), phosphorylated mTOR at serine 2448 (p-mTOR; clone 49F9; cat. no. 2976; Cell Signaling Technology, Inc.; dilution, 1:30; 20 min) and stPTEN (clone 6H2.1; cat. no. M3627; Dako; Cell Signaling Technology, Inc.; dilution, 1:200; 30 min), using the Bond Max™ autostainer (Leica Microsystems, Ltd.) as previously described in detail (31) (Table SI). All sections were stained in one run for each antibody and were evaluated by pathologists experienced in breast cancer and blinded to the patient's clinical and survival data. Positive controls were used for all antibodies from known positive breast cancer cases, while negative controls were obtained by omitting the primary antibody as previously described (26-28,30).

All IHC stains were evaluated according to the formerly outlined interpretation (32). Stromal TIL density was assessed on whole H&E sections according to the guidelines from the International TILs Working Group (33) and was analysed as a continuous variable. HER2 protein expression was scored according to the 2007 ASCO/CAP guidelines (29) (scores between 0 and 3+). A positive for HER2 protein expression (IHC 3+) was defined as uniform intense membrane staining of >30% of invasive tumour cells. Further method details are provided in Data S1.

Fluorescence in situ hybridisation (FISH). TMA sections or whole tissue sections (5-μm-thick) were used for FISH analysis using the ZytoLight® SPEC HER2/TOP2A/CEN17 triple colour probe kit for HER2 (code Z-2093; ZytoVision GmbH). FISH was performed according to the manufacturer's protocol with minor modifications (pepsin solution was applied to the specimens and incubated for 11-12 min at 37˚C in a humidified chamber) in all cases (i.e., not only the HER2 IHC 2+ cases). Digital images were constructed using software specifically developed for cytogenetics (XCyto-Gen, ver 1.2.11; Alphelys) and evaluated as previously described (26,31). For the assessment of HER2 status, the 2007 ASCO/CAP guidelines (29) were used with the addition of the ≥6-HER2-copies criterion (34), as patients had locally received trastuzumab according to this classification. HER2 status was considered positive if HER2 was amplified by FISH and/or a HER2 score of 3+ was obtained by IHC. Representative IHC and FISH images from invasive breast carcinoma cases are presented in Fig. 1. Further method details are provided in Data S1.

Dual nucleic acid extraction and mRNA expression analysis. Simultaneous isolation of DNA and RNA from whole or macrodissected 10-μm paraffin sections, the latter in cases with <50% tumour cell content, was performed for the 145 examined tumours with iron oxide beads coated with a nanolayer of silica using the VERSANT Tissue Preparation Reagents kit (Siemens Healthcare Diagnostics) as previously described (35). The nucleic acid extract of each sample was divided into two aliquots. DNase I was then added to one aliquot in order to remove DNA and ensure the presence of pure RNA for downstream mRNA analyses. cDNA synthesis was performed with random primers and SuperScript III Reverse Transcriptase (catalogue number 48190011 and 18080044; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNAs were assessed in duplicate with quantitative PCR using an ABI7900HT system under default thermal cycling conditions as follows: 50˚C for 2 min, 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 sec and 60˚C for 1 min, respectively.

mRNA expression analysis was performed with pre-made exon-spanning TaqMan-MGB assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) targeting the following gene transcripts (data in parentheses refer to assay ID; exon boundary; amplicon length): AREG Hs00950669_m1; 3-4; 66 bp), BTC Hs01101204_m1; 4-5, 5-6 ; 139 bp), EGF Hs01099999_m1; 19-20, 20-21: 70 bp), EREG ID Hs00914313_m1; 3-4; 65 bp), HBEGF Hs00181813_m1; 3-4; 78 bp), IGFBP4 Hs00181767_m1; 1-2: 91 bp), NRG1 Hs00247620_m1; 2-3: 93 bp), RARA Hs00940446_m1; 4-5, 5-6, 6-7: 68 bp), TGFA Hs00608187_m1; 4-5: 70 bp), TGFB1 Hs00171257_m1; 1-2: 63 bp), THRA Hs00268470_m1; 4-5: 89 bp) (Table I). A TaqMan-MGB expression assay targeting the glucuronidase β (GUSB) gene (Hs99999908_m1; 9-10, 10-11, 11-12; 81 bp) was used as the endogenous reference for relative quantification. The commercially available TaqMan Control Total RNA (cat. no. 4307281; Applied Biosystems; Thermo Fisher Scientific, Inc.) was applied as a positive control for interrun evaluation of the PCR assay efficiency, together with no-template controls. To obtain linear relative quantification (RQ) values, relative expression was assessed as 40-ΔCq, whereby ΔCq was calculated as (average target Cq) - (average GUSB Cq) from all eligible measurements, as previously described (35). Only samples

