

# Evaluation of the effectiveness of serum aromatase activity and tissue polypeptide specific antigen in the diagnosis of breast cancer and prediction of lymph node metastatic status

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## Abstract

*Aim: The aim of this study was to evaluate the effectiveness of serum tissue polypeptide specific antigen (TPS) and aromatase activity in the detection of primary breast cancer and prediction of lymph node metastasis.*

*Subjects and methods: Serum samples were obtained from 35 postmenopausal breast cancer females before surgery and 35 normal healthy volunteers. The clinicopathological data of patients were obtained from their pathological reports. Determination of serum TPS was done using a ready-for-use ELISA kit. Serum total estradiol and total testosterone were determined using ready-for-use RIA kits and the ratio of estradiol to testosterone (E2/T) was used as an indirect expression of aromatase activity. Univariate and multivariate logistic regression analysis were performed to evaluate the association between these biomarkers and breast cancer, as well as lymph node metastasis.*

*Results: For breast cancer prediction, either serum TPS or aromatase activity can be used with significant areas under the ROC curves of 97% & 91.8%, respectively. For lymph node metastasis prediction, serum aromatase activity alone had the best predictive value, with a significant area under the ROC curve of 80.3%. Multivariate logistic regression analysis in which aromatase activity and TPS were included in one model gives 74% adequacy in prediction of lymph node metastasis.*

*Conclusion: To predict breast cancer, either serum TPS or aromatase activity can be applied, while, for prediction of lymph node metastasis serum aromatase activity is recommended.*

*Key words: Aromatase activity, tissue polypeptide specific antigen, breast cancer.*

## Introduction:

Clinical examination, mammography, fine needle aspiration cytology and open surgical biopsy are established diagnostic methods in breast cancer and the final diagnosis of cancer should be based on histopathology.<sup>1</sup> In breast cancer, many tumor markers have been studied in the hope for finding a blood test for cancer diagnosis, but none of them has had such sensitivity and specificity that it could replace conventional diagnostic methods.<sup>2</sup>

A number of serum markers are used as

indicators for breast cancer. Of these, the most widely used are CA 15-3 and CEA. However, lack of sensitivity and specificity preclude the use of these serum markers for the early detection of breast cancer. There is an urgent need for cancer biomarkers with more accurate diagnostic capability, particularly for early-stage cancer.<sup>3</sup> Several clinical studies have been published on tissue polypeptide specific antigen (TPS) determination in women with breast cancer with sensitivity ranging from 31-78%.<sup>4,5</sup>

Tissue polypeptide specific antigen (TPS) is related closely to human cytokeratin 18, which is an essential part of the protein cytoskeleton of epithelial cells.<sup>6</sup> As opposed to CA 15-3, which serum concentration provides data about tumor mass, serum TPS levels indicate proliferative activity of malignant tissue even if the mass is small.<sup>7</sup> Elevated concentrations of TPS have been detected in association with a wide variety of malignancies, including breast cancer.<sup>8</sup>

Human aromatase, a member of the cytochrome P450 superfamily, is a 58 kDa. It catalyzes a complex reaction sequence that results in the conversion of androgens (C19), namely testosterone and androstenedione, into estrogens (C18), namely estradiol, and estrone, respectively.<sup>9</sup> In women of reproductive age, the ovaries express high levels of aromatase and they are the main source of estrogens. After menopause peripheral tissues including breast, lung, brain, and liver become sites of estrogen synthesis as a result of aromatization of circulating androgens.<sup>10</sup> Aromatase expression is elevated in certain malignancies, such as breast and endometrial carcinomas.<sup>11</sup>

The aim of this study was the comparative evaluation of aromatase activity and TPS concentrations in sera from patients with breast cancer before surgery and normal healthy controls. The diagnostic values of the assayed serum parameters were compared using the Receiver Operating Characteristic (ROC) curve analysis. The correlation between biomarker levels and breast cancer histopathological characteristics was determined. We also investigated whether any of the biomarkers or their combined measurement could be used in the prediction of lymph node involvement. Univariate survival analysis of patients was assessed using the Kaplan Meier method.

