



# Increased alpha-fetoprotein receptor in the serum of patients with early-stage breast cancer

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## ABSTRACT

The alpha-fetoprotein (AFP) receptor (RECAF) is an oncofetal antigen found in most types of cancer. Using a competitive radioimmunoassay, we measured the concentration of serum RECAF in three sets of samples.

Set 1 was blind and consisted of 119 normal subjects, 43 breast cancer patients (stages I and II), and 20 patients with benign breast conditions. In this set, the assay discriminated normal from cancer samples with a receiver operating characteristic for the area under the curve (ROC<sub>AUC</sub>) of 0.983; with 95% specificity and 93% sensitivity at a cut-off of 4.6 K (arbitrary) RECAF units; and with 72% sensitivity and 100% specificity at a cut-off of 7.3 K units. At 7.3 K units, the specificity for benign breast conditions was 85%, and the sensitivity was 72% (ROC<sub>AUC</sub> was 0.773). Carcinoembryonic antigen and cancer antigen 15-3 respectively showed 39% and 41% sensitivity, with 95% specificity in comparisons of normal with cancer samples, and 34% and 44% sensitivity, with 85% specificity in comparisons of benign with cancer samples. Set 2 consisted of 353 normal, 30 benign, and 64 cancer samples (stages II and III). The RECAF assay sensitivity in discriminating normal from cancer samples was 97%, with 97% specificity. Benign compared with cancer samples showed 87% sensitivity, with 97% specificity. Set 3 included only 40 normal and 40 cancer samples. The assay sensitivity was 89%, with 100% specificity. Sets 2 and 3 were not tested with carcinoembryonic antigen and cancer antigen 15-3.

These results strongly suggest that the RECAF assay could be used for detecting breast cancer in its early stages.

## KEY WORDS

Alpha-fetoprotein, AFP, AFP receptor, RECAF, CA15-3, CEA, serum, tumour marker, biomarker, oncofetal antigen, sensitivity, specificity, radioimmunoassay, serum test, early-stage, breast cancer

## 1. INTRODUCTION

Alpha-fetoprotein (AFP) is a major fetal serum protein<sup>1-4</sup> that is synthesized mainly by the yolk sac and liver<sup>2,5</sup>. After birth, circulating levels of AFP drop sharply, virtually disappearing from the blood of normal adult individuals<sup>2</sup>.

Immature cells from most fetal structures internalize AFP, and it has been suggested that the purpose of that internalization—and one of the functions of this protein—is to transport and deliver polyunsaturated fatty acids into fetal cells<sup>6,7</sup>. The AFP uptake ceases when embryonic cells and tissue structures approach a high degree of differentiation, even if the blood concentration of AFP is still high or increasing<sup>8-10</sup>.

*In vitro* and *in vivo* studies showed that malignant cells regain the ability to take up AFP via a receptor that would be present in undifferentiated cells of either embryonic or tumour origin<sup>10-15</sup>, but mostly absent in normal adult cells. The existence of such a receptor for AFP (RECAF) was then demonstrated and functionally characterized in several cell lines<sup>16-18</sup>.

Because RECAF is found in a variety of cancers and in fetal cells (but not in their mature counterparts), the receptor falls within the definition of a wide-spectrum oncofetal antigen with potential for cancer diagnosis, screening, and follow-up.

Previous immunohistology work using the same polyclonal antibodies described herein showed a clear difference in RECAF staining between stomach cancer cells and nonmalignant cells<sup>19</sup>, and RECAF concentrations have been reported to be higher in serum from a variety of cancer patients than in serum from patients with benign proliferating diseases or from normal subjects<sup>20</sup>.

In the present study, we describe a serum RECAF radioimmunoassay that can detect the early stages of breast cancer. The test is capable of distinguishing cancer patients from healthy individuals—and from those with benign lesions—with a degree of accuracy so far unattainable with other cancer markers.

## 2. METHODS

### 2.1 Preparation of Mammary Cancer Cell Extracts

The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and grown in RPMI medium containing 10% fetal calf serum. Before extraction, the cells were incubated for 1 hour at 37°C in serum-free RPMI medium to deplete them of bovine AFP. The cells were then trypsinized and re-suspended at a concentration of  $5 \times 10^7$  per millilitre of TBS medium (0.05 mol/L Tris-HCl plus 0.1 mol/L NaCl, pH 7.5). Next, the suspension was sonicated for 2 minutes in an ice bath using a Sonic Dismembrator (Thermo Fisher Scientific, Waltham, MA, U.S.A.) at 32 W, followed by centrifugation for 10 minutes at 14,000 rpm in an Eppendorf Microfuge (Eppendorf Canada, Mississauga, ON). The total protein concentration of the supernatants was 7–12 mg/mL as determined using a commercial protein assay (Total Protein: Bio-Rad Laboratory, Hercules, CA, U.S.A.). After 0.02% thimerosal was added, the cell extracts were stored at –20°C until used in the experiments.

