**ERBB2 and PTPN2 gene copy numbers as prognostic factors in HER2-positive metastatic breast cancer treated with trastuzumab**

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**Abstract.** Trastuzumab has markedly improved the treatment and long-term prognosis of patients with HER2-positive breast cancer. A frequent clinical challenge in patients with relapsing and/or metastatic disease is de novo or acquired trastuzumab resistance, and to date no predictive biomarkers for palliative trastuzumab have been established. In the present study, the prognostic values of factors involved in the HER2-associated PI3K/Akt signalling pathway were explored. The first 46 consecutive patients treated at the Department of Oncology, Linköping University Hospital between 2000 and 2007 with trastuzumab for HER2-positive metastatic breast cancer were retrospectively included. The gene copy number variation and protein expression of several components of the PI3K/Akt pathway were assessed in the tumour tissue and biopsy samples using droplet digital polymerase chain reaction and immunohistochemistry. Patients with tumours displaying a high-grade ERBB2 (HER2) amplification level of ≥6 copies had a significantly improved overall survival hazard ratio [(HR)=0.4; 95% confidence interval (CI): 0.2-0.9] and progression-free survival (HR=0.3; 95% CI: 0.1-0.7) compared with patients with tumours harbouring fewer ERBB2 copies. High-grade ERBB2 amplification was significantly associated with the development of central nervous system metastases during palliative treatment. Copy gain (≥3 copies) of the gene encoding the tyrosine phosphatase PTPN2 was associated with a shorter overall survival (HR=2.0; 95% CI: 1.0-4.0) and shorter progression-free survival (HR=2.1; 95% CI: 1.0-4.1). In conclusion, high ERBB2 amplification level is a potential positive prognostic factor in trastuzumab-treated HER2-positive metastatic breast cancer, whereas PTPN2 gain is a potential negative prognostic factor. Further studies are warranted on the role of PTPN2 in HER2 signalling.

**Introduction**

HER2-positive breast cancer accounts for approximately 15-20% of all breast cancers and has been associated with poor prognosis and decreased survival (1). The outcome of patients with early or HER2-positive metastatic breast cancer (MBC) has been greatly improved following the introduction of trastuzumab, a humanised monoclonal antibody targeting HER2. Despite the success of trastuzumab, the median overall survival of patients with HER2-positive MBC is still limited to 32-42 months (2,3). Furthermore, a significant number of HER2-positive MBC patients exhibit de novo or acquired resistance towards trastuzumab therapy (4). Apart from HER2 itself, there are currently no clinically established biomarkers distinguishing patients who will benefit from trastuzumab treatment from those who will not (2,5-7). With later generations of HER2-targeted drugs now being available, the search for prognostic and predictive biomarkers guiding the patient to the most effective treatment has become increasingly important.

Trastuzumab mainly exerts its anti-tumoural effects via the stimulation of antibody-dependent cell-mediated cytotoxicity (ADCC), the inhibition of ectodomain cleavage, and the inhibition of ligand-independent HER2 signalling (8-10). The latter is dependent on the dimerisation of two receptor units, either homo- or hetero-dimerisation with another HER-receptor family member, with HER2-HER3 being the most potent dimer (11).

HER2 overexpression may induce downstream activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway, a key pathway in carcinogenesis leading to cell proliferation, cell survival, and upregulated protein synthesis (12). The activation of PI3K initiates a downstream chain of phosphorylation events including Akt, mTOR, S6K1 and S6K2, and ultimately

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the upregulation of Cyclin D1, which regulates the G1/S-phase transition through binding to CDK 4/6 and phosphorylation of Rb (13-15). It is hypothesised that dysregulation of PI3K/Akt signalling pathway mediators plays a key role in trastuzumab resistance. PTEN deficiency and/or upregulated Src signalling have been implicated in de novo and acquired trastuzumab resistance in vitro (16-18). Src is activated by upstream receptor tyrosine kinases such as Met or EGFR, but it is also affected by intracellular protein tyrosine phosphatases e.g. protein tyrosine phosphatase non-receptor type 1 (PTPN1) and 2 (PTPN2) (19,20). PTPN1 expression status appears to have no or limited impact in HER2-positive breast cancer or breast cancer patients undergoing neoadjuvant chemotherapy, whilst PTPN2 has been reported to be frequently lost in breast cancer, correlating with poor outcome (21-24).

