

# Comparison between real-time quantitative PCR detection of HER2 mRNA copy number in peripheral blood and ELISA of serum HER2 protein for determining HER2 status in breast cancer patients

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**Abstract.** *Background:* The development of non-invasive procedure to determine HER2 status may represent a powerful method for monitoring disease progression and response to the treatment.

*Methods:* Serum samples and RNA from peripheral blood were evaluated in 85 breast cancer patients (49 HER2 positive and 36 HER2 negative) and 22 healthy controls. HER2 mRNA levels were measured by real-time quantitative PCR (QPCR) and serum HER2 protein by immunoenzymatic assay (EIA). ROC curve analyses were used to determine the optimal cut off values.

*Results:* A statistically significant difference was detected for both QPCR and EIA in HER2 positive patients as compared with both healthy controls and HER2 negative tumours. QPCR showed a 91% (CI95%: 84%–98%) specificity and a 78% (CI95%: 68%–88%) sensitivity for an optimal cut off value of 4.74. The optimal cut off value for EIA was 22 ng/ml yielding a 95% (CI95%: 90%–100%) specificity and a 59% (CI95%: 48%–70%) sensitivity. The QPCR assay was slightly less specific than EIA in discriminating HER2 positive breast cancers from HER2 negative tumours (78% CI95%: 69%–87% versus 86% CI95%: 79%–93%), but it was more sensitive (76% CI95%: 67%–85% versus 55% CI95%: 44%–66%).

*Conclusions:* Our results indicate that QPCR performs better than EIA in the determination of HER2 status of breast cancer patients and could be useful in monitoring the disease during follow up.

Keywords: HER2, breast cancer, QPCR, EIA, RT-PCR, IHC

## 1. Introduction

The human epidermal growth factor receptor 2 (HER2) is a member of the ErbB-like oncogene family,

which consists of four closely related family members, HER2 (neu/ErbB2), epidermal growth factor receptor (EGFR, ErbB1), HER3 (ErbB3) and HER4 (ErbB4) [7]. Active ErbB receptors stimulate signalling pathways such PI3K-Akt and Ras-MAPK that are responsible for cell proliferation, growth and survival. Aberrant signalling through these receptors is believed to play a direct role in malignant transformation and/or progression [3]. Over expression and/or amplification of

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the HER2 oncogene, occur in up to 20–30% of breast cancer patients [8,10,16,22]. Genomic alterations of proto-oncogene HER2 are associated with poor prognosis and more aggressive tumor phenotype. HER2 positive status indicates a poor prognosis, as shorter overall survival time, poorer outcome in node-negative patients when combined with St. Gallen classification, poorer outcome in node-positive patients, and earlier relapse after adjuvant chemotherapy [21]. Patients with HER2 amplification or over expression are eligible for treatment with Trastuzumab (Herceptin, Roche) a humanized monoclonal antibody directed against the extracellular domain of the HER2 receptor. Trastuzumab inhibits cell proliferation both *in vitro* and *in vivo*, and enhances the response to conventional chemotherapy. In combination with chemotherapy, Trastuzumab is currently the standard first line treatment of metastatic breast cancer, and it is also indicated in adjuvant setting, showing a promising activity in neo-adjuvant treatment when combined with ormonotherapy or chemotherapy [1,12,20]. Unlike most pathologic testing, which may only help in establishing the diagnosis, the HER2 status stand alone in determining which patients are likely to respond to Trastuzumab.