#### Subjects and methods:

##### Sample size calculation:

Sample size was calculated using NCSS 2000 (Number Cruncher Statistical System) and PASS (Power Analysis and Sample Size) Program. According to the results obtained by Hang & Rong-cheng for TPS,<sup>12</sup> the minimum sample size required was 40 (20 for breast cancer patients group and 20 for control group)

to achieve 80% power to detect a difference in means of 236 between both groups with estimated group standard deviations of 351 and 35 and with a significance level (alpha) of 0.05 using a two-sided sample t-test.<sup>13</sup>

Seventy postmenopausal females were enrolled in this retrospective case-control study. A female was considered postmenopausal if more than 12 months passed since her last menstrual period. Females were divided into two groups: Group I (breast cancer patient's group): It included 35 female patients with breast invasive ductal carcinoma of clinical stages II and III 14 (recently detected, not receiving surgery or chemotherapy). Their mean age was (51.33±2.12) years. Patients were recruited from the Departments of Experimental and Clinical Surgery and Cancer Management & Research of the Medical Research Institute, Alexandria University in the period from October 2007 to May 2008. Group II (control group): It included 35 normal healthy volunteers of comparable age (52.80±3.50), menstrual cycle and socioeconomic status as patients.

This research protocol was approved by the ethical committee of Medical Research Institute. Each patient underwent full history recording, thorough clinical examination, routine laboratory investigations including complete blood count (CBC), mammography of breast and ultrasonography of abdomen and liver, radiological investigations including X-ray chest, CT scan and bone scan when needed and fine needle aspiration cytology (FNAC) of breast mass to establish the pathological diagnosis in the patients.

Pathologic information was obtained from pathology reports. Collected data included tumor size and grade, lymph node involvement and status of estrogen receptor (ER) and progesterone receptor (PR). Breast cancer was clinically staged by the oncologist according to the tumor-nodes-metastasis (TNM) classification.<sup>14</sup>

All 35 breast cancer patients were subjected to surgery (Modified Radical Mastectomy),<sup>15</sup> then received adjuvant combination chemotherapy [5-Fluorouracil, Adriamycin and Cyclophosphamide (FAC)]<sup>16</sup> for 6 cycles. Breast cancer patients were evaluated after 3

and 6 cycles of chemotherapy clinically, laboratory and radiologically to estimate the clinical response. Also, these patients were followed up till May 2011 for assessment of overall survival.

#### Laboratory investigations:

Blood samples were collected from normal healthy volunteers and breast cancer patients before surgery. Immediately after withdrawing, blood samples were allowed to coagulate and were centrifuged for 20 minutes at 3500 rpm. The separated serum samples were aliquoted, frozen at -80 °C, and stored until assay time. After thawing, each serum aliquot was assayed only once. Determination of serum levels of tissue polypeptide specific antigen (TPS), total testosterone and estradiol were carried out at Radiation Sciences Department, Medical Research Institute, Alexandria University.

#### Determination of serum Tissue Polypeptide Specific Antigen (TPS):

Serum TPS levels were determined using a ready-for-use ELISA kit (DRG diagnostics, Germany) according to the manufacturer's protocol. Briefly, 50 µl of each calibrator and patient's serum sample were added into each well. 50 µl of the diluted HRP-conjugated Anti-TPS solution to each well. Wells were shaken on a rack shaker (600 rpm) for 2 hrs at room temperature. Wells were decanted and washed with 6 x 0.3 ml diluted wash solution. 200 µl tetramethyl-benzidine (TMB) substrate solution were added per well and incubated in darkness for 20 min. 50 µl of stop solution were added per well and the wells were mixed well. Absorbance of every well was read at 450 nm. A standard curve was constructed from which the concentration of TPS in each serum sample was determined. The assay sensitivity was 6 U/L with no detectable cross reactivity to other tumor associated antigens that may be present in serum.

