Evaluation of a Manual ELISA Kit for Determination of HER2/neu in Serum of Breast Cancer Patients

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Abstract. Background: Our aim was to conduct an analytical validation in a routine laboratory setting of the cerb-B2/c-neu ELISA assay kit from Calbiochem® used to measure the extracellular domain (ECD) of HER2/neu in the serum of breast cancer patients. Materials and Methods: The evaluation was based on three different production lots used in a routine laboratory setting. The reference value was based on a population of 217 patients with breast cancer not overexpressing HER2. Results: The detection limit, below that given by the manufacturer, was 0.34 ng ml⁻¹ and the quantification limit was 0.90 ng ml⁻¹. Reproducibility and repeatability were at least 95%, precision coefficients of variation varied between 6 and 8.5% and trueness measured by dilution tests and the standard additions method varied between 97 and 107%. The threshold was estimated at 5 ng ml⁻¹. Conclusion: This technique presents satisfactory levels of accuracy for routine laboratory use.

As a transmembrane protein, the HER2 receptor may undergo proteolysis, a physiological process, resulting in cleavage between a 97 to 115 kDa extracellular domain (ECD), called p105 or HER2/neu ECD, which is released into the extracellular sector, and the remaining 95 kDa protein, p95, which remains embedded in the plasma membrane. The p95 portion may be associated with increased tyrosine kinase activity and thus with a greater signaling potential. This HER2 receptor is normally present at the surface of the cells of numerous tissues: the gastrointestinal, respiratory and urogenital epithelium, as well as skin, mammary, placental and cardiac cells. In the presence of a tumor, expression of the receptor can increase 40-fold, from 5,000 HER2 receptors per cell to over 200,000, as observed in numerous types of cancer, including breast, ovarian, stomach, lung, prostate and colon cancer (1).

The involvement of HER2 at different levels of the tumor process has been demonstrated in numerous in vitro and in vivo studies: in cell proliferation, malignant transformation of cultured cell lines and cell motility. It is a determining factor in the metastatic potential of tumor cells. Amplification and/or overexpression of HER2 has been found to be one of the causes of transformation of cells into cancer cells, via a mechanism whereby cell growth signals are amplified through the intrinsic tyrosine kinase activity of the receptor when it binds to another receptor in the HER family or, in the case of overexpression, through the possible formation of HER2-HER2 dimers. Overexpression and/or amplification of HER2 (or p185) is found in approximately 20-30% cases of human breast cancer (2). No correlation has been established between evaluation of HER2/neu overexpression or amplification in tumor tissue and the release of fragments into circulating blood, which explains the diverging results observed between immunohistochemical (IHC) or fluorescence in situ hybridization (FISH) techniques and serum assays. However, in 3 to 5% of cases, IHC-negative patients were found to develop an increased level of circulating HER2/neu ECD (significant if 10-fold baseline) when metastases overexpressing HER2 occurred (3, 4). A high level of circulating HER2/neu ECD could, like increased overexpression of HER2 detected in IHC, indicate a poor prognosis in breast cancer patients.

Measurement of circulating HER2/neu ECD can also be an effective way of monitoring treatment by trastuzumab (Herceptin®), which specifically targets the HER2 receptor. Studies on HER2/neu ECD, used as a tumor marker, have shown that 20 to 40% of metastatic breast cancer cases present high serum concentrations (5, 6). Information on HER2/neu ECD is useful not only for monitoring patients with metastatic breast cancer, but also in its use as a predictive marker when assessing targeted therapy (7-10).
Several serum assays have been proposed for the assessment of HER2-2 ECD, but only the Bayer immunoassay (ADVIA Centaur; Bayer Diagnostics, Tarrytown, NY, USA) has been validated and approved by the FDA. Calbiochem® (ex Oncogene Research Products®) has developed a sensitive, specific ELISA for quantification for both p185 and p105 in sera, plasma and other biological fluids (11). Our aim was to conduct an analytical validation in a routine laboratory setting of the cerb-B2/c-neu rapid format ELISA assay kit from Calbiochem® (EMD Biosciences, Inc, an affiliate of Merck KGaA, Darmstadt, Germany) used in research for measuring the ECD of HER2/neu in the serum of breast cancer patients and to determine a reference value for this technique which could be used without any automated system.