In the present study, we explored the prognostic values of intra-tumoural biomarkers believed to be involved in trastuzumab resistance in a long-term follow-up cohort of the first consecutive patients receiving palliative trastuzumab treatment at our department.

Materials and methods

Patient material. All patients diagnosed with HER2+ MBC and treated with trastuzumab at Linköping University Hospital between 2000 and 2007 were included in the cohort. Treatment decisions were based on HER2-positive disease as determined by IHC and/or FISH diagnostics performed on primary tumours or, if available, biopsies from metastatic lesions. Exclusion criteria from the present study were incomplete key data (i.e., data related to survival, HER2 status, and/or trastuzumab treatment) and previous anti-HER2 treatment. Primary tumour material was available for all patients and material from metastases was available in one-third of the cases. Formalin-fixed paraffin-embedded (FFPE) tissue specimens, obtained from surgery, were stored at room temperature until DNA extraction. Two reviewers (SE and AM) extracted clinical and pathological data from the medical records. As per clinical routine, results from metastatic lesions were used for final analysis when available. The primary endpoint was overall survival (OS), defined as the time from start of trastuzumab treatment until time of death. The secondary endpoint was progression-free survival (PFS) as measured from the start of trastuzumab treatment to time of death, or at the first sign of radiological and/or clinical progression. Analyses were performed and reported following the REMARK guidelines (25).

DNA extraction. Genomic DNA was extracted from FFPE tumour tissue specimens containing at least 50% tumour cells using the QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Hilden, Germany). The manufacturer's protocol was followed except for the paraffin removal procedure, which was performed in Tissue-Tek Tissue-Clear (Sakura Finetek Europe B.V., Flemingweg, The Netherlands). DNA concentration was measured using Quantifluor® ONE dsDNA Dye kit (Promega Corporation, Madison, WI, USA) on a Quantus™ Fluorometer (Promega Corporation). DNA samples were stored at -70°C during long-term storage and at -20°C for short-term storage.

Droplet digital polymerase chain reaction (PCR). Copy numbers of CCND1, ERBB2, MET, PTPN2, and RP6SKB1 were measured with droplet digital PCR (ddPCR) using AP3BI as a reference gene. The amplicon length of the reference gene was matched with the amplicon length of the gene of interest; two different primer-probe assays were therefore used for AP3BI. The choice of reference gene and the protocol were previously described, with the appropriate annealing temperatures as shown in Table I (26). Gene copy numbers were determined by analysing the distribution of the ddPCR values, with care taken for the distribution being skewed towards two gene copies due to any non-tumour cells in the samples. PTPN2, MET, RP6SKB1, and CCND1 gene copy number analyses were approached as follows: less than two gene copies was considered as gene copy loss, two copies as normal, and three or more was considered as gene copy gain. The samples were subsequently dichotomised into gain/no gain (≥3 vs. <3 copies; Fig. 1). As patients in this cohort were all HER2-positive, ERBB2 gene copy number was divided into low- and high-grade amplification. After the level of ERBB2 amplification was assessed, tumours were partitioned into tertiles, with the lower tertile showing less than six gene copies and the upper tertile showing more than 16 gene copies. This was later dichotomised and the cut-off for high-grade ERBB2 amplification was set to six or more gene copies.

Tissue microarray. Sections cut from tumour donor blocks were stained with haematoxylin and eosin, and morphologically representative regions were selected in each tumour sample. Three tissue cores with a diameter of 0.8 mm were taken from these regions and mounted in recipient blocks, forming tissue microarrays (TMAs) created with a manual arrayer (Beecher Instruments Inc., Sun Prairie, WI, USA). The TMA blocks were cut into 5 μm sections and transferred to frost-coated glass slides for immunohistochemistry (IHC).

Immunohistochemistry. Deparaffinisation, rehydration, and antigen retrieval were performed on the TMA sections using DAKO PT link (Dako; Agilent Technologies GmbH, Waldbronn, Germany) with either high or low pH buffer (EnVision FLEX target retrieval solution low/high pH; Dako; Agilent Technologies GmbH) in most cases. Otherwise, these steps were performed in xylene, serial dilutions of ethanol, and in 10 mM citrate buffer (pH 6) in a pressure cooker (Digital Decloaking Chamber, Biocare Medical, Concord, CA, USA), respectively. TMA sections were blocked in serum-free protein block (Spring Bioscience, Fremont, CA, USA) for 10-60 min followed by overnight incubation at 4°C with the primary antibody. The sections were then incubated for 30 min at room temperature with the appropriate secondary antibody (EnVision+System-HP; Dako; Agilent Technologies GmbH), stained with 3'-diaminobenzidine tetrahydrochloride (DAB/H2O2) solution, counterstained with Mayer's Haematoxylin (Fluka Analytical; Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany), and dehydrated using a series of increasing ethanol dilutions.