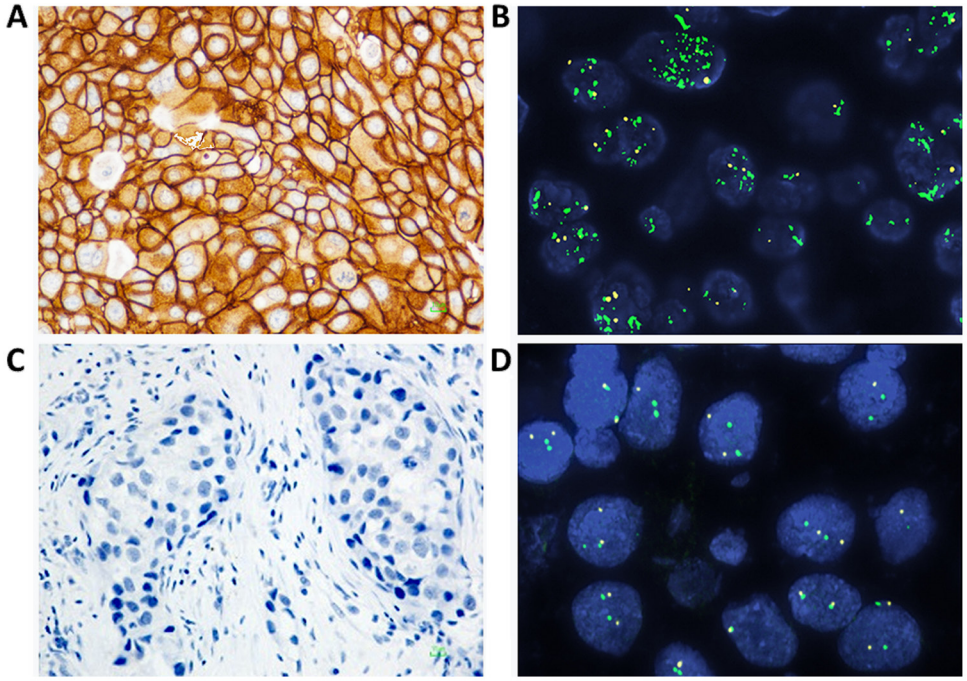

Figure 1. Representative IHC and FISH images from IBC cases. (A and B) In the first two panels an IBC case with (A) HER2 upregulation and (B) gene amplification is shown. (C and D) IBC HER2-negative case stained by (C) IHC and (D) FISH. HER2/TOP2A/CEP17 triple-colour probe: HER2 gene probe, green; CEP17 probe, yellow. The TOP2A gene probe (red) was excluded in the images using the XCytoGen software for cytogenetics. Magnification, x1,000. CEP17, centromeric region of chromosome 17; FISH, fluorescence *in situ* hybridization; IBC, invasive breast carcinoma; IHC, immunohistochemistry; TOP2A, DNA topoisomerase II α.

with average GUSB Cq values <36 and a ΔRQ value for each duplicate sample pair (intrarun variation) of <1 were considered eligible for further analysis in the present study.

Statistical analysis. Frequencies with the corresponding percentages and medians with range were used to summarize categorical and continuous variables, respectively. Comparisons of categorical data were performed using the χ^2 or Fisher's exact test (if more appropriate), while the non-parametric Wilcoxon rank-sum test was applied for the comparison of categorical with continuous variables. Associations of the markers of interest were assessed using Spearman's correlations.

The median value of the mRNA expression of the examined markers was used as the optimal cut-off to classify tumours into high- and low-expression groups and assess their prognostic significance and the associations with several clinicopathological characteristics (including age, menopausal status, histological grade, TIL density, PTEN expression and bone metastasis). Additionally, we evaluated the upper and lower quartiles of the mRNA distribution as potential thresholds. Time to progression (TTP) was defined as the time interval between the initiation of trastuzumab-based treatment for advanced disease (with or without parallel administration of chemotherapy or hormonal therapy) and the first documented disease progression. Non-progressors were censored at the date of last follow-up. Survival was also estimated from the initiation of trastuzumab-based therapy until death (from any cause) or last follow-up. Time-to-event distributions were estimated with the Kaplan-Meier method and comparisons of groups were performed with the log-rank test. Cox proportional hazard regression models (univariate and multivariate) were applied to estimate the effect of the examined markers on TTP and survival.

The TTP and survival analyses were conducted separately in the subgroup of HER2-positive and HER2-negative patients (as defined by HER2 central assessment), while the interactions with the ER/PgR status among patients with HER2-positive tumours and with the disease presentation status in the entire study population were also assessed for all examined markers with respect to TTP and survival to detect whether the effect of the marker expression on patients' outcome varied between the subgroups defined by ER/PgR (positive vs. negative) and disease presentation status [relapsed MBC (R-MBC) vs. *de novo* MBC].

Model choice was performed in multivariate analyses using the following variables in the first step: Menopausal status, performance status, mTOR protein expression, PTEN protein expression and each one of the markers that showed significance in univariate analysis. The final model was selected using backward selection criteria with P<0.10. All tests were two-sided and significance was set at 5%. The SAS software (version 9.3; SAS Institute, Inc.) was used for the statistical analyses.

Results

Patient characteristics and trastuzumab exposure. A total of 145 patients with at least one measurement in the markers of interest, including HER family receptor ligands and other potential prognostic biomarkers, were included in the present study. Among them, 109 (75.2%) had available mRNA data

Table I. Premade TaqMan-MGB assays for mRNA expression analysis.