Materials and Methods

Quantification of serum HER2/neu ECD. HER2/neu ECD levels were measured by ELISA using the cerb-B2/c-neu rapid format ELISA kit from Calbiochem®. The test was performed on 96-well microplates coated with anti-p185 murine monoclonal antibodies. The standard used was a lyophilized recombinant p185HER2/neu protein that was reconstituted in diluent. The detector antibody was a biotinylated murine antibody, anti-human cerb-B2/c-neu. The conjugate was streptavidin-peroxidase conjugate and the chromogenic substrate was tetramethylbenzidine (TMB). The microplate was read within 30 min of adding the stop solution using a spectrophotometer set at dual wavelength 450/595 nm. The standard curve was constructed using 7 calibrators: 0 (S0), 0.075 (S1), 0.15 (S2), 0.3 (S3), 0.9 (S4), 1.2 (S5) and 2.1 (S6) ng mL⁻¹ obtained by diluting the 3 ng mL⁻¹ standard S, supplied with the kit, in diluent. The “0” standard was also treated as a reaction “blank”. Considering the initial dilution factor of the sera, we shall distinguish the concentration measured on the basis of the standard curve (denoted by Cm) from the real concentration of the serum (Cr=20 x Cm).

Analytical validation of assay. Nine assay kits were assessed from three different production lots as follows: 4 kits from lot 1 (L1), 2 kits from lot 2 (L2) and 3 kits from lot 3 (L3). L1 assays were performed by the same operator and L2 and L3 assays were performed under so-called routine conditions (different operators, different intervals between series).

Biological controls. Four biological controls at four different concentration levels, prepared for each lot, were obtained by mixing the sera of breast cancer patients. These pools are denoted by Pij, where “i” is the lot number (i=1, 2, 3) and “j” the concentration level number (j=1, 2, 3, 4). For each lot, the controls for concentration levels 1 and 2, after dilution at 1/20 in accordance with the method described in the technique, were in the lower part of the standard curve: absorbance between 0.050 and 0.150, that is a C between 1.0 ng mL⁻¹ and 3.0 ng mL⁻¹ for level 1; absorbance between 0.15 and 0.35, that is between 2.5 and 7.0 ng mL⁻¹ for level 2. The level 3 control was in the mean absorbance zone between 0.45 and 1.00, that is between 8.0 and 18.0 ng mL⁻¹ and the level 4 control in the upper part of the absorbance range between 1.00 and 2.50, that is between 18 and 30 ng mL⁻¹. Each control was assayed eight times in each assay series in repeatability conditions. The different aliquots of the pools were stored at –20°C during the validity period of the kit.

Validation method. To assess the analytical performance of the assay, the following study protocol was used for analytical examinations of immunoassay kits. The calibration curve was studied for all nine series and the assay of each standard was repeated five times in each series, with determination of: repeatability and reproducibility; response error relationship (RER); linearity and minimum detectable amount or limit of detection (LD); accuracy, (precision and trueness), precision assessment for all three lots, with assessment of intra-series repeatability and intermediate reproducibility, i.e. intra-lot – inter-series in the same laboratory, assessment of trueness for L1 and L2 with on the one hand a dilution test and on the other hand a standard additions or recovery test. For both of these tests, each assay was performed three times in repeatability conditions. The dilution test (P1.4 and P2.4) was assayed at dilutions of 1, 4/5 (0.8), 3/5 (0.6), 1/2 (0.5) 2/5 (0.4), 1/5 (0.2), 1/10 (0.1), 1/15 (0.0667), and 1/20 (0.05). For the recovery tests, the pool P1.2 was spiked volume to volume with each of the standards (0.15, 0.3, 0.9, 1.2, 2.1 and 3 ng mL⁻¹) in l1 and l2.

The analytical specificity of the assay with an analytical interference study was performed.

Study of analytical specificity. Hemolysis: Five whole blood samples from 5 breast cancer patients were centrifuged and 500 μl of non-hemolysed serum were removed. The pellet was resuspended and maintained at –20°C for 30 minutes in order to achieve complete hemolysis. The degree of hemolysis obtained was estimated semi-quantitatively: from + for a low degree to +++ for a high degree. Each serum was assayed for HER2/neu ECD before and after hemolysis.