#### Determination of serum total testosterone:

Serum total testosterone levels were determined using a ready-for-use solid-phase radioimmunoassay kit (RIA, Siemens, USA) according to the manufacturer's protocol. Briefly, 50 µl of each calibrator and patient's

serum sample were pipetted into the test tubes. 1.0 ml of <sup>125</sup>I-labeled total testosterone tracer was added to every tube. Tubes were mixed well and incubated for 3 hrs at 37 °C, and then tubes were decanted thoroughly. Bound radioactivity in each tube was counted for 1 minute in a gamma counter (Perkin Elmer, Finland). A calibration curve was constructed from which the concentration of total testosterone (ng/dl) in each serum sample was determined. The assay sensitivity was 4 ng/dl with high specificity for testosterone.

#### Determination of serum total estradiol:

Serum total estradiol levels were determined using a ready-for-use solid-phase radioimmunoassay kit (RIA, Siemens, USA) according to the manufacturer's protocol. Briefly, 100 µl of each calibrator and patient's serum sample were added into the test tubes. 1.0 ml of <sup>125</sup>I-labeled estradiol tracer was added to every tube. Tubes were mixed well and incubated for 3 hrs at room temperature, and then tubes were decanted thoroughly. Bound radioactivity in each tube was counted for 1 minute in a gamma counter (Perkin Elmer, Finland). A standard curve was constructed from which the concentration of estradiol (pg/ml) in each serum sample was determined. The assay sensitivity was 8 pg/ml with high specificity for estradiol.

#### Estimation of serum aromatase activity:

Each serum value of estradiol (pg/ml) was multiplied by 10 to be converted into its corresponding ng/dl unit. According to several studies, aromatase activity in each serum sample from every patient and normal healthy volunteer was estimated indirectly as the ratio of the total estradiol (ng/dl) level divided by total testosterone level (ng/dl); (E2/T).<sup>17-19</sup>

#### Statistical analysis:

##### A- Data processing:

Data processing had two major objectives namely clean data by performing a series of comprehensive checks and producing analytic results which involved the recoding of variables.

i. Recoding of variables: The quantitative variables were recoded using different cut

off points defined by ROC analysis to include them in the analysis.

- ii. Exploration of the quantitative data for normality distribution.

B- Data analysis included:

- 1- Kolmogorov- Smirnov test for data exploration.
- 2- Calculation of the median and range for the quantitative variables.
- 3- Mann Whitney test was performed to compare between the medians of the two groups.
- 4- Logistic regression analysis is used to assess the impact of interaction between two predictors as TPS and aromatase on the lymph node involvement in breast cancer patients.
- 5- ROC curve analysis was done to detect the area under the curve (overall accuracy) and different cut off points with the associated sensitivity and specificity.
- 6- Survival analysis was done including

survival life tables, Kaplan Meier's curves and Log Rank test to compare between two factors regarding the survival. P value less than 0.05 was considered statistically significant.

Results:

1- The diagnostic value of semm TPS and aromatase activity:

Serum TPS level and aromatase activity were significantly elevated in the breast cancer patients group compared with the control group Table(1). Comparing the diagnostic values of serum TPS and aromatase activity using the receiver operating characteristic (ROC) curve analysis showed significant greater area under the curve for TPS (97%) compared with that for aromatase activity (91.8%). The sensitivity and specificity were 97.1% and 85.7% at cut-off 67.5 U/L for TPS and 86% and 89% at cut-off 0.09 for aromatase activity Figure(1) and Table(2).

Table (1): TPS and aromatase activity in the breast cancer patients and normal healthy control groups.