### 2.2 RECAF Purification

Affinity chromatography on an AFP-agarose column was used to purify the RECAF. The AFP was purified from the supernatant of HEP-2 culture medium by affinity chromatography using an anti-AFP monoclonal antibody. One milliliter MCF-7 extract was incubated for 4 hours, at room temperature and under gentle agitation, with 10 mL AFP-agarose in 0.05 mol/L Tris-HCl (pH 6.5). After thorough washing, the bound RECAF was eluted using 0.8 mol/L KCl in the same buffer. The eluates were concentrated to 300–1000 µg/mL using a Centricon spin filter (Millipore Corporation, Billerica, MA, U.S.A.).

### 2.3 Preparation and Purification of Polyclonal Rabbit Antibody Against RECAF

Rabbits were immunized intradermally with a 50:50 emulsion of 1 mg purified RECAF in 1 mL of phosphate-buffered saline (PBS) and 1 mL Freund's complete adjuvant. Two boosts, each containing 0.5 mg RECAF in incomplete Freund's adjuvant, were administered at 2-week intervals. The rabbits were bled from the ear vein 1 week after the last boost. The blood was allowed to clot overnight at room temperature, and the serum was centrifuged and stored at –30°C until further use. The immunoglobulin G fraction of the anti-RECAF rabbit antiserum was isolated on a protein A Sepharose column (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) according to the manufacturer's instructions.

### 2.4 Polyacrylamide Gel Electrophoresis and Western Blot

Polyacrylamide gels containing 10% sodium dodecylsulfate were used in a Bio-Rad polyacrylamide gel electrophoresis apparatus following the Laemmli procedure. The sample buffer consisted of 4 mL distilled water, 1 mL 0.5 mol/L Tris-HCl, 0.8 mL glycerol, 1.6 mL 10% sodium dodecylsulfate, 0.4 mL 2-mercaptoethanol, and 0.2 mL 0.05% (weight:volume) bromophenol blue. Gels were run for 2 hours at a constant current of 0.2 A (70–100 V) in a 1× Tris-glycine running buffer in a Bio-Rad system (Mini-Protein: catalog number 165-3301). Western blots followed standard procedures, using a Bio-Rad Mini-Transblot apparatus (catalog number 170-3930) and nitrocellulose membranes (0.45-µm pore: Bio-Rad, catalog number 162-0145). After blocking the membranes with 3% fish gelatin or 1% bovine serum albumin in TBS, the RECAF bands were evidenced by incubating the membrane with a suitable concentration of anti-RECAF antiserum or pure AFP biotinylated with Sigma-Aldrich NHS-biotin according to the manufacturers' instructions. Color development was obtained using diaminobenzidine and H<sub>2</sub>O<sub>2</sub> after the membranes had been incubated with a commercial conjugate (Sigma-Aldrich) of either streptavidin (for the biotinylated AFP) or anti-rabbit immunoglobulin labeled with horseradish peroxidase.

### 2.5 RECAF labeling with <sup>125</sup>I

The RECAF was radioiodinated with Na<sup>125</sup>I using the chloramine T method<sup>21</sup>, with minor modifications. Briefly, 40 µg pure RECAF in a volume of 435 µL was mixed with 127 µL 0.4 mol/L sodium phosphate buffer (pH 7.4), to which was then added 37 MBq of Na<sup>125</sup>I in a volume of 10 µL. Then, 20 µL chloramine T 1 mg/mL in H<sub>2</sub>O was added, and the mixture was incubated at room temperature for 60 seconds. The reaction was stopped with 20 µL sodium metabisulfite 2.5 mg/mL in phosphate buffer. After 60 seconds, 40 µL 1% Ovalbumin (Sigma-Aldrich) in PBS and 40 µL blue dextran solution were added. The <sup>125</sup>I-RECAF was then separated from free iodine on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) made with a disposable 5-mL pipette equilibrated with PBS, pH 7.4. The specific activity of the labeled <sup>125</sup>I-RECAF ranged from 74 kBq to 370 kBq per microgram of protein (3.7–11.1 kBq/µL).

