Clinical and technical evaluation of ACS® BR serum assay of MUC1 gene-derived glycoprotein in breast cancer, and comparison with CA 15-3 assays

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The mucin glycoprotein-detecting assay CA 15-3 is a valuable tool for monitoring the course of disease in breast cancer patients. Assays of CA 15-3 are based on the use of two MAbs to polymorphic epithelial mucin (PEM). We evaluated the technical and clinical performance of the Chiron ACS® BR, an automated competitive chemiluminescence assay using a single MAb, B27.29, and compared the assay’s results with those of the Centocor CA 15-3 RIA, the Abbott IMx CA 15-3, and the Boehringer Mannheim Enzymun-Test CA 15-3. The study population consisted of 253 healthy women, 66 patients with benign breast disease, 168 breast cancer patients, and 76 patients with other carcinomas. In the technical evaluation, we assessed the precision and linearity on dilution of the ACS BR assay. Cutoff values (upper limits of values seen in healthy subjects) were determined for all four assays. Agreement between the assays was studied by linear regression analysis. The ACS BR assay gave within- and between-assay CVs of 2.2% and 3.9%, respectively. Three samples from healthy women gave discordant values by ACS BR and were not included in the calculations. All four assays exhibit a highly similar pattern when monitoring breast cancer disease; the closest agreement of values was obtained between ACS BR and Centocor CA 15-3. We conclude that the ACS BR assay is a fast and reliable immunoassay for measuring PEM in serum. Although it detects a slightly different epitope on the PEM molecule than is targeted in other assays, for cancer serum samples it agreed better with the original Centocor CA 15-3 assay than did the other two CA 15-3 assays tested.

INDEXING TERMS: polymorphic epithelial mucin • mucin glycoproteins • tumor markers • chemiluminescence immunoassay • monoclonal antibodies • method comparisons • ROC curve analysis

The human MUC1 gene codes for a mucin glycoprotein that is expressed on the ductal cell surface of most glandular epithelia [1]. This glycoprotein, also called polymorphic epithelial mucin (PEM) [2], plays a role in cell protection and lubrication. In malignant conditions, cell polarity is lost such that PEM is expressed on the entire cell surface and is shed into the circulation. PEM may also interfere with cellular adhesion and thus play a role in the metastatic process [3]. Various monoclonal antibodies (MAbs) have been raised against epitopes present on this heavily glycosylated protein. These MAbs form the basis for the development of serum assays valuable in the management of, among others, breast cancer patients [4].

The first commercially available PEM assay is the Centocor (Malvern, PA) CA 15-3® RIA [5]. The assay uses two MAbs, directed to epitopes located on the tandem repeat forming the peptide core of the extracellular domain of the PEM molecule. One MAb, DF3 [6], is directed to an epitope in the DTRPAGS region of the tandem repeat; the other MAb, 115D8 [7], is directed to a peptide carbohydrate epitope on the same repeat [8]. To improve the analytical performance of the Centocor CA 15-3 RIA, researchers developed automated assays that were based on ELISA or microparticle enzyme immunoassay (MEIA) technologies.

The Chiron Diagnostics (formerly Ciba Corning, E. Walpole, MA) ACS® BR, a single-determinant assay, is based on MAb B27.29 [9], which recognizes the 8-amino-
Materials and Methods

SERUM SAMPLES AND STUDY POPULATION

The clinical evaluation included a total of 693 serum samples. These were obtained from 253 apparently healthy women (mean age, 53; median, 56; range, 37–76 years), and from patients admitted to our hospital between 1987 and 1992 for treatment of benign (n = 66; mean age, 49; median, 48.5; range, 18–76 years) or malignant breast disease (n = 168). Breast cancer serum samples were obtained before treatment at the time of diagnosis (n = 127; mean age, 59.5; median, 61; range, 29–87 years). Serial serum samples (n = 136) were taken from 30 breast cancer patients (3–11 per patient) being monitored during the course of disease and under treatment for a recurrence. We also analyzed 16 serum samples from breast cancer patients with advanced stage disease and 19 serum samples from breast cancer patients with no evidence of disease. For comparison, sera obtained from 76 patients with various other malignancies were also included. Samples were aliquoted and stored frozen at –70 °C until assayed.