Gene symbol	Assay ID, Hs	Size, bp	Exons	Gene name	Genbank ref
AREG	Hs00950669_m1	66	3-4	Amphiregulin	NM_001657.3
BTC	Hs01101204_m1	139	4-5, 5-6	Betacellulin	NM_001316963.1, NM_001729.3
EGF	Hs01099999_m1	70	19-20, 19-20, 20-21	Epidermal growth factor	NM_001178130.2, NM_001178131.2, NM_001963.5
EREG	Hs00914313_m1	65	3-4	Epiregulin	NM_001432.2
HBEGF	Hs00181813_m1	78	3-4	Heparin binding EGF like growth factor	NM_001945.2
IGFBP4	Hs00181767_m1	91	1-2	Insulin like growth factor binding protein 4	NM_001552.2
NRG1	Hs00247620_m1	93	2-3	Neuregulin 1	NM_001159995.2, NM_001159999.2, NM_001160001.2, NM_001160002.1, NM_001160004.2, NM_001160005.1, NM_001160007.1, NM_001160008.1, NM_013956.4, NM_013957.4, NM_013958.3, NM_013960.4, NM_013962.2, NM_013964.4, NM_004495.3
RARA	Hs00940446_m1	68	4-5, 5-6, 6-7, 6-7	Retinoic acid receptor α	NM_001145302.2, NM_001024809.3, NM_001145301.2, NM_000964.3
TGFA	Hs00608187_m1	70	4-5	Transforming growth factor α	NM_001099691.1, NM_001308158.1, NM_001308159.1
TGFB1	Hs00171257_m1	63	1-2	Transforming growth factor β1	NM_000660.5
THRA	Hs00268470_m1	89	4-5	Thyroid hormone receptor α	NM_001190918.1, NM_001190919.1, NM_003250.5, NM_199334.3
GUSB	Hs99999908_m1	81	9-10, 9-10, 10-11, 11-12	Glucuronidase β	NM_001284290.1, NM_001293105.1, NM_001293104.1, NM_000181.3

for all 11 markers. Although all patients were found to be HER2-positive when assessed at the local institutions and, therefore, were treated with trastuzumab, only 98 patients (67.6%) were classified as HER2-positive by the central re-evaluation of HER2 status. Therefore, 47 patients (32.4%) were treated with trastuzumab despite being HER2-negative (Fig. 2).

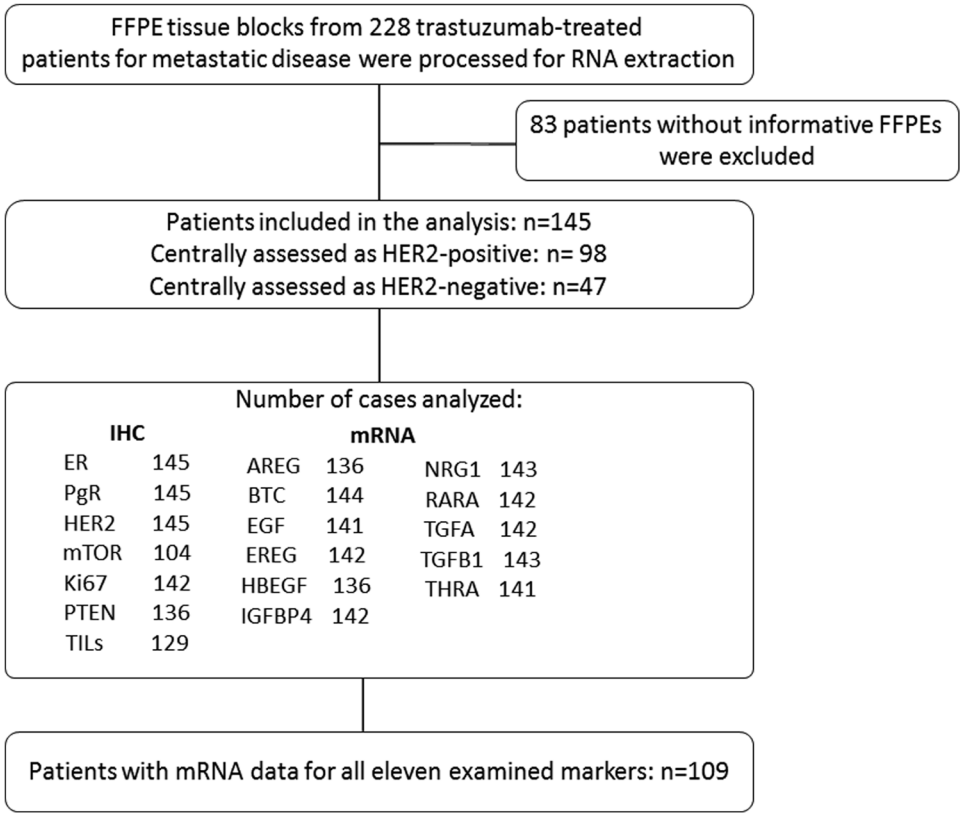

Figure 2. Reporting recommendations for tumour marker prognostic studies (REMARK) diagram. AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; ER, oestrogen receptors; EREG, epiregulin; FFPE, formalin-fixed paraffin-embedded; HBEGF, heparin Binding EGF like growth factor; IGFBP4, insulin-like growth factor binding protein 4; IHC, immunohistochemistry; NRG1, neuregulin 1; PgR, progesterone receptors; RARA, retinoic acid receptor α; TGFA, transforming growth factor α; TGFB1, transforming growth factor β1; THRA, thyroid hormone receptor α; TILs, tumour-infiltrating lymphocytes.

Selected patient and tumour characteristics for the entire study population and by HER2 status, as defined by the central assessment, are shown in Table II. A total of 69 patients (47.6%) had stage IV disease at the time of diagnosis (*de novo* MBC), while 52.4% had R-MBC. Most women were of postmenopausal status at the time of trastuzumab initiation (72.4%), while the majority of the patients with R-MBC had received adjuvant chemotherapy (85.5%). The median age at the initiation of trastuzumab-based therapy was 56 years (range, 29-86 years).