HER2 determination on tumour tissues is routinely performed by immunohistochemistry (IHC) or fluorescence *in situ* hybridization analysis (FISH) and recently by MLPA (Multiplex Ligation-dependent Probe Amplification) [15]. IHC is the choice method, with 0/1+ signifying HER2 negative status, 3+ signifying HER2 positive status. Tumours showing a 2+ score by IHC are considered undetermined and need to be tested by FISH to determine HER2 status [9,19]. However, these techniques only inform about HER2 status at the time of the diagnosis, but cannot be used to monitor patients during the follow up or to determine whether they are responding to trastuzumab. We have recently demonstrated that HER2 mRNA is detectable by specific real-time quantitative reverse transcription PCR (QPCR) in the peripheral blood of breast cancer patients and that this mRNA levels correlate with HER2 status by IHC [5]. In the present study, we developed a Taqman chemistry-based approach for QPCR analysis of HER2 expression in the blood of breast cancer patients [4,5,7,8,13,14,16,18,20]. QPCR analysis together with HER2 serum protein determination, were used to determine HER2 status in 85 newly diagnosed breast cancer cases and 22 healthy controls. Results from these analyses were correlated with HER2 status in the tissue as determined by IHC.

## 2. Materials and methods

### 2.1. Patients and samples

The analysis was performed on blood samples and serum obtained from 85 breast cancer cases (mean age  $58 \pm 12$ ) without distant metastases. As control, serum and blood samples were collected from 22 healthy females (mean age  $42 \pm 10$ ) without malignant disease or family history of cancer. All the subjects provided written informed consent and the study was approved by local ethical committee.

Since we used routine IHC analysis determination of HER2 status, only cases showing a 3+ score were included in the study as HER2 positive tumours ( $n = 49$ ). The 36 breast cancers negative for HER2 overexpression scored 0 or 1+ at IHC. The clinicopathological features of the 85 patients with breast cancer are shown on Table 1.

### 2.2. Total RNA isolation and reverse transcription

Total RNA was extracted from 2.5 ml of peripheral blood collected in Pax gene tubes (Bender Dickinson, UK) using the Pax gene blood RNA kit (Pre-Analytix A Qiagen/BD Company, CH). RNA concentration was quantified by the absorbance measurement at 260 and 280 nm using the Nanodrop spectrophotometer, whereas RNA integrity was determined on an Agilent 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA, USA) and only RNA with RIN (RNA integrity number)  $\geq 7.0$  were processed. Five-hundred nanograms of total RNA were reverse transcribed by using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA).

### 2.3. Real-time quantitative PCR determination of HER2 mRNA copy number

PCR primers for Her2neu were designed across exon 5–exon 6 junction as follows: forward primer in the exon 5 (position 775-798): 5'-GGCTCTCACACT GATAGACACC-3', reverse primer in the exon 6 (position 841-821): 5'-TCCCCAGCAGCGGGAGCCCTT AC-3', probe across exon 5–exon 6 junction (position 803-815): 5'-FAM-TCTCGGGCCTGCCACCCCTG-3'-TAMRA, resulting in an amplicon size of 66 bp. As endogenous control a primer/probe set was designed for the 28S rRNA (28SEC). Primers and probe were designed straddling the exon 2–exon 3 junction as follows: forward primer across exon 2–exon 3 junction

Table 1  
Clinical characteristics of the cancer patients

Tumor dimension <sup>a</sup>		Lymph node status, no. of patients (%)	
T1	39 (46%)	Positive	36 (42%)
T2	35 (42%)	Negative	41 (48%)
T3	2 (2%)	Unknown	8 (9%)
T4	9 (10%)		
Grade, no. of patients (%)		Estrogen receptor, no. of patients (%)	
I	4 (5%)	Positive	38 (45%)
II	39 (46%)	Negative	32 (38%)
III	26 (31%)	Unknown	15 (18%)
Unknown	16 (19%)		
HER2 immunohistochemistry		Progesteron receptor, no. of patients (%)	
IHC 3+	49 (58%)	Positive	29 (34%)
IHC 0/1+	36 (42%)	Negative	49 (47%)
		Unknown	16 (19%)

<sup>a</sup>Tumor dimension according to UICC-TNM classification (International Union Against Cancer).