Biomarker		Control group (n=35)	Breast cancer patient's group (n=35)
TPS (U/L)	Median	33.0000	143.0000*
	Range	(10.00-100.00)	(60.00- 243.00)
Aromatase activity (E2/T)	Median	0.0667	0.1600*
	Range	(0.01- 0.09)	(0.00 – 1.10)

\*:Significance was considered at P-value < 0.05  
n: Number of subjects in each group

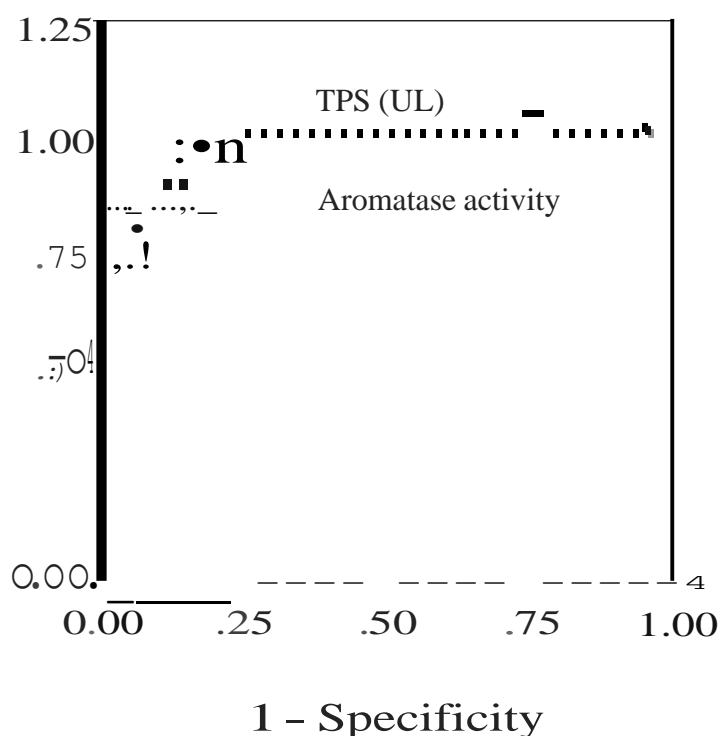


Figure (1): Graphical representation of the ROC curves for serum TPS and aromatase activity for detection of breast cancer.

Table (2): Area under the curve (AUC), cut-off, sensitivity and specificity for serum TPS (U/L) and aromatase activity for detection of breast cancer.

Variables	AUC (%)	P-value	Cut-off	Sensitivity (%)	Specificity (%)
TPS (U/L)	97	0.02*	67.5	97.1	85.7
Aromatase activity (E2/T)	91.8	0.01*	0.09	86	89

\*:Significance was considered at P-value < 0.05

#### IT-Correlation of serum TPS and aromatase activity with breast cancer clinicopathological characteristics:

Serum TPS has no correlation with any of the clinicopathological characteristics of breast cancer patients. While aromatase activity was significantly correlated with lymph node involvement and tumor clinical stage Table(3). Comparison of the power of each serum marker to predict lymph node metastatic status using ROC curve yield a significant greater AUC for aromatase activity (80.3%) than that of TPS (38.9 %). Also, from ROC

curves, the sensitivity and specificity of aromatase activity were 76.2% & 85.8% at cut-off 0.155, while the sensitivity and specificity of TPS were 71.4% & 21.5% at cut-off 92.5 U/L Figure(1) and Table(4). Multivariate logistic regression analysis in which TPS and aromatase activity were included in one model gives AUC of 74%.

Relationship between serum TPS and aromatase activity and patients' overall survival:

Kaplan-Meier analysis revealed that patients with elevated levels of serum TPS and

aromatase activity had a survival non-significantly shorter than those who had lower

levels of these biomarkers as shown in Table(S).

Table (3): Serum levels of TPS and aromatase activity [median (range)] in relation to clinicopathological characteristics of breast cancer patients.