### 2.6 Immunoassays

**2.6.1 Carcinoembryonic Antigen and Cancer Antigen 15-3**  
Circulating levels of carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3) were measured at the Institut für Klinische Chemie, Munich, using the AxSYM system (Abbott Laboratories, Abbott

Park, IL, U.S.A.) for CEA and the Elecsys system (Hoffmann–La Roche, Nutley, NJ, U.S.A.) for CA15-3.

### 2.6.2 Radioimmunoassay Using $^{125}\text{I}$ -RECAF

The RECAF determinations were made at the Pacific Biosciences Research Centre facilities in Vancouver, British Columbia.

The test was designed as a solid-phase competitive immunoassay in which a constant amount of  $^{125}\text{I}$ -RECAF competed with RECAF in the serum sample for binding to the anti-RECAF antibody immobilized on the plastic plate. The 96-well plates (LockWell MaxiSorp; Nalge Nunc International, Rochester, NY, U.S.A.) were coated overnight, at 4°C, with 100  $\mu\text{L}$ /well of 20  $\mu\text{g}/\text{mL}$  rabbit anti-RECAF protein A purified immunoglobulin G in 0.1 mol/L carbonate buffer (pH 9.5). All subsequent steps were carried out at room temperature. After being washed 3 times with  $\text{dH}_2\text{O}$ , the wells were blocked for 2 hours with 3% fish gelatin (Sigma–Aldrich) in TBS. A mixture of 50  $\mu\text{L}$  serum and 50  $\mu\text{L}$   $^{125}\text{I}$ -RECAF at 100 ng/mL was then transferred to the wells and incubated for 2 hours. The wells were washed 3 times with  $\text{dH}_2\text{O}$ , each well was separated from the plastic frame, and radioactivity was measured on a gamma counter (ISODATA 20/10; Global Medical Instrumentation, Ramsey, MN, U.S.A.).

### 2.7 Human Sera

Blood was collected and processed according to approved standard protocols. The study included blind

samples and open samples (in the latter, the diagnosis was previously known to the person conducting the assay). All samples were collected before treatment was administered; the diagnoses were histologically confirmed before the start of the present study.

In a routine clinical laboratory procedure, blood that had been drawn in sterile tubes without chemicals or in S-Monovette SST tubes (Sarstedt, Nümbrecht, Germany) was allowed to clot and was then centrifuged. Aliquots of serum were stored at  $-20^\circ\text{C}$ . Before samples were tested for RECAF, they were thawed, heated for 30 minutes at  $56^\circ\text{C}$ , and supplemented with 0.02% thimerosal.

### 2.8 Age and Circulating RECAF

Levels of RECAF had previously been measured in 260 healthy girls and women 13–96 years of age, distributed as follows: <20 years ( $n = 2$ ), 20–29 years ( $n = 22$ ), 30–39 years ( $n = 41$ ), 40–49 years ( $n = 70$ ), 50–59 years ( $n = 64$ ), 60–69 years ( $n = 30$ ), 70–79 years ( $n = 18$ ), and  $\geq 80$  years ( $n = 13$ ). Linear regression analysis of RECAF against age yielded  $R^2 = 0.01$  ( $F = 0.11$ ), indicating that age does not influence the level of circulating RECAF. Table 1 gives the age data for each group.

The three sample sets used in the study were:

- Blind samples (Set 1)  
The samples in Set 1 were collected and blinded at the Institut für Klinische Chemie in Munich before being sent to be tested for RECAF. The 182 blinded serum samples included 43 samples from

TABLE 1 Descriptive statistics<sup>a</sup> for the receptor for alpha-fetoprotein (RECAF) in normal, benign, and cancer samples