(position 277-301): 5'-TTCCTTAAGATCATCCAAC TATTGG-3', reverse primer in the exon 3 (position 371-353): 5'-ATGCGGATCTGCTGCATCT-3', probe in the exon 3 (position 320-339): 5'-FAM-TCATTGTG GGAGCAGACAAT-3'-TAMRA resulting in an amplicon size of 94 bp. PCR fragments for all target genes and 28SEC were cloned in the pCR<sup>®</sup> 4-TOPO<sup>®</sup> Vector (Invitrogen, USA) and introduced in *Escherichia coli* DH5 $\alpha$ <sup>TM</sup>. Plasmid DNA from the selected transformant cells was isolated by using the QIAprep<sup>®</sup> Spin Miniprep Kit (Qiagen, CH). Recombinant vectors, linearised with Not I, were serially diluted. Six plasmid dilutions (in the range of  $1 \times 10^6$  copies to ten copies) were used to construct calibration curves for real-time PCR.

Real-time quantitative PCR were carried out in triplicates in a volume of 20  $\mu$ l containing 3  $\mu$ l cDNA, 4.5  $\mu$ M of each primer, 2  $\mu$ l of PCR buffer [18] and 1 U PlatinumTaq DNA polymerase (Invitrogen, USA). Amplifications were performed in 96-well plates in a 7700 Sequence detector (Applied Biosystems, Carlsband, CA, USA) using the following conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C 15 s, 60°C 1 min. Data were analyzed by SDS 1.9.1 software (Applied Biosystems, Carlsband, CA, USA). Each plate included calibration curves for the *HER2* and 28SEC (28S rRNA) transcripts, patient cDNA samples, positive and negative controls, and multiple water blanks. Calibration curves were constructed by plotting the threshold cycle versus the logarithm of the relative copy number. For each sample, the level of *HER2* mRNA transcript was determined as the ratio of the number of copy of *HER2* to the number of copies

of 28SEC. The sensitivity of the assay was evaluated using DNA extracted from  $10^6$ – $10^1$  sixfold dilutions. A minimum detection limit of 10 copy number was established in the PCR reaction mixture.

#### 2.4. ELISA measurement of serum HER2 protein

HER2 was measured in serum samples from the 49 patients with HER2 status 3+ at immunohistochemistry (IHC), the 39 samples from HER2 negative patients and the 22 serum samples from controls, using the human sp 185 HER-2 Instant EIA (Bender MedSystems, CA, USA), an enzyme-linked immunoassorbent assay for the quantitative detection of soluble p 185 HER2 levels in serum. An anti-human sp 185 HER2 monoclonal coating antibody adsorbed onto microwells and a lyophilized HRP-conjugated monoclonal anti-human sp 185 HER2 were incubated with 100  $\mu$ l of diluted (1:20) sample serum at room temperature for 3 h on a microplate shaker at 100 rpm. Following 3 times washing, the unbound enzyme conjugate anti-human sp 185 HER2 is removed and 100  $\mu$ l of TMB (tetramethyl-benzidine) substrate solution reactive with HRP is added to the wells. After incubation at room temperature for 10 min, the reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven human sp 185 HER2 standard dilution (from 10.00 to 0.16 ng/ml) and human sp 185 HER2 sample concentration determined. The manufacture recommended cut off value for EIA determination was 15 ng/ml with expected sp 185 HER2 values that ranged between 3.1 and 30.5 ng/ml and with a mean level of 6.8 ng/ml and a standard deviation of  $\pm 6.4$  ng/ml.

### 2.5. Imprecision study

RNA obtained from 1 breast cancer tissues IHC 3+ and 1 breast tissues from reductive mammoplastic, were used to estimate the imprecision of QPCR analysis. We assessed within run imprecision by analyzing several control samples in the same day. Between run imprecision studies were conducted over a 15 days period by measuring HER2/28SEC ratios in the control samples. The within and between run CVs were determined. Total imprecision was then calculated according to the Clinical and Laboratory Standards Institute (CLSI) recommendation.