Influence of human anti-mouse antibodies (HAMA): The diluent supplied with the kit contains mouse antibodies that react with any HAMA present in the sample. By using the “HAMA-ELISA Medac Diagnostika®” assay kit (Medac®, Wedel, Germany) to detect HAMA we were able, in a patient who presented HAMA in immunospectroscopy, to check the absence of analytical interference in the presence of HAMA. HER2/neu ECD concentrations were measured in samples containing HAMA before and after pretreatment of these samples in a heterophilic blocking tube (HBT, Scantibodies®).

Statistical analysis and expression of results. The precision studies were based on International Standard ISO 5725. For the standard range, corresponding to a hierarchical two-factor plan, the series factor being nested within the lot factor, the absorbance reproducibility variation (SDa)² was (SDa)²=(SDL)² + (SDR)² where (SDa)² is repeatability variation, (SDL)² inter-series variation and (SDR)² inter-lot variation.

For the serum pools, the reproducibility variation of the concentrations was determined for each lot, the sera pools differing from one lot to the next, and consequently was the sum of repeatability variation and interseries variation. The detection limit was defined in the usual manner by the concentration corresponding to a signal equal to the mean of the absorbances of the zero concentration standard (C0) plus 3 standard deviations.
The trueness study was based on the one hand on looking for a constant absolute error by comparing with zero the Y-coordinate at the origin of the regression line of measured concentration vs. dilution factor and, on the other hand, on looking for a constant relative error, in the recovery test, by comparing with 1 the slope of the regression line of measured concentration vs. expected concentration. In the study of the standard curve, results are presented as means, standard deviations and coefficients of variation (CV) of absorbances and as percentage of recovery of standard concentrations based on the regression curve equation. In the study of the precision of the control pools assay, results presented are the means of the concentrations of the pools, \( C_m \) and \( C_r \), the repeatability and reproducibility CV, and the repeatability limit \( r \) (\( r=2.8 \, \text{SD}_r \)) and reproducibility limit \( R \) (\( R=2.8 \, \text{SD}_R \)). In the trueness study, results are presented as recovery percentages for the measured concentrations vs. the expected concentrations.

Data were processed using EXCEL® software.

After testing the homogeneity of intra-group variances by Cochran’s test and using Grubbs test to eliminate outliers within groups, analysis of variance (ANOVA) was performed for the precision studies. Analysis of variance of weighted regression was used for the standard curve and trueness studies, a Fisher-Snedecor \( F \)-test was used to study regression linearity, if the coefficient of determination \( r^2 \) was less than 0.998. For a higher value (\( r^2 \geq 0.999 \)), the linear regression model was adopted. Student’s \( t \)-test was used to compare slopes with the value 1 and Y-coordinates at the origin with 0. In all analyses, the significance level \( \alpha \) was 0.05 and \( p \)-values were considered significant at values below or equal to 0.05.

Bioclinical validation. Characteristics of control population: A total of 217 sera samples were taken during the latter stages of breast cancer surgery in patients presenting with breast cancer not overexpressing HER2 (Table I). All of these patients provided informed consent. Eleven samples were also obtained from patients with benign pathologies of the breast and from patients with other types of cancer. Definition of reference value: HER2/neu ECD level were measured in these sera in order to determine a reference value in a population of patients with breast cancer not overexpressing HER2.

Results

Analytical validation. Standard curve: Analysis of variance for hierarchical models was performed to estimate the standard deviation of repeatability and reproducibility of absorbances for each standard. The results obtained enabled us to determine the 95% reproducibility envelope in relation to the mean standard curve (Figure 1). Examination of the different components of variance in this reproducibility revealed that inter-series variation was greater than variation between lots: inter-series CVs expressed in percent were, for standards \( S_1 \) to \( S_6 \) respectively, 19.1, 15.3, 18.9, 15.5, 14.7 and 13.1%, while the inter-lot values were 13.6, 18.2, 14.2, 12.8, 10.1 and 7.6%. Furthermore, these results were used to establish the RER defining absorbance repeatability variance (SD\(_r\))^2 as a function of absorbance \( A \). Several equation models were tested and the best was found to be the parabolic equation model (Figure 2) with a coefficient of determination \( r^2 \) of 0.9884, compared with \( r^2 \) of 0.9523 for the power function and \( r^2 \) of 0.8484 for the exponential function. It was clear that there was no homogeneity of the

\[ \text{Table I. Anatomical and pathological characteristics of control population.} \]