Sample description	Samples (n)	Age <sup>b</sup> (mean/median)	Mean	SD	Percentile		Min	Max	Median
					5	95			
<i>Set 1 (blind)</i>									
Normal	119	Not available	3.19	0.9	1.6	4.52	1.1	5.00	3.24
Benign	20	45.0/47.0	6.04	1.55	4.25	9.08	3.14	9.77	5.57
Cancer	43	59.2/58.0	7.83	1.88	4.25	10.5541	3.3	11.30	8.04
<i>Set 2 (open)</i>									
Normal	353	48.8/48.0	3.66	0.63	2.65	4.47	2.36	5.3	3.71
Benign	30	45.6/45.5	4.35	0.71	3.27	5.26	3.13	5.3	4.35
Cancer	64	55.1/55.0	6.67	1.35	4.92	10.11	4.45	10.66	6.49
<i>Set 3 (open)</i>									
Normal	40	60.7/62.0	3.07	0.63	1.94	4.08	1.73	4.16	3.04
Cancer	40	56.1/55.0	6.71	1.73	4.19	9.39	3.19	10.75	6.39
<i>All sets combined</i>									
Normal	504	50.0/49.0 <sup>c</sup>	3.51	0.74	2.36	4.47	1.10	5.30	3.59
Benign	50	45.4/46.0	5.03	1.39	3.27	7.44	3.13	9.77	5.00
Cancer	147	56.6/55.0	7.02	1.70	4.35	10.30	3.19	11.30	6.77

<sup>a</sup> RECAF values expressed in (arbitrary) K units.

<sup>b</sup> Not related to circulating RECAF (see text).

<sup>c</sup> From Sets 2 and 3.

SD = standard deviation.

patients with stage I and II breast cancer, 20 from patients with breast benign conditions (fibroadenoma,  $n = 12$ ; galactorrhea,  $n = 1$ ; nonmalignant suspicious mammography,  $n = 2$ ; mastopathy,  $n = 1$ ; papilloma of the lactiferous ducts,  $n = 1$ ; cysts and abscesses of the breast,  $n = 3$ ), and 119 from healthy control subjects.

- Open samples (Sets 2 and 3)

To increase the number of cases tested with RECAF, two additional sample sets were tested. Sets 2 and 3 were obtained at a site different from that where Set 1 had been collected. In Sets 2 and 3, neither CEA nor CA15-3 was measured. Set 2 consisted of 447 serum samples: 64 from patients with breast cancer stages II and III, 30 from patients with various benign conditions (fibroadenoma, breast keratosis, chronic mastitis, cysts, papilloma of the lactiferous ducts, fibrocystic disease—henceforth called “benign” samples), and 353 from healthy control subjects (henceforth called “normal” samples). Set 3 consisted of 40 samples from healthy control subjects and 40 from breast cancer patients at unspecified stages. No benign samples were included in Set 3.

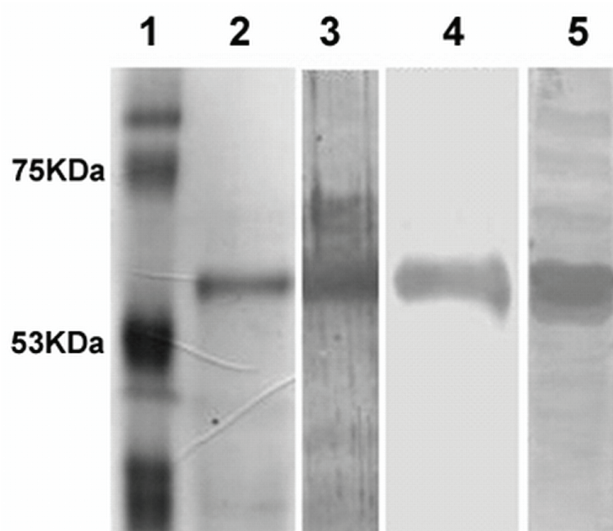
## 2.9 Standard Curve

Several dilutions of the MCF-7 cell extract were used to create a standard curve calibrated in arbitrary “RECAF units” that allowed for the RECAF measurements to be normalized from one experiment to another. The dilutions were carried out in 3% fish gelatin–TBS and were processed in the same manner as the serum samples. All sample readings were within the range of the standards. To extrapolate values from the standard curve, we used the Logit/Log function.

## 3. RESULTS

### 3.1 Purified RECAF and Anti-RECAF Polyclonal Antiserum Characterization

The radioimmunoassay design required pure RECAF. Figure 1 shows that the RECAF preparation used for rabbit immunizations and for radiolabelling has only 1 band with a molecular weight of approximately 62 kDa. That band is recognized by both biotinylated-AFP and the rabbit anti-RECAF antiserum. The monospecificity of the antiserum was verified against a total cell extract of MCF-7 as shown in lane 5 of Figure 1. The biotinylated-AFP also detected a faint 67-kDa band that was absent from the Western blot done with the antiserum. (It is worth noting that the AFP receptor has previously been described as a 62/67 kDa doublet found both in soluble form and associated to membranes<sup>22,23</sup>.) The rabbit antiserum also inhibited the binding of biotinylated-AFP to RECAF (data not shown), which indicates that they both recognize the same RECAF epitope.