All clinical charts were reviewed for diagnosis, histology, and staging; only patients who were thoroughly documented were included in the current study. Procedures followed were in accordance with the Helsinki declaration of 1975, as revised in 1983, and in accordance with the guidelines for research of our institute.

PEM ASSAYS

ACS BR. The ACS BR assay is a competitive, chemiluminescence immunoassay for detecting PEM in serum. Purified antigen, isolated from ZR-75-1 breast cancer cells and immobilized on paramagnetic microparticles, competes for binding to MAb B27.29 with the MUC1 gene-derived antigen present in calibrators and samples [9]. The MAb is conjugated to acridinium ester as a tracer (BR Lite reagent), and the signal is measured by a luminometer. The assay is run on the Chiron ACS:180® system, a fully automated, random-access immunoassay system, for which no predilution of samples is required. The ACS BR assay is calibrated against a master curve. The concentrations of the seven calibrators used in the master curve range from 0 to 475 kU/L (U = arbitrary units); the range is extended to 2250 kU/L by utilizing the autodilute feature of the ACS:180. The bar-coded master curve information is supplied with each new lot number of solid phase and Lite reagent. The system requires a two-point recalibration every 7 days to adjust it to the master curve. For this purpose, purified breast cancer antigen at low (~29 kU/L) and high (~250 kU/L) concentration is dispersed into a reaction cuvette simultaneously with the paramagnetic particles and Lite reagent. After incubation at 37 °C for 7.5 min, during which the competition reaction takes place, the microparticles are separated and washed twice. Starter reagent is then added and the emitted light is measured with a luminometer. The relative light units measured are in inverse correlation to the concentration of PEM in the tested sample. The ACS:180 system can be operated either in a batch-processing mode, if large numbers of samples are to be assessed, or in a random-access mode, for single analyses. In both cases, the analyzer is capable of testing 180 samples per hour.

Centocor CA 15-3. The CA 15-3 RIA is a nonautomated heterologous double-determinant RIA with the capture antibody MAb 115D8 (raised against human milk fat globule membranes) coated onto a solid phase of plastic beads and the tracer 125I-labeled MAb DF3 (raised against a membrane-enriched fraction of a human breast carcinoma [5]). In a first step, a specimen is prediluted 1:50 in kit diluent and then is added to the solid phase. After a 2-h incubation, unbound material is removed by washing. In a second step, 125I-labeled MAb DF3 is added and, after a 3-h incubation and washing, the bound radioactivity is quantified with a gamma counter. The signal is proportional to the concentration of PEM in the specimen.

Enzymun-Test CA 15-3. The Enzymun-Test CA 15-3 assay (Boehringer Mannheim) is a heterologous MEIA in which MAb 115D8-coated microparticles are mixed and incubated with prediluted patients’ sera.

Fig. 1. Amino acid sequence in the epitope region to which MAb B27.29 and MAb DF3 are directed, present on the peptide tandem repeat as part of the MUC1 gene-derived mucin molecule.
Alkaline phosphatase-conjugated DF3 tracer antibody is added, which then complexes with the antigen bound to MAb 115D8. The assay is run on an automated IMx test system [12].

All assays were performed in one laboratory according to the manufacturers’ instructions. The laboratory staff had no knowledge of the corresponding clinical data while performing this analysis.

**TECHNICAL EVALUATION**

The analytical performance of the ACS BR was assessed by evaluating (a) the within-run imprecision (three samples, at low, middle, and high PEM concentrations, run 20 times in duplicate); (b) the between-run imprecision (the same three samples run on 23 consecutive working days); and (c) the linearity on dilution (analysis of seven samples with concentrations of 222-1012 kU/L, diluted stepwise as many as eight times).

