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.

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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

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 *AP3B1* 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 *AP3B1*. 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, RPS6KB1,* 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. The 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-HRP; Dako; Agilent Technologies GmbH), stained with 3'-diaminobenzidine tetrahydrochloride (DAB/H_2O_2) 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'-GGTCCTGGAAGGCCACAAGG-3' RP: 5'-GGTTTTCCCACCACATCCTCt-3' Probe: 5'ACACAACACATCCCCCTCCTTGACTA TCAA-3'	Sigma-Aldrich; Merck KGaA Applied Biosystems; Thermo Fisher Scientific, Inc.	61	80
CCND1	11q13	dHsaCP2500371	Bio-Rad Laboratories, Inc.	55	64
PTPN2	18p11	FP: 5'-AAGCCCACTCCGGAAACTAAA-3' RP: 5'-AAACAAACAACTGTGAGGCAATCTA-3' Probe: 5'-TGAGGCTCGCTAACC-3'	Sigma-Aldrich; Merck KGaA Applied Biosystems; Thermo Fisher Scientific, Inc.	64.2	65
RPS6KB1	17q23.1a	Hs04469680_cn	Thermo Fisher Scientific, Inc.	60	87
AP3B1	5q14.1	dHsaCP2500348	Bio-Rad Laboratories, Inc.		60
		dHsaCP1000001	Bio-Rad Laboratories, Inc.		85

ddPCR, 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	Company	Antigen retrieval	Blocking time	Antibody dilution	Scoring nuclear	Scoring cytoplasm	Scoring membrane
p4EBP1-S65	Phospho-4E-BP1 (Ser65) (174A9)	Cell signaling	10 mM citrate buffer, pH6 pressure cooker	10 min	1:100	Low: Weak-moderate in >50% High: Strong in >50%	Low: Weak High: Moderate in >50%-strong in >50%	N/A
pAkt-T308	Phospho-Akt (Thr308) (244F9)	Cell signaling	10 mM citrate buffer, pH6 pressure cooker	10 min	1:25	Low: Negative High: Positive	Low: Negative High: Weak/moderate/strong	N/A
pAkt-S473	Phopsho-Akt (Ser473) (D9E) XP®	Cell signaling	10 mM citrate buffer, pH6 pressure cooker	10 min	1:50	Low: Negative High: Weak/strong	Low: Negative High: Weak/moderate/strong	N/A
Cyclin D1	Cyclin D1 SP4	ThermoFisher scientific	PT-link, high pH	10 min	1:100	Low: Negative/weak High: Moderate/strong	Low: Negative High: Positive	N/A
HER3	erbB3/Her3 clone 5A12	Nano tools	PT-link, high pH	60 min	1:20	N/A	Low: Negative/moderate High: Strong	Low: Negative High: Weak/ moderate-strong
HER4	ERBB4 polyclonal antibody	Abnova	PT-link, low pH	60 min	1:20	N/A	Low: Negative/weak High: Moderate/strong	Low: Negative-weak High: Moderate/ strong
Met	Met (D1C2) XP®	Cell signaling	PT-link, high pH	60 min	1:100	N/A	Low: Negative/weak High: Moderate/strong	Low: <10% High: >10%
pMet-Y1349	Anti-Met (c-Met) (phospho Y1349)	Abcam	PT-link, high pH	60 min	1:25	N/A	Low: Negative High: Weak/moderate-strong	Low: <10% High: >10%
PTEN	PTEN (138G6)	Cell signaling	10 mM citrate buffer, pH6 pressure cooker	10 min	1:50	N/A	Low: Negative/weak High: Normal/strong	N/A
PTPN2	PTPN2 polyclonal antibody	Proteintech	PT-link, low pH	60 min	1:400	N/A	Low: Negative/weak High: Moderate/strong	N/A
S6K1	P70 S6 kinase (49D7)	Cell signaling	PT-link, low pH	10 min	1:100	Low: Weak-moderate in >50% High: Strong in >50%	Low: Weak High: Moderate or strong in >50%	N/A
pS6K1-T389	Phospho-p70 S6 kinase (Thr389) (1A5)	Cell signaling	PT-link, low pH	10 min	1:100	Low: 0-25% High: 26-100%	Low: Negative High: Positive	N/A

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

Clinicopathological variable	n (%)
Pre-Dominant metastatic site	
Visceral	38 (82.6)
Non-visceral[a]	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)

[a]Lymph node or bone.