**FIGURE 1** Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis and Western blot of rabbit antibody to receptor for alpha-fetoprotein (RECAF). Lane 1: Molecular weight (MW) markers stained with Coomassie blue. Lane 2: Pure RECAF preparation (MW: approximately 62 kDa) used for labeling. Lane 3: Western blot of the pure RECAF preparation using biotinylated alpha-fetoprotein (AFP). Lane 4: Same as lane 3, using the rabbit anti-RECAF antibody instead of AFP to detect RECAF. Lane 5: Western blot on whole extract of the MCF-7 cell line, developed with the rabbit anti-RECAF antibody, showing that the antiserum is monospecific (only 1 major stained band at approximately 62 kDa).

## 3.2 Data Analysis

### 3.2.1 Assay Reproducibility and Precision

Ten blind samples distributed within the range of measured RECAF values were repeatedly tested to determine the intra- and inter-sample variability of the assay. The intra-sample coefficient of variation was  $\leq 6\%$  and the inter-assay coefficient of variation was  $< 10\%$ .

### 3.2.2 Serum RECAF Values and Cut-Off Value Determination

All 3 sample sets showed large differences in serum RECAF when normal or benign samples were compared with samples from cancer patients (Table 1 and Figure 2).

**Set 1 (Cancer Stages I and II, Blind Samples):** The  $p$  value of an independent  $t$ -test (assuming unequal variances) comparing normal with cancer samples was  $4.78 \times 10^{-22}$ . When benign and cancer samples were compared, the  $p$  value of the  $t$ -test was  $1.3 \times 10^{-4}$ .

Figure 3 depicts the receiver operating characteristic (ROC) curves for CEA, CA15-3, and RECAF when normal samples were compared with samples from cancer patients. For RECAF, the area under the curve (AUC) was 0.983. Using a cut-off value of 4.6 K RECAF units, the sensitivity was 93%, with 95% specificity. At 95% specificity, the CEA sensitivity was 39% (AUC: 0.723) and the CA15-3 sensitivity was 41% (AUC: 0.739).