Scoring was done by two independent investigators per antigen, without any knowledge of clinical data. The expression levels were based on staining intensity (negative, weak, moderate, and strong) and percentage of positive cells. Staining intensity was evaluated in the nucleus, the cell
Table I. Gene copy number assay overview.

| Gene    | Chromosome | ddPCR assay     | Company                                | Annealing temperature (˚C) | Amplicon length (nt) |
|---------|------------|-----------------|----------------------------------------|-----------------------------|----------------------|
| MET     | 7q31       | dHsaCP2500321   | Bio-Rad Laboratories, Inc.             | 60                          | 62                   |
| ERBB2   | 17q12      | FP: 5'-GTCTTGGGACAGCTGAGG-3’ RP: 5'-GGTTTCCCACACATGCTCT-3’ | Sigma-Aldrich; Merck KGaA  | 61                          | 80                   |
|         |            | Probe: 5'ACACAACATCCCTCCGGACTA TCA-3’ | Applied Biosystems; Thermo Fisher Scientific, Inc. |                             |                      |
| CCND1   | 11q13      | dHsaCP2500371   | Bio-Rad Laboratories, Inc.             | 55                          | 64                   |
| PTPN2   | 18p11      | FP: 5'-TGGCCCACTCCGGAAACTAAA-3’ RP: 5'-AAACAAACAACTGGAGGCAATCTA-3’ | Sigma-Aldrich; Merck KGaA  | 64.2                        | 65                   |
|         |            | Probe: 5'-TGAGGTCTCGCTAACC-3’ | Applied Biosystems; Thermo Fisher Scientific, Inc. |                             |                      |
| RPS6KB1 | 17q23.1a   | Hs04469680_cn   | Thermo Fisher Scientific, Inc.         | 60                          | 87                   |
| AP3B1   | 5q14.1     | dHsaCP2500348   | Bio-Rad Laboratories, Inc.             | 60                          | 60                   |
|         |            | dHsaCP1000001   | Bio-Rad Laboratories, Inc.             |                             | 85                   |

dPCR, droplet digital polymerase chain reaction.

membrane, and the cytoplasm depending on the localisation of the respective protein. If discordant scorings were found, the slide was re-examined in tandem and a joint score was made. The antibodies, the conditions used, and how the scoring was performed are summarised in Table II. Phospho-specificity of the pAkt-S473, p4EBP1-S65, pMet-Y1349, and pS6K-T389 antibodies was previously validated in our lab (26-29).

Statistics. Statistical analyses were carried out using IBM SPSS Statistics v.23 (IBM Corp., Armonk, NY, USA). Univariable hazard ratios (HR) were calculated using Cox regression for all biomarkers and clinicopathological prognostic factors. Parameters found to be significant in the univariable analyses were further analysed with multiple Cox regression analysis. Comparisons of categorical outcomes were analysed using Fisher's exact test. Kaplan-Meier curves were used to visualise survival and progression-free survival and differences between groups were estimated with Mantel-Cox. No imputations for missing data were used and P<0.05 was considered to indicate a statistically significant difference.

Results

Fifty patients with HER2-positive MBC patients who received de novo-treatment with trastuzumab at our institution between 2000 and 2007 were identified and 46 of these were included in this study. Three patients were excluded due to incomplete clinical data and one patient was excluded due to HER2-negative disease. Patient follow-up was performed in 2016 and the results are summarised in Table III. The age of the included patients ranged between 35 and 79 years with a median age of 57 years. Median OS and PFS were 2.23 years (95% CI: 1.63-2.84) and 0.51 years (95% CI: 0.43-0.98), respectively (Fig. 2A and B). All but one patient experienced progressive disease and breast cancer-related death during the follow-up time. The patient who was still alive at follow-up had then been monitored for twelve years and had received a total of 159 cycles of trastuzumab.