In total, 132 patients (91.0%) received trastuzumab as a first-line treatment, while 9 patients (6.2%) were treated with trastuzumab as a second-line treatment. The remaining patients received trastuzumab as third- to seventh-line therapy. In 130 patients (89.7%), trastuzumab was administered with chemotherapy, whereas 13 patients received hormonal therapy at the time of trastuzumab initiation, and 2 patients received trastuzumab as monotherapy. In addition, 6 patients (4.1%) had been previously treated with a trastuzumab regimen in the adjuvant and/or neoadjuvant setting.

Marker distribution by HER2 status. The distribution of the markers of interest based on the normalised expression of mRNA-encoding genes is presented in Fig. S1. The distribution of all examined markers by HER2 status (based on central assessment) is presented in Table SII, while Table SIII presents the distribution of markers by ER/PgR status among HER2-positive patients. Table SIV shows the distribution of markers by disease presentation status for the entire study population. HER2-positive patients presented with higher HBEGF, TGFB1 and THRA mRNA expression compared with patients with HER2-negative tumours (P=0.026, P<0.001 and P<0.001, respectively; Table SII), while IGFBP4 mRNA expression was higher in HER2-positive patients with positive ER/PgR status compared with HER2-positive patients with ER/PgR-negative tumours (P=0.004; Table SII). No significant differences were observed in the distribution of the markers of interest between patients with *de novo* MBC and R-MBC (Table SIV).

Correlations among HER family receptor ligands. IGFBP4 was positively strongly correlated with RARA (rho=0.45; P<0.001), TGFB1 (rho=0.60; P<0.001) and THRA (rho=0.45; P<0.001). In addition, RARA was strongly and positively correlated with THRA (rho=0.52; P<0.001). A strong correlation was also detected between TGFB1 and THRA (rho=0.51; P<0.001) and between BTC and EREG (rho=0.47; P<0.001; Fig. 3).

Association of HER family ligand receptors with clinicopathological characteristics. Patients carrying tumours with high AREG mRNA expression (using the median value as a cut-off point) were of younger age at the time of trastuzumab initiation and were more frequently premenopausal compared

Table II. Selected patient and tumour characteristics in groups of patients divided by HER2 status.

Characteristics	HER2 status (by central assessment)		
	Total (n=145)	Negative (n=47)	Positive (n=98)
Median age, years (range)[a]	56 (29-86)	58 (33-76)	54 (29-86)
De novo MBC, n (%)	69 (47.6)	18 (38.3)	51 (52.0)
R-MBC, n (%)	76 (52.4)	29 (61.7)	47 (48.0)
History of adjuvant CT, n (%)[b]	65 (85.5)	26 (89.7)	39 (83.0)
Anthracycline-based adjuvant CT, n (%)[b]	52 (68.4)	20 (69.0)	32 (68.1)
Taxane-containing CT, n (%)[b]	33 (43.4)	9 (31.0)	24 (51.1)
CMF-based CT, n (%)[b]	32 (42.1)	15 (51.7)	17 (36.2)
History of adjuvant HT, n (%)[b]	46 (60.5)	20 (69.0)	26 (55.3)
History of adjuvant RT, n (%)[b]	43 (56.6)	16 (55.2)	27 (57.4)
Histological grade, n (%)			
I-II	50 (34.5)	19 (40.4)	31 (31.6)
III	88 (60.7)	25 (53.2)	63 (64.3)
Unknown	7 (4.8)	3 (6.4)	4 (4.1)
Menopausal status, n (%)[a]			
Premenopausal	39 (26.9)	13 (27.7)	26 (26.5)
Postmenopausal	105 (72.4)	34 (72.3)	71 (72.4)
Unknown	1 (0.7)	0 (0.0)	1 (1.0)
N of trastuzumab lines, n (%)			
1	59 (40.7)	22 (46.8)	37(37.8)
2	32 (22.1)	10 (21.3)	22 (22.4)
3	24 (16.6)	6 (12.8)	18 (18.4)
≥4	30 (20.7)	9 (19.1)	21 (21.4)
Performance status, n (%)[a]			
0-1	137 (94.5)	41 (87.2)	96 (98.0)
2-3	5 (3.4)	4 (8.5)	1 (1.0)
Unknown	3 (2.1)	2 (4.3)	1 (1.0)
Subtype classification, n (%)			
Luminal A	7 (4.8)	7 (14.9)	0 (0.0)
Luminal B	30 (20.7)	30 (63.8)	0 (0.0)
Luminal-HER2	56 (38.6)	0 (0.0)	56 (57.1)
HER2-enriched	42 (29.0)	0 (0.0)	42 (42.9)
TNBC	8 (5.5)	8 (17.0)	0 (0.0)
Unknown	2 (1.4)	2 (4.3)	0 (0.0)
N of metastatic sites, n (%)[a]			
1-3	126 (86.9)	37 (78.7)	89 (90.8)
≥4	16 (11.0)	8 (17.0)	8 (8.2)
Unknown	3 (2.1)	2 (4.3)	1 (1.0)
Sites of metastasis, n (%)[a]			
Locoregional	44 (30.3)	17 (36.2)	27 (27.6)
Distant	127 (87.6)	41 (87.2)	86 (87.8)
Only locoregional	8 (5.5)	3 (6.4)	5 (5.1)
Only distant	90 (62.1)	27 (27.6)	63 (64.3)
Bones	60 (41.4)	21 (44.7)	39 (39.8)
Nodes	28 (19.3)	10 (21.3)	18 (18.4)
Visceral metastases	98 (67.6)	29 (61.7)	69 (70.4)

[a]At initiation of trastuzumab treatment; bOnly for patients with relapsed metastatic breast cancer. CMF, cyclophosphamide/methotrexate/5 fluorouracil; CT, chemotherapy; HT, hormonal therapy; MBC, metastatic breast cancer; R-MBC, relapsed metastatic breast cancer; RT, radiotherapy; TNBC, triple-negative breast cancer.