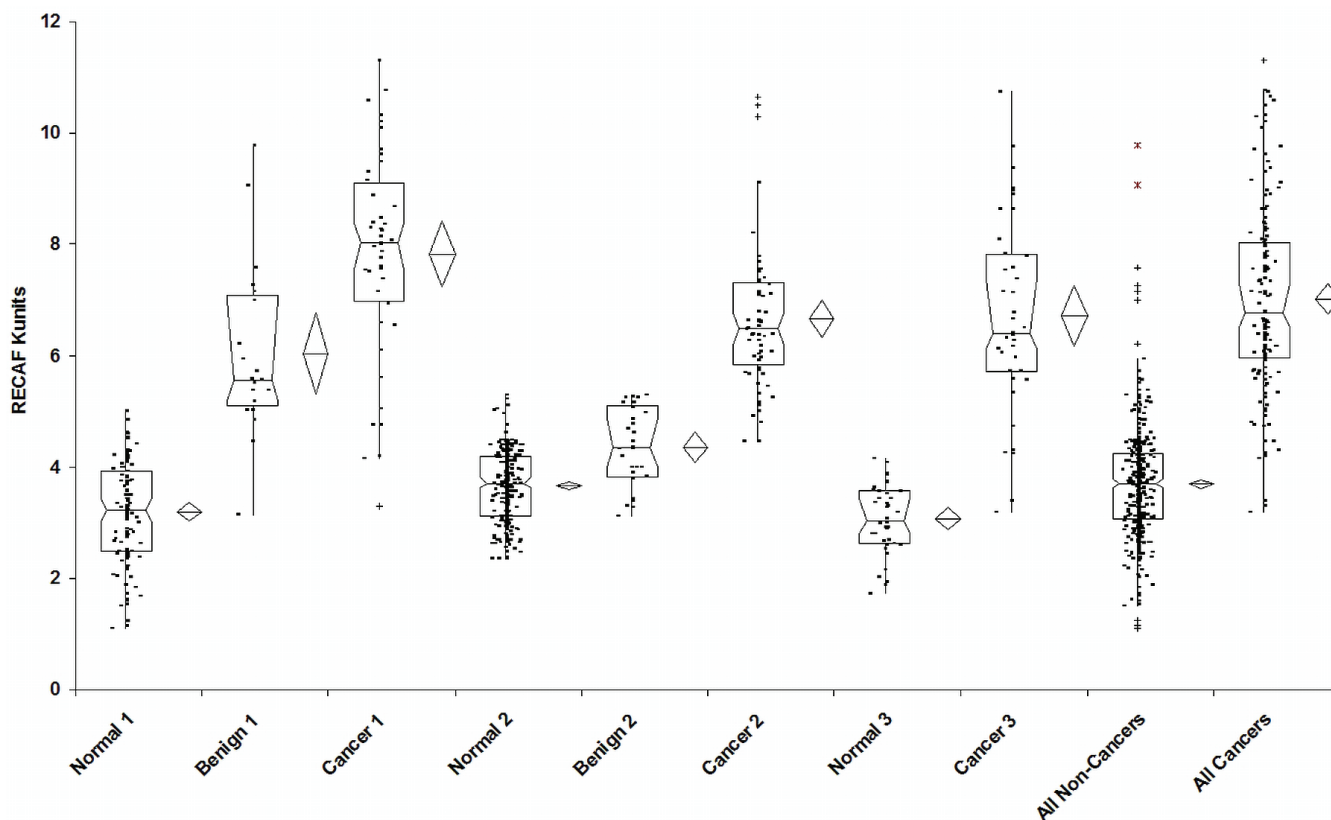


FIGURE 2 Comparative distribution of samples from normal subjects, patients with benign breast lesions, and patients with breast cancer for each set of values and for all values combined. The notched box shows the median, the lower and upper quartiles, and the confidence interval around the median. Whiskers extend to the furthest observations within  $\pm 1.5$  interquartile ranges (IQRs) of the 1st or 3rd quartile. Observations outside 1.5 IQRs are marked (+) as near outliers; those outside 3.0 IQRs are marked (\*) as far outliers.

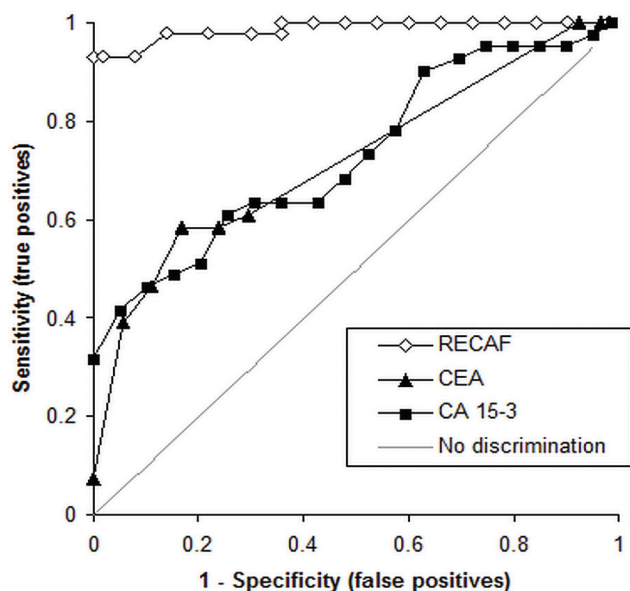


FIGURE 3 Receiver operating characteristic curves for the receptor for alpha-fetoprotein (RECAF) in samples from cancer patients and from normal subjects [area under the curve (AUC): 0.987], for carcinoembryonic antigen (CEA, AUC: 0.723), and for cancer antigen 15-3 (CA 15-3, AUC: 0.739).

Increasing the RECAF assay cut-off to 7.30 K units to attain 100% specificity, 72% detection of early breast cancers resulted. At 100% specificity, the CEA and CA15-3 sensitivities were 32% and 7.3% respectively.

When benign samples were compared with samples from cancer patients (Figure 4), the higher RECAF cut-off fared better. At 7.3 K units, the sensitivity was 72%, with 85% specificity (AUC: 0.773). On the same samples, at 85% specificity, the sensitivities of the CEA and CA15-3 tests were 34% and 44% (AUC: 0.626) and 44% (AUC: 0.685) respectively.