<table>
<thead>
<tr>
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<th>Control population (n=217)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>38-89</td>
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<tr>
<td>Histological type</td>
<td></td>
</tr>
<tr>
<td>CCI(^1)</td>
<td>75%</td>
</tr>
<tr>
<td>CLI(^2)</td>
<td>18%</td>
</tr>
<tr>
<td>CCIS(^3)</td>
<td>2%</td>
</tr>
<tr>
<td>CI(^4)</td>
<td>1%</td>
</tr>
<tr>
<td>Other</td>
<td>4%</td>
</tr>
<tr>
<td>Status of receptors</td>
<td></td>
</tr>
<tr>
<td>ER(^3) and/or PR(^5)&lt;10%</td>
<td>84%</td>
</tr>
<tr>
<td>ER and PR&lt;10%</td>
<td>16%</td>
</tr>
<tr>
<td>Histoprotestic grade SBR(^7)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>25.5%</td>
</tr>
<tr>
<td>II</td>
<td>39.5%</td>
</tr>
<tr>
<td>III</td>
<td>22.5%</td>
</tr>
<tr>
<td>ND(^8)</td>
<td>12.5%</td>
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<tr>
<td>MiB1 or Ki 67 &lt;25%</td>
<td>80%</td>
</tr>
<tr>
<td>&gt;25%</td>
<td>20%</td>
</tr>
</tbody>
</table>

\(^1\)Infiltrating ductal carcinoma, \(^2\)infiltrating lobular carcinoma, \(^3\)ductal carcinoma \textit{in situ}, \(^4\)inflammatory carcinoma, \(^5\)estrogen receptor, \(^6\)progesterone receptor, \(^7\)Scarff–Bloom and Richardson, \(^8\)not defined.
variances, since the Cochran test statistic was 0.595, higher than the critical value of 0.431 at the 5% threshold, and the classic unweighted linear regression model did not fit. Considering the parabolic shape of the equation of absorbance variance as a function of concentration 
\[
(SD_r)^2 = 0.0025c^2 + 0.0011c, \quad r^2 = 0.9843
\]
it was decided to weight each point on the standard curve by the inverse square of the concentration relative to the mean of the weights, i.e. for standard
\[
S_i \ (i=1,2,\ldots,6): \quad w_i = \frac{1}{s_i^2}
\]
Since this weighting did not take into account the zero concentration standard, weighting by inverse variance relative to the mean of the weights was also performed:
\[
\frac{1}{w} = \frac{1}{s_i^2}
\]
Through calculations based on the mean standard curve, we were able to verify its linearity, all the coefficients of determination being above 0.995, and to determine the recovery percentages of the standard concentrations obtained with the three types of regression. Without weighting, the first two calibration points S_1 and S_2 were underestimated, and inverse variance gave an overestimation, in particular of the standard S_1 concentration. These results were substantiated by determining the means of the percentages obtained on the basis of all 9 series. They indicated that inverse square weighting of the concentration seemed to be the most appropriate weighting method both for the mean recovery percentages and the corresponding coefficients of variation, except in the case of the last standard. Inverse square weighting of the concentration was used in the next part of the study. The absorbance detection limit was 0.024, corresponding to a concentration detection limit of the order of 0.017 ng ml\(^{-1}\), i.e. a C_r of 0.34 ng ml\(^{-1}\). The absorbance standard quantification limit, which was ten times the standard deviation of the blanks added to their mean, was 0.051, corresponding to a concentration limit of 0.042 ng ml\(^{-1}\), i.e. a C_r of 0.84 ng ml\(^{-1}\).