Concomitant treatment with trastuzumab and chemotherapy was prescribed to 38 patients of whom 32 received vinorelbine, three received docetaxel, and three paclitaxel. Seven patients were treated with single trastuzumab and one patient with trastuzumab and concomitant aromatase inhibitor (AI). Trastuzumab treatment was continued beyond progression in 27 of the 46 patients (58.7%). During the disease course, 28 of the 46 patients (60.8%) developed metastases in the central nervous system (CNS).

Clinicopathological variables and survival. Patients with more than one metastatic site at the start of trastuzumab therapy, as compared with patients with a single metastatic site, displayed significantly shorter OS and PFS in univariable analysis (HR=1.50; 95% CI: 1.05-2.15, P=0.025 and HR=1.45; 95%: 1.02-2.06, P=0.037, respectively). Patients
Table II. Overview of the antibodies used for immunohistochemistry.

| Protein | Antibody | Antigen retrieval | Blocking time | Antibody dilution | Scoring nuclear | Scoring cytoplasm | Scoring membrane |
|---------|----------|-------------------|---------------|-------------------|-----------------|------------------|-----------------|
| p4EBP1-S65 | Phospho-4E-BP1 (Ser65) (174A9) | 10 mM citrate buffer, pH6 pressure cooker | 10 min | 1:100 | Low: Weak-moderate in >50% | Low: Weak | N/A |
| pAkt-T308 | Phospho-Akt (Thr308) (244F9) | 10 mM citrate buffer, pH6 pressure cooker | 10 min | 1:25 | Low: Negative High: Moderate in >50% | Low: Negative | N/A |
| pAkt-S473 | Phospho-Akt (Ser473) (D9E) XP® | 10 mM citrate buffer, pH6 pressure cooker | 10 min | 1:50 | Low: Negative High: Moderate/strong | Low: Negative | N/A |
| Cyclin D1 | Cyclin D1 SP4 | PT-link, high pH | 10 min | 1:100 | Low: Negative/weak High: Moderate/strong | Low: Negative | N/A |
| HER3 | erbB3/Her3 clone 5A12 | PT-link, high pH | 60 min | 1:20 | N/A | Low: Negative/weak High: Moderate/strong | Low: Negative |
| HER4 | ERBB4 polyclonal antibody | PT-link, low pH | 60 min | 1:20 | N/A | Low: Negative/weak High: Moderate/strong | Low: Negative |
| Met | Met (D1C2) XP® | PT-link, high pH | 60 min | 1:100 | N/A | Low: Negative/weak High: Moderate/strong | Low: <10% |
| pMet-Y1349 | Anti-Met (c-Met) (phospho Y1349) | PT-link, high pH | 60 min | 1:25 | N/A | Low: Negative/weak High: Moderate/strong | Low: <10% |
| PTEN | PTEN (138G6) | 10 mM citrate buffer, pH6 pressure cooker | 10 min | 1:50 | N/A | Low: Negative/weak High: Moderate/strong | N/A |
| PTPN2 | PTPN2 polyclonal antibody | PT-link, low pH | 60 min | 1:400 | N/A | Low: Negative/weak High: Moderate/strong | N/A |
| S6K1 | P70 S6 kinase (49D7) | PT-link, low pH | 10 min | 1:100 | Low: Weak-moderate in >50% High: Strong in >50% | Low: Weak | N/A |
| pS6K1-T389 | Phospho-p70 S6 kinase (Thr389) (1A5) | PT-link, low pH | 10 min | 1:100 | Low: 0-25% High: 26-100% | Low: Negative | N/A |
receiving lapatinib after failure on trastuzumab therapy had a significantly improved OS (HR=0.39; 95% CI: 0.18-0.85, P=0.019) vs. those not receiving lapatinib. Combination therapy (trastuzumab and chemotherapy/AI) was significantly associated with improved PFS (HR=0.40; 95% CI: 0.17-0.92, P=0.031) but not with OS (HR=0.50; 95% CI: 0.22-1.20, P=0.11). First-line vs. second or later line of trastuzumab showed a trend towards improved PFS (HR=0.57; 95% CI: 0.31-1.05, P=0.071). Lapatinib treatment after trastuzumab vs. those receiving other or no therapy after trastuzumab was the only clinicopathological variable keeping a significant association with OS in the multiple Cox regression analysis. Likewise, concomitant treatment was the only clinicopathological variable that remained significant regarding PFS.

The results of the multivariable analyses are summarised in Table IV.