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 VI). However, gene copy number variation of the S6K1-encoding gene *RPS6KB1* 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 (30), 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 uni- 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 (31-33). 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

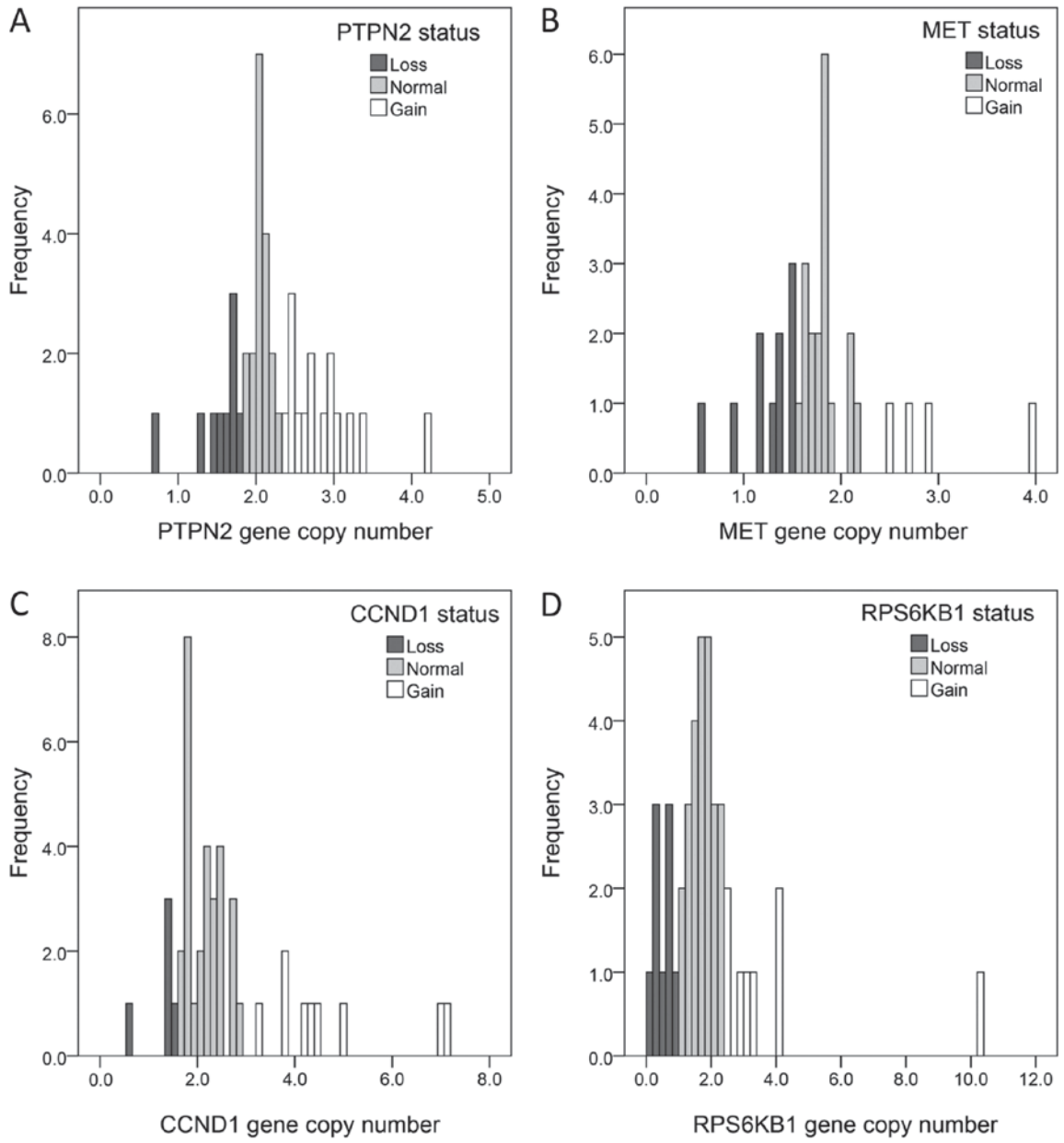

Figure 1. Results of the ddPCR analysis for all patients and their distribution. Gene copy number status loss, normal and gain were defined using this distribution bearing in mind a small skewness towards two gene copies due to any non-tumour cells in the sample. (A) PTPN2, (B) MET, (C) CCND1 and (D)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

Table IV. Multiple Cox regression analyses of clinicopathological parameters, and *ERBB2* and *PTPN2* gene copy number.

		Overall survival		Progression-free survival	
Variables	n	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[a]
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
ERBB2 high-grade	39	0.37 (0.18-0.79)	0.010[a]	0.35 (0.16-0.73)	0.006[a]
PTPN2 gain	39	3.40 (1.53-7.57)	0.008[a]	2.72 (1.30-5.71)	0.008[a]
Lapatinib after trastuzumab	39	0.29 (0.12-0.73)	0.008[a]	-	-

HR, hazard ratio; CI, confidence interval. [a]P<0.05.