### 2.6. Statistical analyses

Multiple comparison among patients and controls in terms of HER2 expression were carried out through the non-parametric Dunn and Kruskal–Wallis tests. The non-parametric choice was due to the results of the Kolmogorov–Smirnov and Shapiro tests allowing us to reject the Gaussian distribution assumption. The optimal cut-off in terms of sensitivity and specificity for detecting HER2-positive tumours by real-time QPCR and EIA was determined by drawing the ROC curve considering only HER2 positive tumours and controls. In both cases the area under the ROC curve, computed numerically and tested for statistical significance, was assumed as a measure of goodness of the test. The comparison between the two ROC curves was carried out testing the difference between the two areas under the ROC curve using the Gaussian approximation. All statistical procedures were implemented in the R statistical software. Results from the Taqman-based assay and the Syber green test used in our previous work [5] were compared by using the Z-test for proportion.

## 3. Results

### 3.1. Analytical assessment of imprecision

The mean  $\pm$  SD values, ranges, and within-between-run CVs for the QPCR, and the total imprecision for each of the control samples, are shown in Table 2.

### 3.2. HER2 determination in peripheral blood samples by real-time QPCR

Peripheral blood RNAs from 85 patients were analyzed by QPCR assay for HER2 gene. For 49 (58%) of those patients tumour specimens were IHC 3+ positive whereas 36 (42%) were IHC negative (Table 1). As control peripheral blood obtained from 22 healthy individuals were tested. The median value of the HER2/28SEC copy number ratios for the three groups was as follows: 14 (5–36.5) for the IHC positive patients, 3 (2–4) for IHC negative patients and 2 (1.8–4) for the healthy control group ( $p < 0.001$ , Kruskal–Wallis) (Fig. 1(A)). The Dunn test demonstrated statistically significant differences between IHC positive patients and both healthy controls and IHC negative patients ( $p < 0.001$  and  $p < 0.001$ ). No statistical difference was found between gene copy number ratios of HER2 negative breast cancer patients and healthy controls.

The gene copy number ratios in healthy controls and in IHC 3+ positive cases was used to design ROC curve (Fig. 1(A)), the AUC value of the curve was 0.85 ( $p < 0.001$ ). Based on the ROC curve an optimized gene copy number ratio of 4.74 yielded a 78% (CI95%: 68%–88%) sensitivity and a 91% (CI95%: 84%–98%) specificity as compared with a sensitivity of 60% (CI95%: 42%–78%,  $p = 0.04$ , Z-test) and

Table 2  
Analytical assessment of imprecision

	Between-run imprecision		Within-run imprecision	
	Normal	Tumour	Normal	Tumour
Mean $\pm$ SD	4.77 $\pm$ 0.61	795.46 $\pm$ 47.26	3.88 $\pm$ 0.49	783.40 $\pm$ 75.36
Minimum	4.16	748.20	3.39	708.04
Maximum	5.37	842.72	4.37	858.76
CV%	12.76	5.94	12.56	9.62
Total imprecision				
	Normal	Tumour		
	16.35%	11.18%		

Note: Means, standard deviation (SD) and ranges are expressed in relative copy number.