**Receptor tyrosine kinases, PTPN2, and survival.** High-grade 
**ERBB2** amplification, defined as ≥6 gene copies, was found in 27 of the 40 (67.5%) tumours available for ddPCR analysis and was significantly associated with improved OS and PFS in univariable analysis (HR=0.49; 95% CI: 0.25-0.99, P=0.045 and HR=0.44; 95% CI: 0.22-0.89, P=0.022, respectively; Table V; Fig. 2C and D). Of the patients with high-grade **ERBB2** amplification, 78% (21/27) developed CNS metastases compared with 31% (4/13) of the patients with low-grade amplification (Fisher’s exact test, P=0.006).

**PTPN2** gain, defined as three or more gene copies, was found in 15 of 42 (35.7%) patients available for ddPCR analysis and was significantly associated with shorter OS and PFS in univariable analysis (HR=2.0; 95% CI: 1.0-4.0, P=0.040 and HR=2.1; 95% CI: 1.0-4.1, P=0.041, respectively; Table V, Fig. 2E and F). Analysis of Met, pMet, HER3 and HER4 did not reveal any significant prognostic values (Tables V and VI).

In the multiple Cox analysis, high-grade **ERBB2** amplification was significantly associated with improved prognosis both in terms of OS and PFS. In contrast, **PTPN2** gain was significantly associated with shorter OS and PFS (Table IV).

**PI3K/Akt pathway and survival.** High cytoplasmic expression level of the S6K1 protein was significantly associated with improved OS in univariable analysis (Table V). However, gene copy number variation of the S6K1-encoding gene **KPS65KB1** did not show a significant prognostic value and high S6K1 expression in the cytoplasm was not prognostic in the multiple Cox analysis (Tables V and VI). Protein expression levels of **PTEN, pAkt, p4EBP1**, and **Cyclin D1** had no significant prognostic value (Table VI).

**Discussion**

The present study was performed on a cohort of the first 46 consecutive patients with recurring HER2-positive breast cancer who received palliative treatment with trastuzumab at Linköping University Hospital. The results reveal that the prognosis remains poor for patients with HER2-positive MBC even after the introduction of trastuzumab therapy. While the overall survival, in general, was comparable with the outcome data of the early trastuzumab studies (28), a small number of long-term responders were identified in the present cohort.

Analyses of the tumour material with ddPCR revealed that high-grade **ERBB2** amplification and gain of **PTPN2** appear as prognostic biomarkers in patients with HER2-positive MBC. High-grade **ERBB2** amplification was significantly associated with prolonged OS and PFS in both univariable and multivariable analyses. Gene copy number analysis with ddPCR has been confirmed to have a high correlation with FISH/ISH when determining the level of **ERBB2** amplification (29-33). The cut-off determined for high-grade **ERBB2** amplification in this study was derived from the distribution of data and could be argued to be low when compared with the current cut-off to determine HER2-positivity with ISH/FISH (>4 gene copies). Both similar and higher cut-offs have been used to define high-grade **ERBB2** amplification and further research

Table III. Clinicopathological variables at trastuzumab start and treatment regimen.

| Clinicopathological variable                        | n (%)  |
|----------------------------------------------------|--------|
| Pre-Dominant metastatic site                       |        |
| Visceral                                           | 38 (82.6) |
| Non-visceral                                      | 8 (17.4)  |
| Number of metastatic sites                        |        |
| 1                                                  | 20 (43.5)  |
| 2                                                  | 16 (34.8)  |
| 3+                                                 | 10 (21.7)  |
| ER status                                          |        |
| Negative                                          | 34 (73.9)  |
| Positive                                          | 12 (26.1)  |
| PR status                                          |        |
| Negative                                          | 32 (69.6)  |
| Positive                                          | 14 (30.4)  |
| Trastuzumab therapy                               |        |
| Single therapy                                     | 7 (15.2)   |
| Combination therapy                               | 39 (84.8)  |
| Trastuzumab as first-line                         |        |
| No                                                | 25 (54.3)  |
| Yes                                               | 21 (45.7)  |
| Lapatinib                                         |        |
| No                                                | 36 (81.8)  |
| Yes                                               | 8 (18.2)   |
| Nottingham histological grade                     |        |
| 1                                                 | 2 (6.1)    |
| 2                                                 | 15 (45.4)  |
| 3                                                 | 16 (48.5)  |
| Concomitant treatment                             |        |
| None                                              | 7 (15.2)   |
| Vinorelbine                                       | 32 (69.6)  |
| Paclitaxel                                        | 3 (6.5)    |
| Docetaxel                                         | 3 (6.5)    |
| Aromatase inhibitor                               | 1 (2.2)    |

*1Lymph node or bone.*
Figure 1. Results of the ddPCR analysis for all patients and their distribution. Gene copy number status loss, normal and gain were defined using this distribution bearing in mind a small skewness towards two gene copies due to any non-tumour cells in the sample. (A) PTPN2, (B) MET, (C) CCND1 and (D) RPSKB1.