**STATISTICAL CONSIDERATIONS AND DATA ANALYSIS**

The agreement between the four assays was evaluated by least-squares linear regression analysis [13] with SPSS-PC software (SPSS, Chicago, IL). The relative difference between the results obtained by the ACS BR assay and by each of the three CA 15-3 assays taken individually was calculated as \[ \frac{[(\text{CA 15-3 assay value} - \text{ACS BR value})/\text{CA 15-3 assay value}] \times 100\%} \]

The upper limit of the reference interval for healthy individuals was determined, and the diagnostic value of each assay was also evaluated by receiver-operating characteristic curve analysis [14]. Monitoring graphs obtained from individual patients with all four assays were compared.

**Results**

**TECHNICAL EVALUATION**

Reproducibility of ACS BR assay. Within- and between-assay CVs are shown in Table 1. Within-assay imprecision was lowest in the high-concentration range (CV 2.2%) and highest in the median range (CV 3.9%). Interassay precision experiments gave CVs ranging from 5.6% to 3.9% for the low- and high-concentration control samples, respectively.

ACS BR linearity on dilution. The correlation coefficients for the seven serially diluted serum samples ranged from 0.8615 to 0.9893, with recoveries ranging from 95% to 105% (Fig. 2).

**Correlation between the assays.** As Table 2 shows, the linear regression analysis correlation coefficients \(r\) for ACS BR vs Centocor CA 15-3 range from 0.8567 in healthy controls to 0.9845 in all cancer patients. For Enzymun-Test CA 15-3 vs ACS BR, \(r\) ranged from 0.8763 to 0.9781 in healthy controls and in all cancer patients, respectively. For IMx CA 15-3 vs ACS BR, \(r\) ranged between 0.9070 in healthy controls to 0.9818 in pretreatment breast cancer patients.

The relative difference between the results obtained in benign breast disease and in cancer patients with the ACS BR assay and each of the three other PEM assays taken individually is illustrated in Fig. 3. The trend of relative differences was closest to zero over the whole range of values when comparing ACS BR with Centocor CA 15-3, whereas IMx CA 15-3 gave lower PEM values than ACS BR throughout this range. In the low-concentrations range, ACS BR gave higher results than Enzymun-Test CA 15-3, whereas for high PEM concentrations ACS BR results were lower than Enzymun-Test CA 15-3 results (Fig. 3).

**CLINICAL EVALUATION**

Cutoff values. According to the 95th percentile of results for healthy women, the cutoff values (kU/L) for each assay were as follows: ACS BR, 39; Centocor CA 15-3, 35; Enzymun-Test CA 15-3, 30; IMx CA 15-3, 26. These values were calculated from the results obtained from 250 healthy women, after exclusion of 3 samples with outlier ACS BR values, which were reanalyzed separately (Table 3). ACS BR assay results for these three samples were discordant with those obtained by the CA 15-3 assays and were therefore not included in the calculations. Analysis performed on samples obtained from the same women in two subsequent years again gave discordant above-normal ACS BR test results. All samples were rerun by ACS BR at Chiron Diagnostics, which show less-discordant ACS BR results in all three series. Western blot analysis revealed low concentrations of PEM in all samples. Sample 1 (Table 3), first year, was serially diluted to test for
Recovery in the 1:2 dilution was 96%, but results for further step-wise dilutions were not linear and showed increasingly lower recoveries (i.e., 65%, 57%, 33%, and 8%, respectively). Possible interfering factors such as high concentrations of protein or triglycerides were not found in the samples.

Benign breast diseases. Measurements in sera obtained before treatment from patients with benign breast disease (Table 4) revealed median assay values ranging from 15 kU/L for IMx CA 15-3 to 19 kU/L for ACS BR. At the cutoff value of 30 kU/L advised by the manufacturers, the lowest number of false-positive test results was obtained with the IMx CA 15-3 assay (1.5%), whereas the highest number of false positives (6.1%) was found with both the Centocor CA 15-3 and the Enzymun-Test CA 15-3 assays. The number of false-positive test results with ACS BR was 4.5%. At the cutoff values based on the 95th percentile of results for healthy controls determined in this study, however, we obtained true-positive rates ranging between 26% for the Centocor CA 15-3 and 7.9% for the ACS BR. The diagnostic value of the assays for detecting breast cancer, with a cutoff value based on the 95th percentile of results for healthy subjects, is given in Table 5.