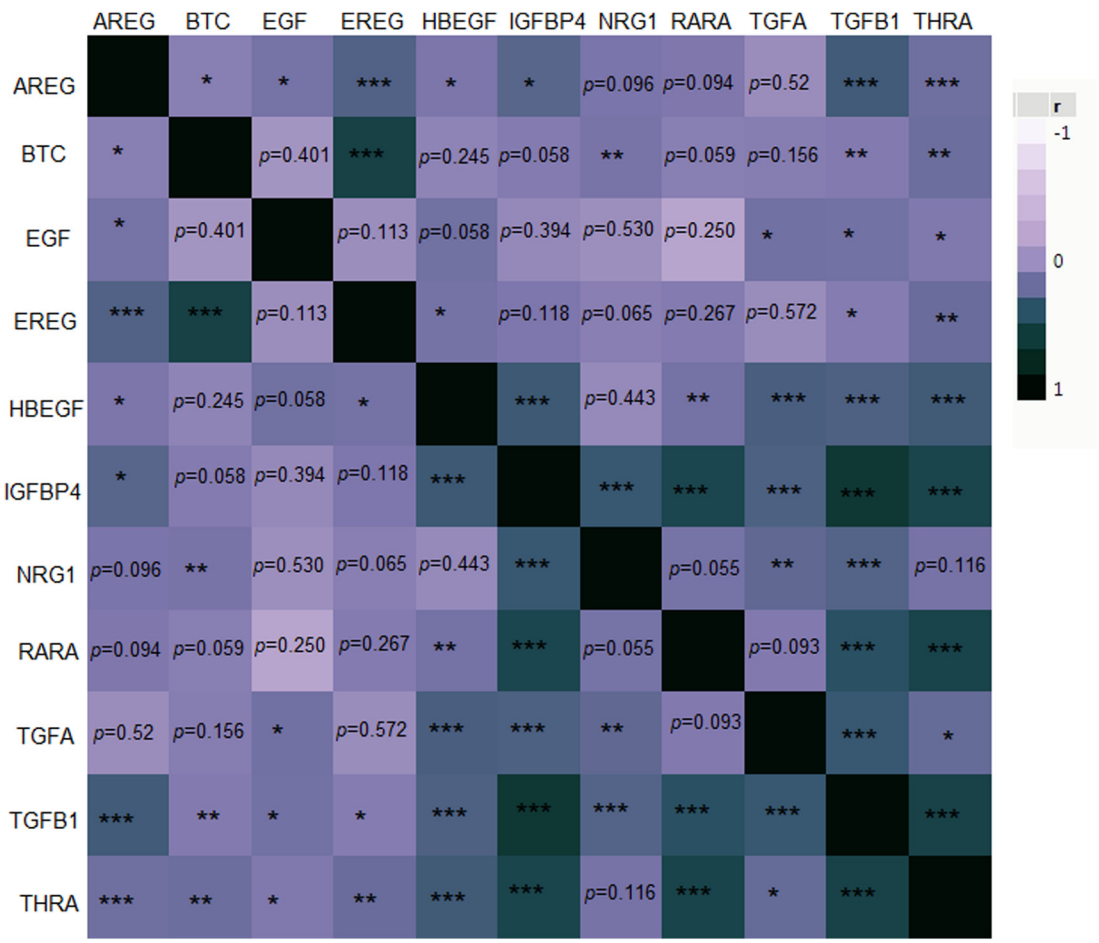

Figure 3. Heatmap matrix plot showing Spearman's correlation coefficients (rho) among the examined markers in the entire cohort. The value of r ranges between -1 (light purple) and 1 (dark green) as explained in the legend corresponding to negative or positive correlations between the markers. *P<0.050, **P<0.010 and ***P<0.001, respectively. Non-significant P-values are stated explicitly. AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EREG, epiregulin; HBEGF, heparin Binding EGF like growth factor; IGFBP4, insulin-like growth factor binding protein 4; NRG1, neuregulin 1; r, Spearman's correlation coefficient; RARA, retinoic acid receptor α; TGFA, transforming growth factor α; TGFB1, transforming growth factor β1; THRA, thyroid hormone receptor α.

with those with low AREG mRNA expression (P=0.002 and P=0.002, respectively). Younger age was also associated with high NRG1 mRNA expression (P=0.030), while lower histological grade was associated with high IGFBP4 mRNA expression (P=0.008). Patients with high mRNA expression levels of RARA, as compared with those with low expression (using the median value as a cut-off point), more frequently exhibited bone metastasis (P=0.012). Higher TIL density was observed in tumours with high TGFA mRNA expression (using the median value as a cut-off point), while PTEN loss was associated with low mRNA expression levels of TGFA and THRA, using the median values as cut-off points (P=0.043, P=0.045 and P=0.003, respectively; Table SV).