Table IV. Multiple Cox regression analyses of clinicopathological parameters, and \textit{ERBB2} and \textit{PTPN2} gene copy number.

| Variables                             | n   | Overall survival | Progression-free survival |
|---------------------------------------|-----|------------------|---------------------------|
|                                       |     | HR (95% CI)      | P-value                   | HR (95% CI)      | P-value |
| First-line trastuzumab                | 39  | 1.03 (0.48-2.20) | 0.95                      | 0.62 (0.28-1.37) | 0.23    |
| Number of metastasis at trastuzumab start | 39  | 1.33 (0.81-2.18) | 0.26                      | 1.10 (0.67-1.80) | 0.70    |
| Combination therapy                   | 39  | 0.55 (0.20-1.39) | 0.20                      | 0.31 (0.12-0.82) | 0.018*  |
| Visceral metastasis                   | 39  | 0.56 (0.23-1.36) | 0.20                      | 0.71 (0.26-1.96) | 0.51    |
| High S6K1 expression in cytoplasm     | 39  | 0.85 (0.39-1.83) | 0.67                      | 1.04 (0.53-2.04) | 0.91    |
| \textit{ERBB2} high-grade             | 39  | 0.37 (0.18-0.79) | 0.010*                    | 0.35 (0.16-0.73) | 0.006*  |
| \textit{PTPN2} gain                   | 39  | 3.40 (1.53-7.57) | 0.008*                    | 2.72 (1.30-5.71) | 0.008*  |
| Lapatinib after trastuzumab           | 39  | 0.29 (0.12-0.73) | 0.008*                    | -              | -       |

HR, hazard ratio; CI, confidence interval. *P<0.05.
is needed to determine the optimal cut-off for FISH and ddPCR (34-36).

The positive prognostic value of high-grade ERBB2 amplification is in concordance with previous studies on MBC, although, to our knowledge, no previous study has utilised ddPCR quantification of ERBB2 amplification to discriminate between patients with good vs. poor prognosis in this setting (34,35,37,38). A recent meta-analysis, focused on ERBB2 amplification status in
patients with loco-regional HER2-positive breast cancer treated with adjuvant trastuzumab, co-analysed three cohort studies with 1360 patients in total. In contrast to our results, Xu et al. (35) concluded that ERBB2 amplification level was not prognostic in the adjuvant setting. This implies that the prognostic value of ERBB2 amplification status appears to vary depending on the disease stage. Further studies should focus on the prognostic impact of ERBB2 amplification status in metastatic rather than loco-regional HER2-positive breast cancer.

The high prevalence of CNS metastases (61%) in our cohort may be explained by the long follow-up period and the nature of HER2-positive disease; similar results have been found in other long-term follow-up studies (3). Interestingly, high-grade ERBB2 amplification was associated with increased prevalence of CNS-metastases (78% vs. 31%), but not the time to the first presentation of CNS-metastasis (data not shown). If confirmed in other studies, the high prevalence of CNS-metastasis found in this population could warrant routine radiological evaluation of the brain in suitable intervals for all HER2-positive MBC patients, especially in patients with high-grade ERBB2 amplification.