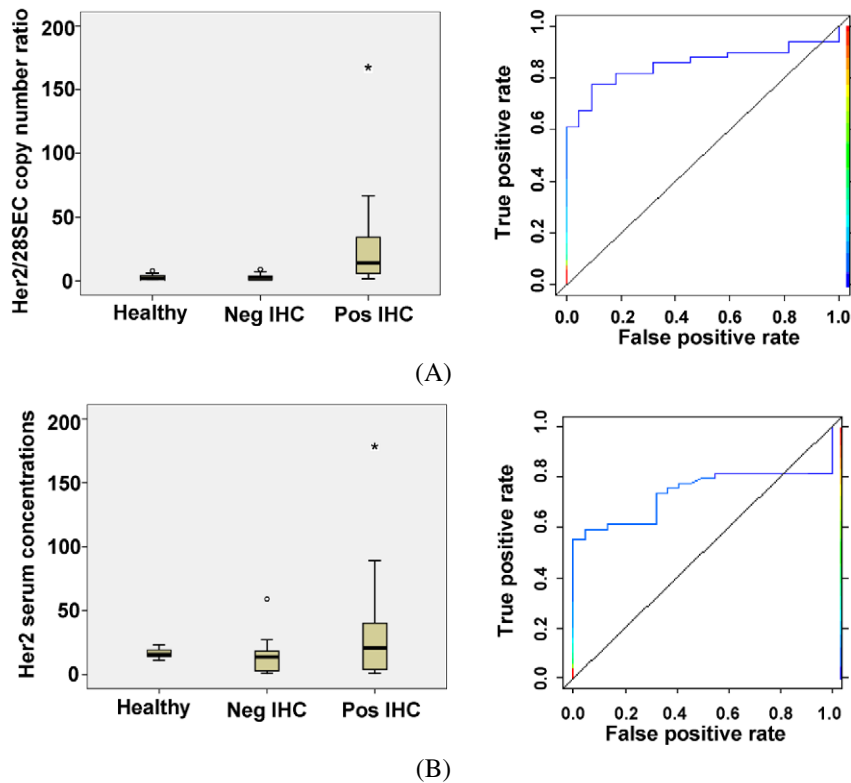


Fig. 1. (A, left panel) Box plot for the HER2/28SEC copy number ratios determined by QPCR for the three groups of patients. The lines inside the boxes denote median values. The whiskers represent the interval between the 10th and 90th percentiles. The empty circle indicate the outliers values between 1.5 and 3 length upper or down from the interquartile range. “\*” Indicates the extreme cases with more than three boxes length upper or down from the interquartile range. (A, right panel) ROC curve for QPCR assay was designed on the basis of HER2/28SEC copy number ratios in IHC 3+ positive tumours and healthy controls. The area under the curve is 0.85 ( $p < 0.001$ ). (B, left panel) Box plot for the HER2 serum concentrations determined by EIA for the three groups of patients. The lines inside the boxes denote median values. The whiskers represent the interval between the 10th and 90th percentiles. The empty circle indicate the outliers values between 1.5 and 3 length upper or down from the interquartile range. “\*” Indicates the extreme cases with more than three boxes length upper or down from the interquartile range. (B, right panel) ROC curve for QPCR assay was designed on the basis of HER2/28SEC copy number ratios in IHC 3+ positive tumours and healthy controls. The area under the curve is 0.85 ( $p < 0.001$ ).

specificity of 83% (CI68%–95%,  $p = 0.12$ ,  $Z$ -test) obtained in our previous study by using a Syber green-based approach [5]. The sensitivity and specificity of the Taqman-based assay in discriminating between IHC negative and IHC 3+ tumours were 76% (CI95%: 67%–85%) and 78% (CI95%: 69%–87%), respectively (Fig. 2(A)).

### 3.3. HER2 protein determination in serum by ELISA

HER2 protein serum levels were determined in all patients and controls by ELISA. The median values of the serum HER2 concentration was significantly higher in IHC 3+ positive cases than in IHC negative cases and in healthy controls and were as follows: 27 (18–40) for the IHC positive patients, 15 (7–19) for IHC negative patients and 15.5 (14–19.3)

for the healthy control group ( $p < 0.001$ , Kruskal–Wallis) (Fig. 1(B)). Statistically significant differences were detected between IHC positive patients and both healthy controls and IHC negative patients ( $p = 0.01$  and  $p < 0.001$ , Dunn test). No statistical difference was found between gene copy number ratios of HER2 negative breast cancer patients and healthy controls.