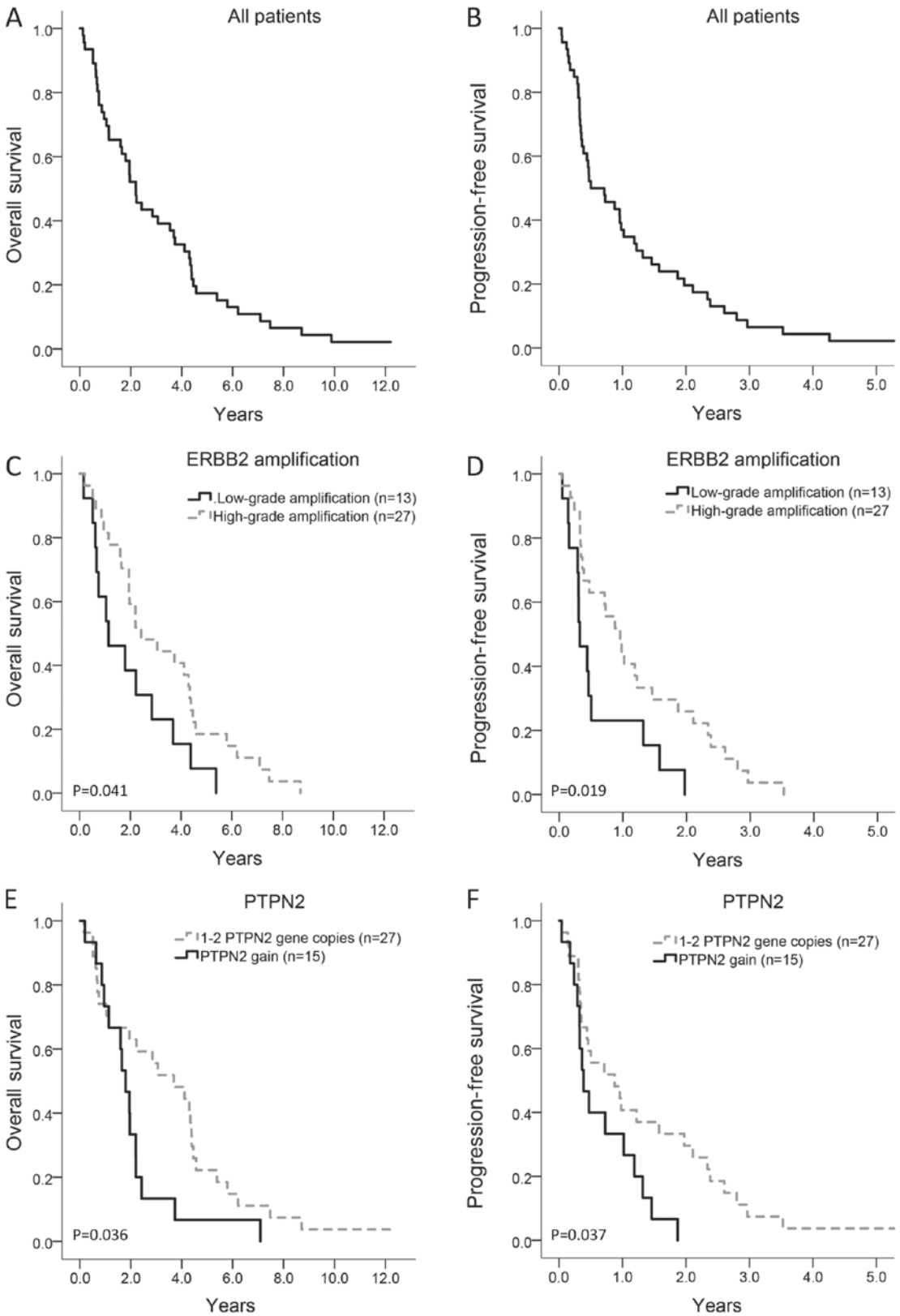

Figure 2. Kaplan-Meier curves based on overall survival and progression-free survival. (A) OS for all patients (n=46). (B) PFS for all patients (n=46). (C) OS characterised by low-grade and high-grade ERBB2 amplification. (D) PFS characterised by low-grade and high-grade ERBB2 amplification. (E) OS characterised by PTPN2 gene copy number. (F) PFS characterised by PTPN2 gene copy number.