Association of markers with patient outcome. The median follow-up time for HER2-positive and HER2-negative patients was 120.3 and 114.2 months, respectively. During this time, 108 patients (74.5%) died (72 HER2-positive, 73.5%; 36 HER2-negative, 76.6%), while 113 (77.9%) experienced disease progression (76 HER2-positive, 77.6%; 37 HER2-negative, 78.7%). The median TTP was 15.1 months (95% CI, 12.6-19.6) and 11.6 months (95% CI, 7.1-17.6) for HER2-positive and HER2-negative patients, respectively. The median survival was 48.5 months (95% CI, 39.2-59.8) for HER2-positive patients and 38.1 months (95% CI, 25.8-49.1) for HER2-negative patients, while no significant differences were observed between HER2-positive and HER2-negative patients in terms of TTP or survival (P=0.33 and P=0.26, respectively).

High NRG1 mRNA expression (using the median value as a cut-off) and high BTC mRNA expression (using the upper quartile as a cut-off) was associated with an increased risk of progression in the HER2-positive population (Table III; Fig. 4). In the HER2-negative population, high EREG mRNA expression (using the median value as a cut-off) was univariately associated with a decreased risk of progression (hazard ratio = 0.45; 95% CI, 0.23-0.90; P=0.025). However, this was not retained upon the adjustment for clinicopathological parameters (P=0.12).

A significant interaction was observed between the disease presentation status and EGF mRNA expression (using the median value as a cut-off) for TTP (interaction P=0.037). High EGF mRNA expression was associated with a decreased risk of progression among patients with *de novo* MBC (Table III), while the hazard ratio was of the opposite direction in the subgroup of R-MBC women, even though significance was

Table III. Hazard ratios and 95% confidence intervals estimated by univariate and multivariate Cox regression analyses for TTP and survival.

	Univariate			Multivariate[a]		
Parameter, endpoint	HR	95% CI	P-value	HR	95% CI	P-value
TTP						
HER2-positive patients[b]						
NRG1 mRNA expression (median as high vs. low)	1.63	1.03-2.57	0.035	1.78	1.03-3.09	0.040
BTC mRNA expression (upper quartile as cut-off value; high vs. low)	1.90	1.12-3.24	0.018	2.00	1.02-3.93	0.043
Patients with *de novo* MBC						
EGF mRNA expression (median value as cut-off; high vs. low)	0.55	0.32-0.94	0.029	0.52	0.25-1.08	0.080
Survival						
Patients with *de novo* MBC						
EGF mRNA expression (lower quartile as cut-off; high vs. low)	0.46	0.24-0.87	0.017	0.40	0.19-0.87	0.020

[a]Results of backwards selection models. [b]According to HER2 central re-evaluation. BTC, betacellulin; CI, confidence interval; EGF, epidermal growth factor; HR, hazard ratio; MBC, metastatic breast cancer; NRG1, neuregulin 1; TTP, time to progression.

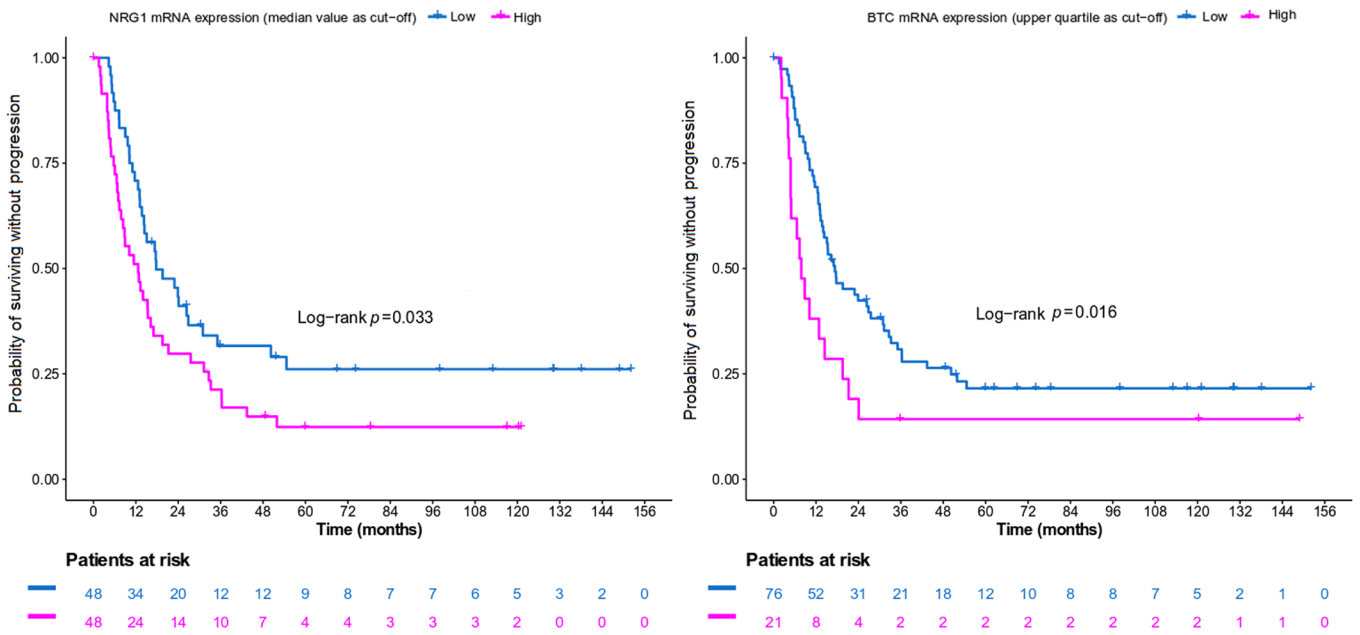

Figure 4. Kaplan-Meier curves with respect to TTP based on NRG1 and BTC mRNA expression in HER2-positive patients. BTC, betacellulin; NRG1, neuregulin 1; TTP, time to progression.

not reached (P=0.43). After adjustment for clinicopathological parameters, a non-significant trend towards improved TTP was observed for patients with *de novo* MBC carrying tumours with high EGF mRNA expression compared with those with low expression.