### Table V. Gene copy numbers in relation to overall survival and progression-free survival.

| Gene (protein) | Copy number high | High/n (%) | HR (95% CI) | P-value | HR (95% CI) | P-value |
|---------------|------------------|------------|-------------|---------|-------------|---------|
| CCND1 (Cyclin D1) | ≥3 | 9/42 (21.4) | 1.6 (0.7-3.4) | 0.23 | 1.3 (0.6-2.8) | 0.46 |
| ERBB2 (HER2) | ≥6 | 27/40 (67.5) | 0.5 (0.3-0.9) | 0.045<sup>a</sup> | 0.4 (0.2-0.9) | 0.022<sup>a</sup> |
| MET (Met) | ≥2 | 31/42 (73.8) | 1.2 (0.6-2.4) | 0.65 | 0.9 (0.5-1.9) | 0.84 |
| PTPN2 (PTPN2) | ≥3 | 15/42 (35.7) | 2.0 (1.0-4.0) | 0.040<sup>a</sup> | 2.1 (1.0-4.1) | 0.041<sup>a</sup> |
| RP56K1 (S6K1) | ≥3 | 8/42 (19.0) | 1.7 (0.8-3.8) | 0.19 | 2.0 (0.9-4.5) | 0.10 |

HR, hazard ratio; CI, confidence interval. <sup>a</sup>P<0.05.

### Table VI. Protein expression levels in relation to overall survival and progression-free survival.

| Protein | Localisation | High/n (%) | HR (95% CI) | P-value | HR (95% CI) | P-value |
|---------|--------------|------------|-------------|---------|-------------|---------|
| p4EBP1-S65 | Cytoplasmic | 34/40 (85.0) | 0.9 (0.4-2.1) | 0.75 | 0.8 (0.3-1.9) | 0.61 |
| Nuclear | 15/40 (62.5) | 1.2 (0.6-2.3) | 0.55 | 1.0 (0.5-1.9) | 0.98 |
| pAkt-S473 | Cytoplasmic | 36/42 (85.7) | 1.7 (0.7-4.1) | 0.23 | 1.8 (0.7-4.3) | 0.20 |
| Nuclear | 33/42 (78.6) | 1.2 (0.5-2.5) | 0.69 | 1.2 (0.6-2.6) | 0.60 |
| pAkt-T308 | Cytoplasmic | 15/41 (36.6) | 1.1 (0.6-2.2) | 0.75 | 1.0 (0.5-2.0) | 0.96 |
| Nuclear | 36/40 (90.0) | 1.9 (0.7-5.4) | 0.24 | 2.0 (0.7-5.9) | 0.18 |
| Cyclin D1 | Nuclear | 26/43 (60.5) | 1.1 (0.6-2.1) | 0.69 | 1.2 (0.7-2.3) | 0.49 |
| HER3 | Membranous | 33/44 (75.0) | 1.4 (0.7-2.9) | 0.32 | 1.3 (0.6-2.6) | 0.49 |
| Cytoplasmic | 15/44 (34.1) | 1.2 (0.6-2.3) | 0.57 | 1.0 (0.5-1.9) | 0.93 |
| HER4 | Membranous | 6/43 (14.0) | 1.4 (0.6-3.3) | 0.46 | 1.0 (0.4-2.3) | 0.93 |
| Cytoplasmic | 28/43 (65.1) | 1.0 (0.5-1.9) | 1.00 | 1.2 (0.7-2.4) | 0.51 |
| pMet-Y1349 | Membranous | 19/43 (44.2) | 1.1 (0.6-2.0) | 0.78 | 1.1 (0.6-2.0) | 0.84 |
| Cytoplasmic | 29/43 (67.4) | 0.7 (0.4-1.4) | 0.33 | 0.8 (0.4-1.5) | 0.48 |
| Met | Membranous | 7/40 (17.5) | 1.2 (0.5-2.8) | 0.66 | 1.3 (0.5-1.0) | 0.60 |
| Cytoplasmic | 22/40 (55.0) | 0.6 (0.3-1.2) | 0.17 | 0.5 (0.3-1.0) | 0.06 |
| PTEN | Cytoplasmic | 21/43 (48.5) | 1.1 (0.6-2.1) | 0.68 | 1.0 (0.5-1.8) | 1.0 |
| PTPN2 | Cytoplasmic | 31/42 (73.8) | 1.6 (0.8-3.2) | 0.19 | 1.6 (0.8-3.3) | 0.19 |
| pS6K1-T389 | Cytoplasmic | 16/43 (37.2) | 1.1 (0.6-2.1) | 0.71 | 1.2 (0.7-2.3) | 0.50 |
| Nuclear | 12/43 (27.9) | 0.6 (0.3-1.3) | 0.19 | 0.7 (0.3-1.4) | 0.30 |
| S6K1 | Cytoplasmic | 22/43 (51.2) | 0.5 (0.3-1.0) | 0.04<sup>a</sup> | 0.7 (0.4-1.3) | 0.24 |
| Nuclear | 13/43 (30.2) | 0.8 (0.4-1.6) | 0.57 | 1.1 (0.6-2.1) | 0.79 |