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

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

			Overall survival		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[a]	0.4 (0.2-0.9)	0.022[a]
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[a]	2.1 (1.0-4.1)	0.041[a]
RPS6KB1 (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. [a]P<0.05.

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

			Overall survival		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 (0.5-19)	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[a]	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. [a]P<0.05.

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 *ERRB2* 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.

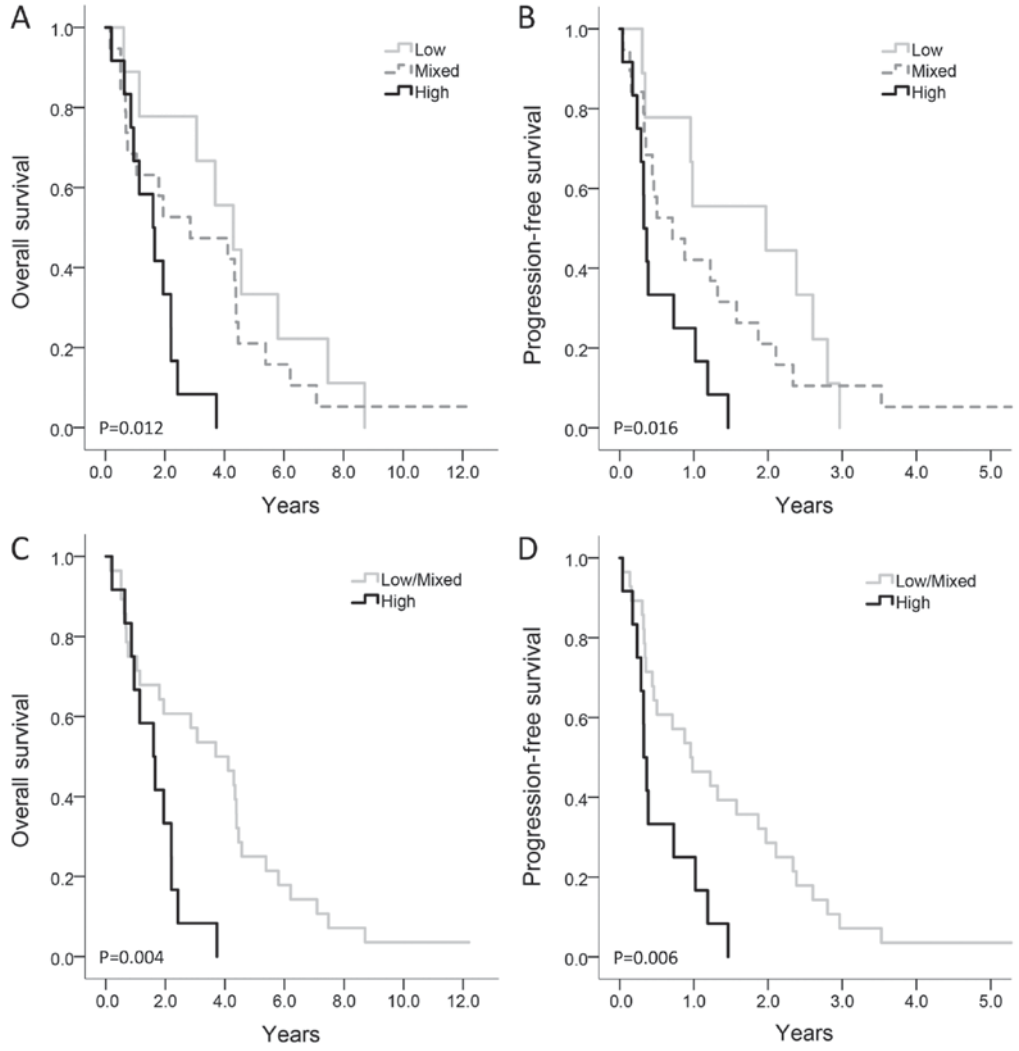

Figure 3. Kaplan-Meier curves based on overall survival and progression-free survival in regards to combined protein and GCN PTPN2 status. (A) OS characterised by PTPN2 status. (B) PFS characterised by PTPN2 status. In A and B low PTPN2 status is defined as no PTPN2 gain and low PTPN2 protein expression in IHC (n=9), mixed PTPN2 status is defined as either PTPN2 gain and low protein expression or no PTPN2 gain and high protein expression (n=19) and high PTPN2 status is defined as both PTPN2 gain and high protein expression (n=12). (C) OS characterised by PTPN2 status. (D) PFS characterised by PTPN2 status. In C and D high PTPN2 (n=12) is compared to the low and mixed status combined (n=28) (C, D).

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 sensitise 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. 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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