HER2 serum levels in healthy individuals and IHC 3+ positive patients were used to design a ROC curve, the AUC value was 0.73 ( $p = 0.001$ ) (Fig. 1(B)). Based on the ROC curve obtained, an optimal serum HER2 level of 22 ng/ml yielded a 59% (CI95%: 48%–70%) sensitivity and a 95% specificity (CI95%: 90%–100%). The sensitivity and specificity in discriminating between IHC negative and IHC 3+ tumours were 55% (CI95%: 44%–66%) and 86% (CI95%: 79%–93%), respectively (Fig. 2(B)).

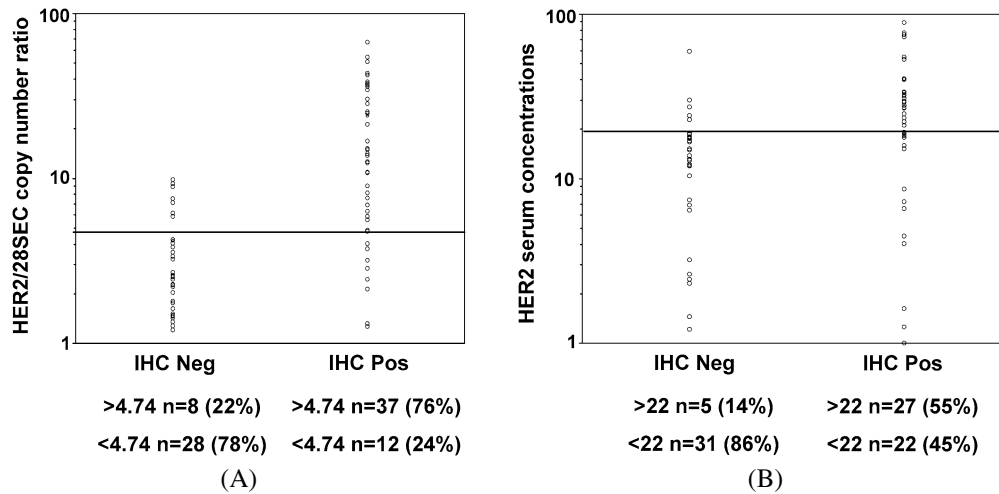


Fig. 2. (A) Scatter plot for HER2/28SEC copy number ratios for patients with positive (score 3+) and negative (scores 0 and 1+) IHC. The horizontal line indicates the optimal cut off for the HER2/28SEC copy number ratios (4.74), which gave a sensitivity of 76% and specificity of 91%. (B) Scatter plot for HER2 serum concentrations for patients with positive (score 3+) and negative (scores 0/1+) IHC. The horizontal line indicates the optimal cut off for the HER2 serum concentrations (22), which gave a sensitivity of 59% and specificity of 95%.

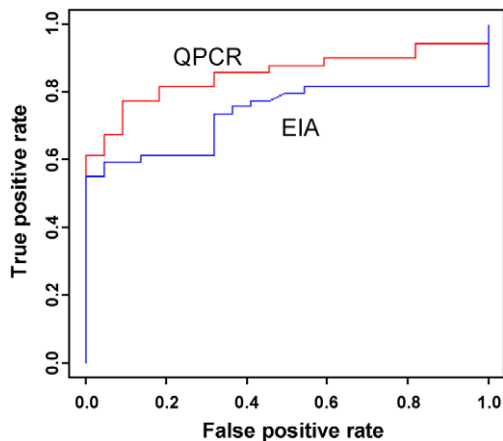


Fig. 3. Comparison of the ROC curves for QPCR and EIA were designed on the basis of HER2/28SEC copy number ratios in IHC 3+ positive tumours and healthy controls.

### 3.4. Comparison of ELISA and QPCR and correlation with clinical features

The comparison of the ROC curves obtained for QPCR and ELISA indicated that QPCR performs better than ELISA although this difference did not reach statistical significance (Fig. 3).