**Accuracy study. Precision:** For measured concentrations between 0.060 and 0.140 ng ml\(^{-1}\) (P_1), the CV_r (repeatability coefficients of variation) varied between 6.0 and 8.5%, and CV_R (reproducibility coefficients of variation) between 12 and 13%. The limit of repeatability r varying between 0.012 and 0.023 ng ml\(^{-1}\) and the limit of reproducibility R between 0.025 and 0.042 ng ml\(^{-1}\), these two limits obviously increased with the concentration. For measured concentrations between 0.15 and 0.40 ng ml\(^{-1}\) (P_2), CV_r were around 7%, CV_R between 10 and 15%, r between 0.028 and 0.040 ng ml\(^{-1}\) and R between 0.042 and 0.129 ng ml\(^{-1}\). For measured concentrations between 0.45 and 0.90 ng ml\(^{-1}\) (P_3), CV_r was on average 6%, CV_R was between 5 and 10%, r between 0.073 and 0.128, and R between 0.078 and 0.213 ng ml\(^{-1}\). Finally, for concentrations between 0.9 and 1.5 ng ml\(^{-1}\) (P_4), CV_r varied between 3 and 8%, CV_R between 8 and 15%, r between 0.087 and 0.290 ng ml\(^{-1}\) and R between 0.221 and 0.506 ng ml\(^{-1}\).

**Trueness of assay method:** (i) Dilution test (Figure 3). The observed recovery percentages, Rec(%) = 100 C_m/C_ex, were acceptable, varying between 90 and 110% for the two dilution tests performed. The regression of C_m as a function of the dilution factor was weighted, considering the unequal variances within the dilution factors. The Cochran test statistic, established by inverse variance relative to the mean
of the weights, was 0.654 for L₁ and 0.646 for L₂, higher than the critical value, which was 0.478 at the 5% threshold. This regression can be considered linear (F=2.080 and \( p = 0.010 \) for dilution of pool P₁, F=2.322 and \( p = 0.071 \) for dilution of pool P₂). The \( t \)-coordinates at the origin of the regression line with its standard deviation were 0.0062 and 0.0075 for L₁, and −0.0038 and 0.0044 for L₂. These two \( t \)-coordinates at the origin were not significantly different from zero (\( t = 0.820 \) and \( p = 0.420 \) for L₁, \( t = -0.880 \) and \( p = 0.387 \) for L₂). Thus no evidence was found of a constant absolute error in the assay method.

(ii) Standard addition test (Figure 4): Observed recovery percentages Rec were between 97 and 109% for both recovery tests performed. The regression of \( C_m \) as a function of the expected concentration was weighted considering the heterogeneity of the variations depending on supplementation. The Cochran test statistic was 0.634 for L₁ and 0.647 for L₂, higher than the critical value, which was 0.616 at the 5% threshold. This regression can be considered linear (F=2.770 and \( p = 0.0076 \) for L₁, F=0.689 and \( p = 0.613 \) for L₂). The slope of the regression line and its standard deviation were 0.993 and 0.015 respectively for L₁, and 0.984 and 0.0089 for L₂. These two slopes were not significantly different from 1 (\( t = -0.454 \) and \( p = 0.655 \) for L₁, \( t = -1.834 \) and \( p = 0.086 \) for L₂). Thus, no constant relative error was found in the immunoassay method.

Analytical specificity. Hemolysis. No statistically significant differences were observed between assays of hemolysed serum. It was concluded that hemolysis did not interfere with the HER2/neu ECD ELISA assay.

Human anti-mouse antibodies. No significant difference was found between the two HER2/neu ECD measurements for HAMA quantities ranging between 1,500 and 125,000 ng ml⁻¹.

Bioclinical validation. The HER2/neu ECD reference value in this technique was defined on the basis of a population of 217 patients: the mean value±2 standard deviations was 2.6±1.9 ng ml⁻¹, more than 97% of the population (n=211) presented a level <5 ng ml⁻¹, while fewer than 3% (n=6) presented a level between 5 and 9 ng ml⁻¹. The reference value in this technique was <5 ng ml⁻¹. The HER2/neu ECD level of the 11 patients with benign pathologies of the breast, or with other type of cancer was less than 1.8 ng ml⁻¹.