HR, hazard ratio; CI, confidence interval. <sup>a</sup>P<0.05.
Copy gain of *PTPN2* was significantly associated with shorter OS and PFS in both uni- and multivariable analyses. Likewise, high protein expression of *PTPN2* was associated with a shorter OS and PFS, though not statistically significant. Combining protein expression with gene copy number increased the prognostic value (Fig. 3) indicating that further research of both protein and gene expression could be valuable. In contrast with these results, *PTPN2* has previously been described as a tumour suppressor and loss of *PTPN2* was shown to correlate with shorter breast cancer survival in patients with early breast cancer, including those with HER2-positive disease (23). Unlike the patients in our cohort, these patients did not receive trastuzumab. This suggests that the effect seen in our study could be due to an interaction between *PTPN2* gain, HER2 positivity, and trastuzumab treatment. A recent *in vivo* study of malignant melanoma in mice suggested that loss of *PTPN2* may sensitize tumours to immunotherapy through the regulation of antigen presentation and recruitment of cytotoxic CD8+ T-cells (39). Hypothetically, *PTPN2* could have a similar effect on the ADCC caused by trastuzumab, which would explain the negative prognostic value of *PTPN2* gain in this study.

Previous studies report conflicting data regarding the prognostic significance of the PI3K/Akt pathway. *In vitro* and *in vivo* PTEN loss has been previously identified as a mechanism of trastuzumab resistance (40,41). Here, no prognostic value of PTEN was found, in line with a more recent large study of patients treated in the adjuvant setting (42). Furthermore, no prognostic value of other key proteins and genes within the PI3K/Akt pathway was evident in this study, making them less likely to be suitable biomarkers for patients with HER2-positive MBC receiving trastuzumab. Although it would have been relevant to explore the prognostic value of the components of the PI3K/Akt pathway in relation to ER-status, this was beyond the scope of the present study and the limited size of our cohort did not permit such subgroup analyses.

The only clinicopathological variable where a significant impact on OS was evident in multivariable analysis was the presence of later line lapatinib treatment after failure of trastuzumab. Although the number of patients receiving lapatinib
was low (n=8), these data support the idea that the continuation of HER2-targeted therapy is valuable even after progression on trastuzumab (43).

The weaknesses of this study include its retrospective nature and the small population size, and any results should be interpreted in this context. The main strengths of this study are the extensive follow-up duration, with endpoint events (OS and PFS) reported for all but one patient, and the wide inclusion criteria mirroring the real world authentic clinical setting.

In conclusion, our results suggest that high-grade ERBB2 amplification is a potential positive prognostic factor and that PTPN2 gene copy gain is a potential negative prognostic factor in HER2-positive MBC patients receiving trastuzumab treatment. Furthermore, the ERBB2 amplification level may identify patients at high risk of developing CNS-metastases in this subgroup of patients.

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Availability of data and materials

The datasets generated and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contribution

SE, MS, AM, SW and OS conceptualised the design of the study. SE and AM collected clinical data from patient records. SE, OS, CV, GPT, VF and JG performed and analysed immunohistochemistry results. CV and KG performed ddPCR analyses. SE, CV, ALH, NE and OS interpreted the data. SE and CV were major contributors of drafting the manuscript. CV and KG performed the study. SE and AM collected clinical data from patient records. SE, MS, AM, SW and OS conceptualised the design of the study. SE and AM collected clinical data from patient records. SE, OS, CV, GPT, VF and JG performed and analysed immunohistochemistry results. CV and KG performed ddPCR analyses. SE, CV, ALH, NE and OS interpreted the data. SE and CV were major contributors of drafting the manuscript. All authors read and critically revised the manuscript and later approved the final version to be published.

Ethics approval and consent to participate

The present study was approved by the Regional Ethical Review Board in Linköping (M140-06 and updated in 2014, 2014/163-32). The local ethical review board concluded that individual consent was not required.

Patient consent for publication

Not applicable. Due to the retrospective nature of the study, the non-invasive design and the anonymisation of personal data, the local ethics review board concluded that patients would be unlikely to object to publication and that the public interest outweighed the potential harm to the individuals' integrity.

Competing interests

The authors declare that they have no competing interests.

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Methods

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