HER2 expression level obtained from EIA and QPCR were also compared with clinical features (tumour dimension, grading, lymph node status, estrogen and progesterone receptors). When we analyzed the correlation with lymph node status, we found for the QPCR but not for EIA an association between HER2

expression level in lymph node positive cases as compared with lymph node negative cases ( $p = 0.005$ , Mann–Whitney) (Fig. 4), this result was further confirmed in a logistic regression model in which elevated blood mRNA levels of HER2 were associated with a higher probability of a positive lymph node status (Fig. 5).

## 4. Discussion

Genetic alterations lead to marked changes in the expression of many genes at both mRNA and expression level. Real-time quantitative PCR is a high sensitive method able to detect and quantify mRNA expression in tumour tissues and bodily fluids. QPCR analysis has been used to detect HER2 mRNA in the tumour tissues of breast cancer patients [2]. Recently using a Syber green chemistry-based approach we demonstrated that this technique can also be applied to the detection of HER2 mRNA in the blood of breast cancer patients. The HER2 mRNA levels correlated with HER2 status as determined by IHC and with disease outcome [5].

In the present study we developed Taqman chemistry-based assay [1,2,6,9–12,14,15,17,19,21,22] to analyze a cohort of 85 breast cancer patients and 22 healthy controls. We choose the 28S rRNA as internal control because it shows a significant lower level of variability in whole blood samples from healthy donors as compared with other housekeeping genes commonly used for gene expression analysis (e.g.,

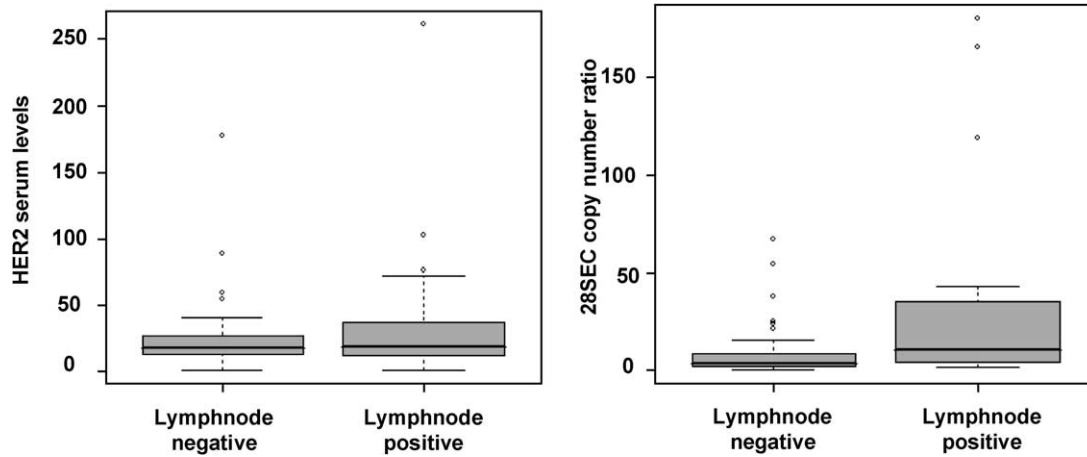


Fig. 4. Box plot for the HER2 serum concentrations determined by EIA and for the HER2/28SEC copy number ratios determined by QPCR in lymph node positive cases as compared with lymph node negative cases. The lines inside the boxes denote median values. The whiskers represent the interval between the 10th and 90th percentiles. The empty circle indicates the outliers values between 1.5 and 3 length upper or down from the interquartile range.