Discussion

Analytical validation. The manufacturer recommends including the 3 ng ml⁻¹ concentration standard S in the calibration curve. We chose not to include it for two reasons: first, because of the heteroscedastic nature of the variables, absorbances greater than 3 were measured in certain series; second, between 2 and 3 ng ml⁻¹, the standard curve becomes concave. We were thus able to use the linear regression model. However, because of the heteroscedasticity, it was necessary to perform inverse square weighting of the standard concentrations relative to the mean of the weights. This type of weighting, which is constant from one series to the next, was satisfactory except for the last calibration point of 2.1 ng ml⁻¹ for which the recovery concentration was underestimated. Therefore, given the satisfactory dilution test results, we recommend that sera with measured concentrations higher than 1.5 ng ml⁻¹ be diluted.
The detection limit, found at 0.017 ng ml⁻¹ i.e. a Cᵣ of 0.34 ng ml⁻¹, was below the manufacturer’s value of 0.024 ng ml⁻¹ i.e. a Cᵣ of 0.48 ng ml⁻¹. The manufacturer’s calculation method is not known. The quantification limit was half way between the zero standard and the 0.075 ng ml⁻¹ standard. The corresponding Cᵣ for patients with HER2/neu ECD levels below this limit was <0.9 ng ml⁻¹, as mentioned in the results. This quantification limit meets precision requirements since for pool P₁,₁ with a mean measured concentration of 0.071 ng ml⁻¹, the CVᵣ was 6% while the CVᵦ was 12.5%. Furthermore, in the dilution test, the 1/15 and 1/20 dilutions of sera pools P₁,₁ and P₂,₁ enabled measured concentrations between 0.040 and 0.090 ng ml⁻¹, gave recovery values of between 93 and 94%, showing the trueness of the assay in this zone despite a slight underestimation.

Precision was found to be acceptable, regardless of the measured concentration, with CVᵣ values no greater than 8.5%. CVᵦ values were no greater than 15% and, as expected, CVᵦ<10% corresponded to concentrations close to the detection limit. The best CVᵣ and CVᵦ, observed in L₁, might be due to the fact that the same operator performed all four series. In terms of trueness, the dilution test did not reveal the existence of a constant absolute error, nor did the standard additions method reveal the existence of a constant relative error. Thus, the accuracy of the HER2/neu ECD assay using this technique was considered satisfactory for routine laboratory use. The threshold estimated at 5 ng ml⁻¹ confirmed the previous level described with this technique (12, 13).

**Bioclinical validation.** HER2/neu ECD as a circulating tumor marker has considerable clinical potential for monitoring metastatic breast cancer, early detection of relapse and breast cancer progression, monitoring response to hormone therapy or chemotherapy, but not as a predictive factor or for selecting patients for targeted therapy on monitoring response to trastuzumab.

Elevated HER2/neu ECD was correlated in 3 cases out of 4 with HER2-positivity using IHC (14). However, cases of high HER2/neu ECD levels have been observed without HER2 overexpression in IHC and *vice versa*: a difference in the level of activation of receptor cleavage could account for variations in HER2/neu ECD concentrations (15). The HER2/neu ECD level not only reflects expression of HER2 and tumor size, but also activation of receptor cleavage.

HER2/neu ECD levels have been found to be predictive of the progression of HER2-overexpressing breast cancer. In 22 studies involving over 4,000 patients, a correlation was found in 85% of patients between HER2/neu ECD levels, relapse, metastasis and reduced survival (16). Patients with tumors not overexpressing HER2 may, in the early stage or infra-clinical stage of metastatic progression, have circulating HER2/neu ECD levels which rise in correlation with the reduction in disease-free survival and overall survival (17-19).

The use of HER2/neu ECD levels to predict response to chemotherapy or hormone therapy is still a controversial issue (20). Discrepancies between the results of various studies point to the need for standardization of studies and validation of the various assay methods (21-24). High HER2/neu ECD levels have been associated with a reduction in response duration (p<0.005) and early recurrence of metastatic disease (p<0.005) in a phase II multicenter trial to assess the efficacy and tolerance of docetaxel-epirubicin in first-line therapy in metastatic breast cancer (25). Biological monitoring of HER2/neu ECD levels can be used to predict response to trastuzumab and progression-free survival (26). Patients whose HER2/neu ECD levels returned to normal 12 weeks after the start of trastuzumab therapy responded better than those whose HER2/neu ECD levels remained high (p=0.005) (27).

**Conclusion**

In patients with metastatic breast cancer, monitoring that includes measurement of HER2/neu ECD levels should be validated in multicenter studies. The possibility of predicting response to therapy is of interest on an individual level but also more generally as a way of reducing treatment costs.

**References**


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