Receiver-operating characteristic curves comparing all four assays for breast cancer patients vs healthy controls and patients with benign breast disease are given in Fig. 4. The greatest area under the curve was found with the Centocor CA 15-3 assay (0.64); all three automated assays had essentially similar areas (0.5), but these were not significantly different from that for the Centocor assay. Table 4 lists results according to stage for breast cancer patients sampled before treatment (n = 127). Serum concentrations for all four assays in node-negative and node-positive patients are also given. Comparison is made between serum PEM concentrations in breast cancer patients with recurrent or progressive disease and a high tumor load, and those in breast cancer patients with no evidence of disease. The lowest median pretreatment concentrations determined by ACS BR were found in those patients having carcinoma in situ of the breast (median 14 kU/L); the highest were in the patients with metastatic breast disease (median 111 kU/L). No significant difference in ACS BR-determined PEM serum con-
centrations was seen in relation to the presence or absence of nodal metastasis. However, the difference in ACS BR results for patients with a high tumor load (median 294 kU/L) and those having no evidence of disease (median 25 kU/L) was significant ($P < 0.0001$). Similar results were obtained with the CA 15-3 assays.

Other malignancies. The highest median serum concentrations for all four assays were obtained in patients with ovarian cancer, for whom values ranged between 38 kU/L by ACS BR and 26 kU/L by IMx CA 15-3 (Table 6). The lowest serum concentrations were found in patients with cervical cancer, the median values ranging between 15 kU/L by ACS BR and 12 kU/L by Enzymun-Test CA 15-3 and IMx CA 15-3.

Breast cancer follow-up. Serial serum samples were obtained during follow-up in 30 patients with breast cancer. PEM serum concentrations measured during the course of disease revealed a highly similar pattern for all four assays. Some examples are shown in Fig. 5. Increasing concentrations of this marker correlated with recurrent or progressive disease, whereas decreasing values indicated regressive disease. In none of the patients with no evidence of disease were assay results above normal.

Discussion
PEM, derived from the MUC1 gene located on chromosome 1q21 [2], is overexpressed in malignant mammary epithelial cells and has formed the basis for development of MAb-based serum assays that have been valuable in the management of, among others, breast cancer patients. High serum concentrations of mucin marker correlate with advanced stage of disease, and response to treatment is reflected in decreasing PEM concentrations in serum. In the follow-up of breast cancer patients, increasing concentrations are an indication for disease recurrence [4]. The individual value of such assays in the discrimination between benign and malignant breast tumors is limited because of the low positivity rate in the early stages of breast cancer; i.e., these assays have rather low sensitivity and are specific for neither tumors in general nor breast cancer.

Among the PEM assays included in this study, the one evaluated most extensively in pretreatment sera from patients with breast tumors is the Centocor CA 15-3 RIA. Pons-Anicet et al. [15] found above-normal results by Centocor CA 15-3 in 27% of patients with local disease, compared with 54% in patients presenting with positive lymph nodes and 91% in those with metastatic disease. In patients with metastatic carcinoma, the highest values reported by Centocor CA 15-3 were seen in patients with liver and visceral metastases; the lowest were those for patients with skin and soft tissue metastases [16–19]. High pretreatment serum concentrations of PEM are strongly indicative of the presence of distant metastases and can, therefore, be an important tool in the choice of...
primary therapy, supporting confirmation or exclusion of distant metastases before therapy mode decisions are made [20].