**Set 2 (Cancer Stages II and III, Open Samples):** Figure 5 shows the RECAF ROC curves comparing control and benign samples with samples from cancer patients in Sets 2 and 3. At a cut-off value of 4.61 K RECAF units, the sensitivity was 97% and the specificity was 97.5% (AUC: 0.99). When benign and cancer samples were compared using a cut-off of 5.3 K units, the sensitivity was 87%, with 97% specificity (AUC: 0.97).

**Set 3 (Unspecified Cancer Stages, Open Samples):** Set 3 included only control and cancer samples. In the ROC analysis, at 4.53 K RECAF units, the sensitivity was 89%, with 100% specificity (Figure 5). The AUC was 0.98.

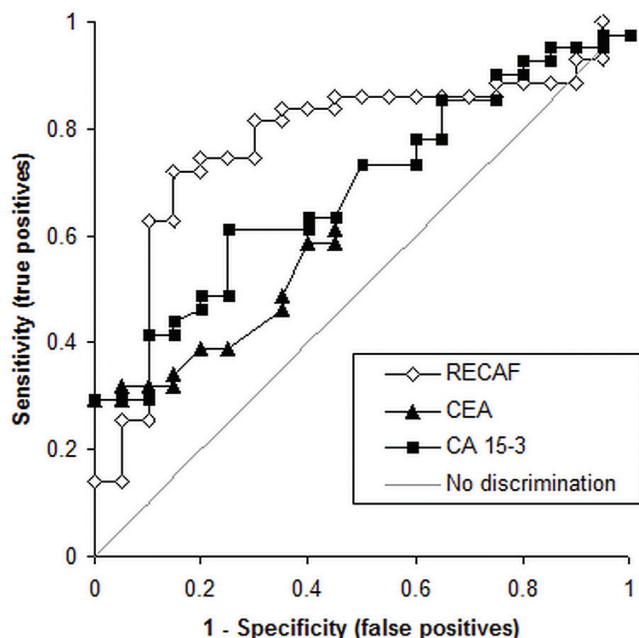


FIGURE 4 Receiver operating characteristic curves for the receptor for alpha-fetoprotein (RECAF) in samples from cancer patients and from patients with benign lesions of the breast [area under the curve (AUC): 0.77], for carcinoembryonic antigen (CEA) (AUC: 0.723), and for cancer antigen 15-3 (CA 15-3) (AUC: 0.739).

Figure 5 also shows the RECAF ROC curves obtained after combining the normal, benign, and cancer samples from all sets.

#### 4. DISCUSSION AND CONCLUSIONS

The results presented here show that a RECAF-based serum immunoassay can discriminate, with high sensitivity and specificity, 504 normal subjects and 50 patients with benign breast lesions from 147 patients with breast cancer. More importantly, the assay can detect the early stages of breast cancer with a sensitivity and specificity unattainable to this point with other cancer markers.

Selecting a low cut-off value (4.6 K RECAF units) maximizes cancer detection: sensitivity of 93%, with 95% specificity against normal subjects. In the blind group (Set 1), increasing the cut-off to 7.3 K units resulted in 100% specificity against normal subjects, and yet, against early cancers, the sensitivity remained relatively high (72%).

The sensitivity and specificity values in the blind samples (Set 1) were obtained when samples from stages I and II breast cancer were tested. At those stages, the 5-year survival rates are 87% and 75% compared with 46% and just 13% for stages III and IV<sup>24</sup>. By contrast, the sensitivity reported by an expert panel from the American Society of Clinical Oncology for CEA in stage I breast cancer was only 10%; for stage II, it was 19% (both measured at 95% specificity<sup>25</sup> against normal subjects). In the same