Clinicopathological characteristic	TPS (U/L)	Aromatase activity (E2/T)
Tumor size		
≤5 Cm3	173 (60- 243)	1.10 (0.0-1.10)
>5Cm3	174 (60- 234)	0.82 (0.10-0.92)
Lymph node involvement		
Negative	159 (75- 234)	0.25 (0.05- 0.30)
Positive	183 (60- 243)	1.10 (0.00-1.10)*
Clinical stage		
II	127(90- 217)	0.06 (0.05- 0.11)
III	183 (60- 243)	1.10 (0.00-1.10)*
Pathological grade		
I&II	183 (60- 243)	0.92 (0.00- 0.250)
III	145 ( 72- 217)	1.00 (0.10- 1.10)
ER		
Negative	134 (100-234)	0.28 (0.10- 0.38)
Positive	183 (60- 243)	1.10 (0.00- 1.10)
PR		
Negative	174 (60- 234)	0.85 (0.07- 0.92)
Positive	173 (70- 243)	1.10 (0.00-1.10)

\*:Significance was considered at P-value < 0.05  
ER & PR: Estrogen and progesterone receptor

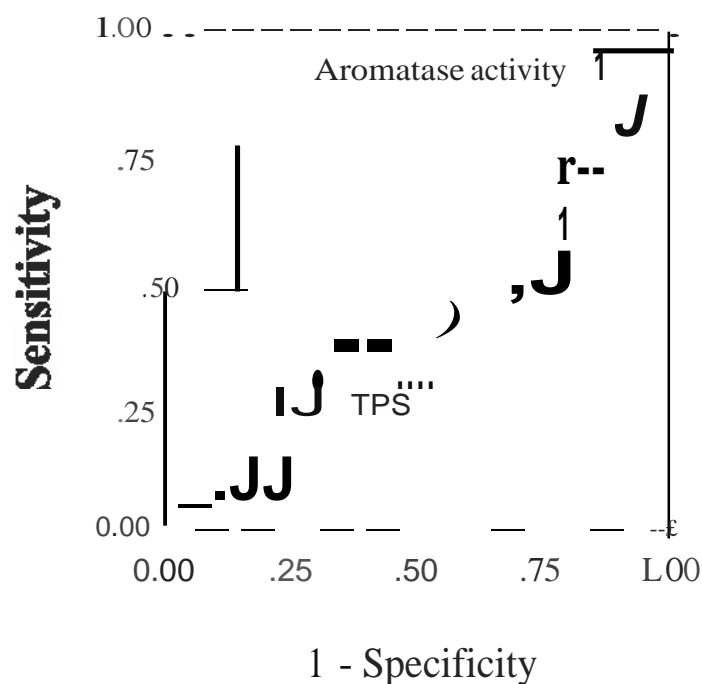


Figure (2): Graphical representation of the ROC curves for serum TPS (U/L) and aromatase activity for prediction of lymph node involvement in breast cancer patients group.

Table (4): Area under the curve (AUC), cut-off, sensitivity and specificity for serum TPS (U/L) and aromatase activity for prediction of lymph node involvement in breast cancer patients group.

Variables	AUC(%)	P-value	Cut-off	Sensitivity (%)	Specificity (%)
TPS (U/L)	38.9	0.027*	92.5	71.4	21.5
Aromatase activity (E21T)	80.3	0.003*	0.155	76.2	85.8

\*:Significance was considered at P-value < 0.05

Table (5): Correlation between serum TPS levels, aromatase activity and overall survival (months) among breast cancer patients group.

	Cut off values	Survival time (months) Mean±SE	95% Confidence Interval		P-Value
			Lower bound	Upper bound	
TPS (UIL)	67.5 (Negative)	38.33 ±6.01	23.30	46.86	P=0.941
	>67.5 (Positive)	35.08 ±2.35	32.47	41.67	
Aromatase activity (E2/T)	<0.09 (Negative)	41.20±1.32	38.60	43.80	p = 0.605
	.09 (Positive)	35.36±3.28	28.93	41.79	