In terms of survival, a significant interaction was identified between disease presentation status and EGF expression (using the lower quartile as a cut-off; interaction P=0.045). In the subgroup of patients with *de novo* MBC, high EGF expression was associated with a decreased risk of death (Table III). No significance was reached among patients with R-MBC (P=0.82).

Discussion

The present study examined the expression of the ERBB family receptor (ERBB1, ERBB3 and ERBB4) ligands and of other putative prognostic biomarkers, including THRA, RARA,

TGFB1 and IGFBP4, and their association with clinicopathological parameters and clinical outcomes in patients treated with trastuzumab-based therapy for MBC. Tissue samples from the primary tumours were examined for all patients in the study. In addition, a cohort of metastatic HER2-negative patients treated with trastuzumab was assessed. In the present study, 47 patients (32.4%) were found to be HER2-positive in the local institution and HER2 negative in the central re-assessment and were treated with anti-HER2 therapy.

In the literature, discordance between local and central laboratories in HER2 results, using either IHC or FISH, has been reported (9). Repeat testing is recommended if results appear contradictory to other histopathologic findings (8). In an N9831 study, the concordance for central HercepTest and central FISH assays was 92%, while a National Surgical Adjuvant Breast and Bowel Project (NSABP-B) prospective adjuvant trastuzumab study revealed an 18% discordance rate between local and central laboratories in HER2 testing (36-38). A number of factors may contribute to this discordance, such as tumour heterogeneity, borderline HER2-positive samples, difficulty in evaluating tumours with chromosome 17 polysomy, methodologic factors, such as antibody sensitivity, antigen retrieval, tissue processing, lack of concordance between IHC and FISH, and lack of experience of pathologists, especially in the early years of trastuzumab use, and low-volume testing laboratories (36,39,40). In the present study, additional factors, such as the limited experience of local pathologists with the IHC HER2 assessment and the adoption of a 4-point scale of HER2 status for the administration of trastuzumab, especially during the early years of its registration, may have contributed to the observed discordance.

This discordance highlights the main advantage of the central assessment of HER2 testing. Testing must be performed in central laboratories, which are able to show high concordance with a validated HER2 test on a large set of specimens. Expression of HER2 is a predictive factor of response to anti-HER2 therapies and therefore, accurate testing of HER2 is of great importance. According to the NSABP B47 study, HER2-low tumours do not derive any benefit from the addition of 1 year of trastuzumab to standard chemotherapy (41). In the present study, no significant difference was found between HER2-positive and HER2-negative patients in terms of TTP or survival, a finding that can be explained by the improvement of prognosis of HER2-positive tumours due to trastuzumab. It is clear that the use of trastuzumab improved the outcomes of the HER2-positive breast tumours and transformed those tumours into less aggressive ones (42). The question that remains, however, is whether treatment with trastuzumab equalised the prognosis between HER2-positive and HER2-negative MBC.

The amplification or upregulation of the HER2 oncogene identifies patients for whom HER2-directed therapy is appropriate. There is a subset of HER2-positive patients that will not respond to trastuzumab or other approved anti-HER2 targeted therapies due to primary or secondary resistance (19). A number of mechanisms of resistance to trastuzumab have been described, and one of these is increased signalling from other ERBB family receptors (18,43). The initial signal is generated by the extracellular ligands of the ERBB family receptors, which leads to the dimerisation of two HER family receptors and transphosphorylation of their intracellular regions, with subsequent activation of a number of downstream signalling pathways (44).

The present study examined the mRNA expression of the specific HER family receptor ligands and their association with patient outcomes. It was demonstrated that NRG1 had a negative prognostic value in the trastuzumab-treated HER2-positive MBC population, as high NRG1 mRNA expression was associated with an increased risk for disease progression in both univariate and multivariate analyses. In breast cancer, NRG1 is known as a ligand for the HER3 receptor, which has no intrinsic tyrosine kinase activity. When activated by NRG1 binding, the HER3 receptor forms a heterodimer with other HER family receptors and mediates downstream signalling pathways, leading to multiple effects, including growth, proliferation, decreased apoptosis, cellular migration and angiogenesis (45).

In the literature, NRG expression is associated with poor outcomes and high-risk features of breast carcinoma, as this gene promotes metastatic dissemination of breast cancer cells (46). Similarly, a previous study has revealed that NRG1/HER3 activation is one of the key factors inducing primary resistance to trastuzumab in HER2-overexpressing breast cancer cells and that a HER3 antibody may reverse primary trastuzumab resistance by inhibiting the activation of NRG1-dependent HER3 (47). The present results are consistent with the literature and suggest upregulation of NRG1 as a potential mechanism of resistance to trastuzumab that leads to an increased risk of disease progression. This finding is important because it could lead to the development of novel drugs to overcome the resistance to trastuzumab and other anti-HER2 treatments.

Another study demonstrated that upregulation of the NRG1-HER3 axis is a mechanism of resistance in HER2-positive breast cancer cell lines and xenografts treated with anti-HER2 therapy, and that multitargeted antibody mixtures, such as Pan-HER, inhibit the growth of drug-sensitive and drug-resistant HER2+ cancers (48). In addition, in our cohort of HER2-positive patients, BTC mRNA expression was found to have a negative prognostic value for TTP. This was a novel finding regarding the effect of BTC on the clinical outcome of patients with HER2-positive disease that should be taken into consideration and interpreted with caution until it can be further validated in larger cohorts.