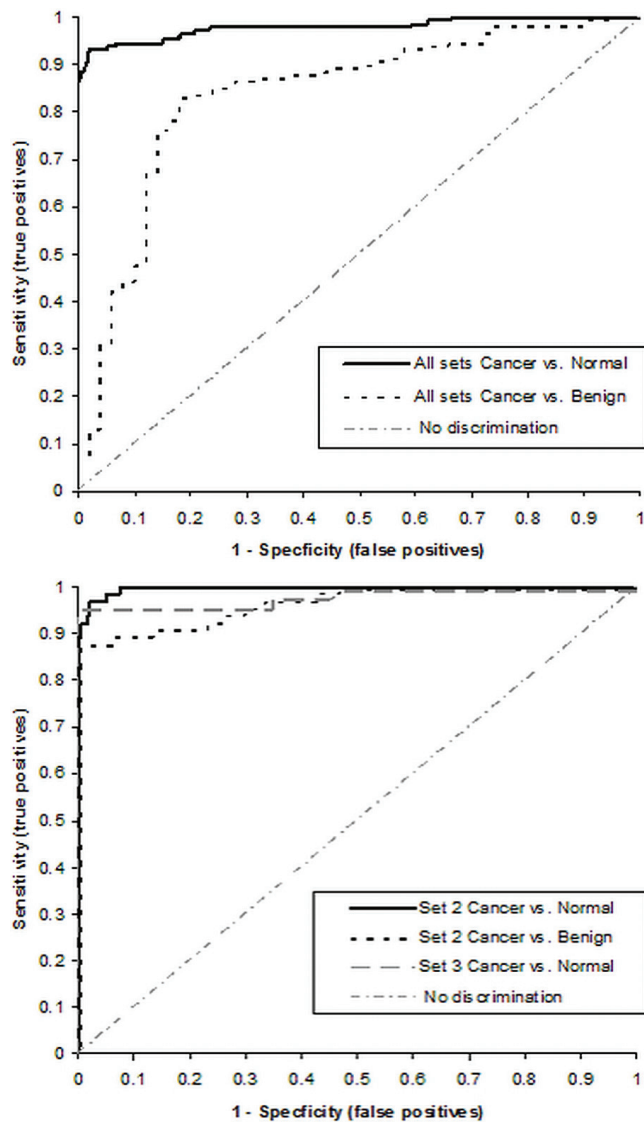


FIGURE 5 Receiver operating characteristic curves for the receptor for alpha-fetoprotein (RECAF) for (A) Sets 2 and 3 (open samples), and (B) for all 3 sets combined.

study, the sensitivity reported for CA15-3 was 9% in stage I cancer and 19% in stage II, with a specificity of 95% against normal subjects and a specificity of 80% against benign breast lesions<sup>26</sup>. In the present study, the sensitivity exhibited by the CEA and CA15-3 tests was higher (39% and 41% respectively, both at 95% specificity). When sera from cancer patients were compared with sera from patients with benign breast lesions, the sensitivities for CEA and CA15-3 were also higher than the published data already mentioned; and yet, the performance of the RECAF assay was significantly better than either one of those two markers.

Using a cut-off value of 7.3 K units, the discrimination of positive cases among benign sera in the blind samples of Set 1 was 15%, which is slightly less

than the 20% reported for CA15-3<sup>25</sup>. In two thirds of benign samples, testing showed levels of less than 6 K RECAF units.

The results from Sets 2 and 3 (open samples) were consistent with those from the blind group (Set 1), thus significantly expanding the number of samples.

It is unclear why some benign lesions are RECAF-positive. It is common knowledge that, in general, cancer markers detect a certain percentage of benign lesions. Several explanations are possible:

- Some benign lesions are considered premalignant, and the expression of cancer-associated markers might precede the morphology changes detected by a pathologist. Using *TP53* as a prognostic marker, Rohan *et al.* concluded that p53 protein accumulation appears to be associated with an increased risk of progression to breast cancer in women with benign breast disease<sup>27</sup>. The most recognizable premalignant lesion of the breast is atypical hyperplasia. However, unfolded lobules and ductal hyperplasia could be considered earlier premalignant epithelial abnormalities<sup>28</sup>. The risk of a benign lesion evolving into cancer appears to be related to the degree of epithelial atypia<sup>29</sup>, with fibroadenomas having the lowest risk. Interestingly, one of the patients in Set 1 had a histologic diagnosis of fibroadenoma, and yet, among the benign sera samples, hers had the highest amounts of circulating RECAF. A year later, this patient was diagnosed with breast cancer. Removing her from the benign group in Set 1 would increase the specificity of the test from 85% to 89%.
- Another reason that the discrimination between benign and cancer samples might be worse than the discrimination between normal and cancer samples is that healthy subjects do not normally undergo biopsy, but patients with benign lesions do. This difference introduces an additional source of error that can be measured by studies of agreement among pathologists examining the same breast lesion slides<sup>30,31</sup>.

Future work on the biology of RECAF might explain why some benign breast lesions test positive and might perhaps provide insight into how to better interpret the results of the RECAF test.

## 5. CONFLICT OF INTEREST DISCLOSURES

RM and JGT receive remuneration from the company that owns the intellectual property rights for use of the RECAF marker. PS has no financial conflicts of interest to disclose.

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