As a double-determinant, nonautomated RIA, the Centocor CA 15-3 assay is time- and sample-consuming and has environmental side effects. Therefore, faster and easier to handle CA 15-3 assays, run on automated systems, have been developed. Further, PEM-based serum immu-

### Table 3. PEM concentrations (kU/L) in sera from healthy women (n = 250).

<table>
<thead>
<tr>
<th></th>
<th>ACS BR</th>
<th>Centocor CA 15-3</th>
<th>Enzymun-Test CA 15-3</th>
<th>IMx CA 15-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>22</td>
<td>19</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Median</td>
<td>21</td>
<td>18</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>SD</td>
<td>8.8</td>
<td>8.8</td>
<td>7.1</td>
<td>6.4</td>
</tr>
<tr>
<td>Range</td>
<td>5.2–58.4</td>
<td>3.7–61.7</td>
<td>0.35–49.2</td>
<td>4.3–45.2</td>
</tr>
<tr>
<td>p95</td>
<td>39</td>
<td>35</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>Discordant valuesa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>416 (327) [310]</td>
<td>19</td>
<td>14 (13) [13]</td>
<td>10 (12) [10]</td>
</tr>
<tr>
<td>2</td>
<td>104 (100) [69]</td>
<td>7</td>
<td>9 (8) [9]</td>
<td>7 (7) [7]</td>
</tr>
<tr>
<td>3</td>
<td>40 (36) [29]</td>
<td>10</td>
<td>8 (10) [8]</td>
<td>7 (8) [7]</td>
</tr>
</tbody>
</table>

*a Serum from three healthy women, measured in three consecutive years: (), 2nd year sample; [], 3rd year sample.

### Table 4. Assay values (kU/L) in sera from patients with benign and malignant breast disease according to stage, nodal involvement, and tumor load.

<table>
<thead>
<tr>
<th>Patient’s status</th>
<th>n</th>
<th>ACS BR</th>
<th>Centocor CA 15-3</th>
<th>Enzymun-Test CA 15-3</th>
<th>IMx CA 15-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment, benign breast disease</td>
<td></td>
<td>Median</td>
<td>Range</td>
<td>% &gt;p95</td>
<td>Median</td>
</tr>
<tr>
<td>Positive</td>
<td>66</td>
<td>19</td>
<td>6–36</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Negative</td>
<td>75</td>
<td>20</td>
<td>1–55</td>
<td>8.3</td>
<td>23</td>
</tr>
<tr>
<td>Follow-up for progressive disease</td>
<td></td>
<td>Median</td>
<td>Range</td>
<td>% &gt;p95</td>
<td>Median</td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
<td>294</td>
<td>49–3184</td>
<td>100.0</td>
<td>216</td>
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<tr>
<td>Negative</td>
<td>19</td>
<td>25</td>
<td>9–47</td>
<td>15.8</td>
<td>19</td>
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</tbody>
</table>

*a All pretreatment breast cancer patients.

### Table 5. Diagnostic value of the assays in breast cancer.

<table>
<thead>
<tr>
<th>Cutoff (p95), kU/L per method</th>
<th>ACS BR: 38.6</th>
<th>Centocor CA 15-3: 35.0</th>
<th>IMx CA 15-3: 26.1</th>
<th>Enzymun-Test CA 15-3: 29.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivitya</td>
<td>7.9</td>
<td>26.0</td>
<td>12.6</td>
<td>13.4</td>
</tr>
<tr>
<td>Specificityb</td>
<td>100.0</td>
<td>97.0</td>
<td>93.9</td>
<td>92.4</td>
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<tr>
<td>Pos. pred. valuec</td>
<td>100.0</td>
<td>94.3</td>
<td>80.0</td>
<td>77.3</td>
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<tr>
<td>Neg. pred. valued</td>
<td>36.1</td>
<td>40.5</td>
<td>35.8</td>
<td>35.7</td>
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<tr>
<td>Test accuracyd</td>
<td>39.4</td>
<td>50.3</td>
<td>40.4</td>
<td>40.4</td>
</tr>
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</table>

*a Percentage of patients with breast cancer with above-normal (>p95) test results.
*b Percentage of subjects with no breast cancer with test results not above normal.
*c Probability that a positive test result will truly be associated with breast cancer.
*d Probability that a negative test result will truly be associated with absence of breast cancer.
*e Proportion of the total test results that are correctly positive or negative.
noassays, which detect other epitopes on the PEM molecule, have also been developed in an attempt to improve the clinical performance of the tumor marker by increasing its sensitivity for breast cancer. Examples of such assays are assays for mucin-like carcinoma-associated antigen [21], based on MAb b-12, and CA M29 and CA M26 [22], which use MAbs M38, M29, and M26 for detection of epitopes on the same PEM molecule. The recently developed ACS BR serum immunoassay uses a single MAb, B27.29 [9], which recognizes a specific 8-amino-acid sequence within the 20-amino-acid tandem repeating sequence of the core protein of PEM [10].