In HER2-negative patients, high EREG mRNA expression was univariately associated with a decreased risk of progression, but this was not retained in the multivariate analysis. At the same time, a significant interaction was detected between EGF mRNA expression and disease presentation status in all patients with respect to TTP. More specifically, the univariate analysis revealed that EGF mRNA might represent a positive prognostic factor for TTP in *de novo* metastatic patients. In the multivariate analysis, a trend associated with a lower risk of progression was observed for high EGF mRNA, while longer survival was confirmed for patients with tumours with high EGF mRNA expression in the same subgroup of patients.

EGF expression in breast cancer is associated with poor outcomes and aggressive phenotypes, such as low hormone receptor levels, high proliferation index and HER2 upregulation (49). In a review article by Ross and Fletcher (50), EGFR expression was associated with a higher risk of relapse in

patients with breast cancer. It has been hypothesized that high EGF mRNA is a potential mechanism of trastuzumab resistance, as the growth inhibition by trastuzumab in HER2-positive breast cancer cell lines is blocked by increased levels of the HER family ligands, such as heregulin and EGF (43,51). Furthermore, there is evidence that EGF stimulates the synthesis of its own receptor (EGFR) in a human breast cancer cell line (52). Additionally, in the North Central Cancer Treatment Group N9831 (Alliance) trial, patients with high expression levels of EGFR derived a decreased benefit from adjuvant trastuzumab administered concurrently with chemotherapy (53).

Based on all the aforementioned results, one can reasonably assume that high EGF mRNA and subsequent activation of EGFR-mediated signalling pathways act as a potential mechanism of resistance to trastuzumab and, therefore, should be related to a higher risk of progression and death in trastuzumab-treated HER2-positive patients. The results of the present study, however, are not consistent with the literature, since in the present study, high EGF mRNA expression was associated with a decreased risk of progression among patients with *de novo* MBC. One explanation could be that *de novo* (vs. recurrent) metastatic patients were not pre-treated with trastuzumab and had not developed resistance to trastuzumab, although this assumption cannot explain the positive prognostic value of high EGF in this subgroup of patients. In addition, only a small subset (4.1%) of patients in the present study received trastuzumab in the adjuvant or neoadjuvant setting.

Another explanation could be based on the 'EGFR paradox' in primary vs. MBC (54). According to this paradox, there are fundamental changes in EGFR signalling in metastases compared with primary breast tumours, such as EGF-induced apoptosis and EGF-induced growth arrest. This means that EGFR acts as a tumour driver in primary breast cancer and as a tumour suppressor in metastatic disease (55). In the present study, in recurrent metastatic patients, tissue from the primary tumour and not from the metastases was examined, which could explain the positive prognostic value of high EGF mRNA only in the *de novo* metastatic patients.

The present study is an exploratory study consisting mainly of hypotheses generated with limited samples. The results should be further validated in a larger cohort. Among the strengths of the present study are the long follow-up of patients and the central assessment of HER2 status, which precludes any false-positive cases. Furthermore, this is the first study to collectively evaluate ligands of all three HER2 receptors in patients with MBC.

In conclusion, the present results provided evidence suggesting that there may be an association between HER family ligand expression and clinical outcome in patients with HER2-positive and HER2-negative MBC who received trastuzumab-based therapy. High NGR1 mRNA expression was a negative prognostic factor for progression among patients with HER2-positive MBC, while high EGF mRNA expression was a positive prognostic factor for progression only in patients with *de novo* MBC. In addition, it was observed that the HER2-negative trastuzumab-treated subgroup of patients had similar TTP and survival compared with the HER2-positive group.

MBC is a heterogeneous disease with poor prognosis. Although survival in HER2-positive breast cancer has been improved due to anti-HER2 directed therapies, further studies are required to improve the prognostic outcomes of patients with this disease.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available at https://files.hecog.gr/RQ_mRNA_data.xls.

Authors' contributions

VR, VK and GF were responsible for the conceptualization of the study. Formal analysis was performed by GAK. Experiments and data collection were completed by KP, MB, KC, SC and VK. VR, EM, IB, GP, DB, CC, IN, MS, CM, AKou, PP, AKot, ERa, AP, DT, DP, ERe, AA and GF were involved in patient provision, data acquisition and analysis, and critical revision of the manuscript. EM, VK and GF supervised the study. VR, EM, GAK, KP, SC, VK and GF were responsible for writing the original draft. KP and GF confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The translational research protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (Protocol #4283; January 14, 2008; Thessaloniki, Greece) under the general title 'Investigation of major mechanisms of resistance to treatment with trastuzumab in patients with metastatic breast cancer'. All patients included in the study from 2005 onwards provided written informed consent for the provision of biological material for future research studies before receiving any treatment. A waiver of consent was obtained from the Bioethics Committee for patients included in the study before 2005.

Patient consent for publication

Not applicable.

Competing interests

EM Advisory boards: Roche; GP advisory role: Roche; research funding: Roche. CC Advisory role: Roche; honoraria: Roche; advisory role: Roche. PP Advisory role: Roche; honoraria: Roche. AK Consulting or advisory role: Roche. ER Travel: Roche. AP Consultation Fees: Roche; honoraria: Roche. DP Advisory role: Roche; honoraria: Roche. GF Advisory Board: Roche.

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