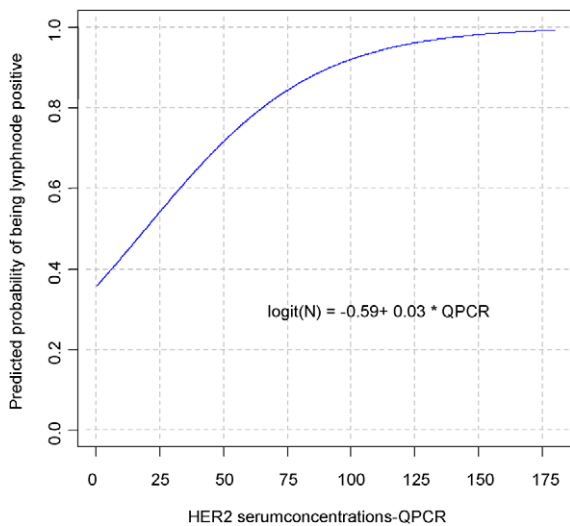


Fig. 5. Logistic regression of lymph node status in terms of HER2 blood mRNA copy number detected by QPCR. The increase in mRNA copy number in the blood is associated with a higher probability to have a positive lymph node status.

$\beta$ -actin and GAPDH) [6]. Quantification of HER2 expression was determined by using serial dilution of plasmid DNA containing the region amplified by the primer/probe set. This method allows a precise determination of the target (HER2) and reference genes (28SEC) copy number with a better relative quantification of the HER2 transcript. As compared with our previous Syber green-based approach [5], Taqman assay showed a similar specificity but a significantly higher sensitivity in discriminate breast cancer cases

from healthy controls.

HER2 serum levels were determined in patients and controls by EIA and the sensitivity and specificity of the two techniques were compared. In our experimental design we choose to analyze newly diagnosed breast cancer without distant metastases. This allowed us to eliminate the bias due to the comparison of HER2 expression in blood and serum at the time of testing, with results from the IHC performed at diagnosis, thus minimizing the discrepancies related to changes in the HER2 status at the time of recurrence. Moreover the blood was drawn to our patients prior to begin Trastuzumab therapy to be sure that protein serum and mRNA levels could not be affected by therapy. Statistically significant differences were detected in HER2 mRNA and protein expression in IHC 3+ positive breast cancer cases as compared with healthy controls and negative IHC tumours.

Although the manufacture recommended cut off value for EIA determination was 15 ng/ml, the specificity was only of 53% (CI95%: 42%–64%), thus an optimal cut off value of 22 ng/ml was determined on the basis of the ROC curve designed by using HER2 protein levels in the serum of healthy individuals and IHC 3+ cases. For QPCR the ROC curve construed by using the HER2/28SEC copy number ratio in healthy individuals and IHC 3+ positive cases allowed the definition of an optimal cut off values of 4.74. Cut off values yielded a specificity of more than 90% for both QPCR and EIA, whereas the sensitivity of the assay was better for QPCR (78% CI95%: 68%–88%) than for EIA (59% CI95%: 48%–70%). The comparison of

ROC curves demonstrated that QPCR performs better than EIA although this difference did not reach statistical significance. These data together suggest that QPCR could be more suitable than EIA for the determination of HER2 status in breast cancer patients. We also determined the sensitivity and specificity of QPCR and EIA in discriminating IHC 3+ breast cancers from IHC negative tumours. The QPCR assay was slightly less specific than EIA (78% specificity as compared with 86% specificity), but it was far more sensitive (76% sensitivity as compared with 55% sensitivity). As shown in Fig. 2 the majority of false positive tumours show for both QPCR and EIA levels very close to the cut off value. Since the specificity of both tests in healthy individuals is high, it is possible that both QPCR and EIA are able to detect very low levels of protein and mRNA copies. As well as false negative rates may result from dilution of cells carrying amplified genes among non-tumour cells [2,5,6,8,10,14,15,17–22]. Interestingly we found for QPCR but not for EIA, a correlation between lymph node status and HER2 mRNA levels suggesting that QPCR correlates better than EIA with the status of the disease.

In summary, QPCR is suitable alternative method for the determination of HER2 status in the blood of breast cancer patients. Overall QPCR performs better than ELISA in terms of sensitivity in discriminating HER2 IHC positive tumours from both healthy individuals and IHC negative breast cancers. QPCR could be used as diagnostic tool when primary tumour samples are unavailable or to monitor the outcome of the disease and the response to therapy during follow up of breast cancer patients.

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