In the present study comparing ACS BR with the original, manual Centocor CA 15-3 RIA and two established automated CA 15-3 assays, the analytical performance of the ACS BR assay appears to be excellent, especially in the higher PEM concentration range (within-assay CV 2.2% and between-assay CV 3.9%). Dilution experiments showed recoveries of between 95% and 105% for concentrations ranging from 222 to 1012 kU/L. ACS BR results for patients with breast cancer correlated best with the original Centocor CA 15-3 RIA results, especially in the high concentration range ($r = 0.9845$). The trend of relative differences was closest to zero over the whole range of values for the comparison of ACS BR with Centocor CA 15-3 (Fig. 3).

The reference value for Centocor CA 15-3 as obtained in healthy women is higher than that recommended by the manufacturer, confirming results of a previous study [23]. Among 253 healthy women, 3 showed discordant ACS BR serum results for which we found no explanation (Western blot analysis revealed normal PEM concentrations in these sera). The discord seems to be specific to the subject rather than to sample, because results obtained in the samples taken from the same women 2 and 3 years later were again discordant (Table 3). Inasmuch as CA 15-3 and ACS BR are not suited for screening purpose, this problem is predominantly of scientific interest. Further research on the subject is ongoing at Chiron Diagnostics and at our institute. No circulating antibodies to MUC-1 were present in these samples.

The clinical performance of the ACS BR assay, which uses a MAb detecting an epitope on the PEM molecule that overlaps with the epitopes recognized in the other assays, was equal to that of the CA 15-3 assays. In sera from patients with malignancies other than breast cancer, the highest values were found in patients with ovarian cancer, which is in concordance with earlier reports [24]. High results were obtained in breast cancer patients with advanced stage of disease and high tumor load, low results in breast cancer patients with an early stage of disease or no evidence of disease and in patients with benign breast lesions. Our findings confirm the study by Dnistrian et al. [25], in which BR27.29, a competitive inhibition RIA using MAb B27.29, was evaluated in breast cancer patients; those authors concluded that increased concentrations determined with the BR27.29 assay, as with CA 15-3 [20], are associated predominantly with late-stage disease. Although the Centocor CA 15-3 assay showed a slightly better performance than the automated assays, the clinical value of PEM-detecting serum immunoassays for the diagnosis of breast cancer is limited (Table 5, Fig. 4).

In the breast cancer patients followed during the course of disease, we observed similar marker patterns for

<table>
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<tr>
<th>Site</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
<th>% &gt;p95</th>
<th>Median</th>
<th>Range</th>
<th>% &gt;p95</th>
<th>Median</th>
<th>Range</th>
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<td>50</td>
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<td>60</td>
<td>26</td>
<td>8–107</td>
<td>50</td>
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<tr>
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<td>26</td>
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<td>5–120</td>
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<tr>
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<td>10–76</td>
<td>18</td>
<td>24</td>
<td>6–31</td>
<td>0</td>
<td>25</td>
<td>6–32</td>
<td>27</td>
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<td>8–45</td>
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<td>6–46</td>
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all four assays. As we saw when monitoring patients during the course of disease, the trend of the values reflects disease activity more accurately than does the absolute value of the marker; we therefore recommend that one and the same assay be used in follow-up of individual patients. Further prospective studies should reveal the clinical significance of ACS BR and CA 15-3 assays in the early detection of recurrent disease in breast cancer patients.

In summary, ACS BR is a fast, reliable, and reproducible assay for quantifying PEM in serum of breast cancer patients. It has an excellent analytical performance and the advantage of an automated easy-to-handle assay system. Although based on a single MAb directed to an overlapping epitope on the PEM molecule, ACS BR correlates better with the original Centocor CA 15-3 RIA for samples from breast cancer patients than do the automated CA 15-3 assays included in this study.

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