**Discussion:**

Early detection of breast cancer can improve cancer mortality, and early prediction of lymph node metastasis may facilitate the choice of operation type as well as use of adjuvant therapy. The use of serum markers has been well established in the screening and post-operative follow-up of different types of malignancies.<sup>20</sup> The present most commonly used breast cancer serum markers, carcinoembryonic antigen (CEA) and breast cancer-specific cancer antigen 15.3 (CA1 5-3), have disadvantages including poor sensitivity, poor specificity and a lack of standard cut-off values for either primary breast cancer detection or metastatic status prediction.<sup>21</sup> Several biomarkers have been proposed as potential new markers for breast cancer, including tissue polypeptide-specific antigen (TPS) which measures a specific epitope structure of soluble fragments of human cytokeratin 18, an acidic-type intermediate filament protein detected in various types of human cancer.<sup>6</sup>

For TPS, at a cut-off level of 75-170 U L-1, the sensitivity and specificity for detection of primary breast cancer were reported to be 30-95% and 75-90%, respectively.<sup>22,23</sup> Kassim et al used TPS as a sensitive proliferative serum marker in screening for those at high risk of breast cancer.<sup>22</sup> Hwa et al. showed that TPS was the

best predictive marker for primary breast cancer at a cut- off value of 69.1 U/L with a sensitivity of 80% and specificity of 67%.<sup>21</sup>

The findings of this study support serum TPS to be a diagnostic serum marker that differentiates breast cancer patients from normal healthy controls. At cut-off value of 67.5 U/L, TPS has sensitivity and specificity of 97.1% & 85.7%, respectively, with AUC of 97%. Our results are in agreement with Kassim et al.<sup>22</sup> who used serum TPS to differentiate Egyptian breast cancer females from females with benign breast diseases. They reported that at a cut-off for TPS of 88 U/L, the sensitivity is 95%, and the specificity is 75% with AUC of 91%. The greater cut-off value in Kassim et al. study<sup>22</sup> may be due to the use of patients with benign breast diseases instead of normal healthy volunteers. Also, different distribution of clinical stages of studied cases may influence the cut-off value.

Concerning the diagnostic value of serum aromatase activity, the present study showed the possibility of using this enzyme to differentiate between breast cancer females and normal healthy volunteers. At cut-off value of 0.09, aromatase activity has sensitivity and specificity of 86 % & 89%, respectively, with AUC of 91.8%. By comparing the diagnostic values of serum TPS and aromatase activity using ROC curve, the AUC of TPS was found



to be significantly greater than that of aromatase activity indicating that TPS is superior to aromatase activity in detecting breast cancer in postmenopausal females. To the best of our knowledge, no other previous or recent work studied the diagnostic role of this enzyme in the sera of breast cancer females.

The present study reveals that serum TPS is correlated with neither clinicopathological characteristics nor overall survival of breast cancer patients. Our study is compatible with the results reported by Hu et al.<sup>24</sup>

In our study, serum aromatase activity was significantly correlated with lymph node metastatic status and patient clinical stage. On the other hand, aromatase activity has no significant correlation with patients' overall survival. The absence of a significant correlation between serum TPS levels and aromatase activity with patients overall survival may be related to the small sample size used in the present study.

Univariate logistic regression analysis for prediction of lymph node metastasis showed that serum aromatase activity has the best predictive value for lymph node metastasis with AUC of 80.3%, the sensitivity and specificity were 76%, 85.8 %, respectively, at a cut-off 0.155.

The multivariate logistic regression model is a simple method for considering multiple continuous and/or categorical factors simultaneously. It can calculate the weighting on the individual prediction. A variety of markers, each of which had been reported to be elevated in patients with cancer of any type, were combined to see if multivariate analysis would yield information not available by the use of one marker alone.<sup>21</sup> The developed multivariate logistic regression equation used in the present study presents the degree of contribution of each variable to the prediction.

To study the effect of combination of aromatase activity and TPS on the prediction of lymph node metastatic status, multivariate logistic regression analysis showed that combination of serum levels of aromatase activity and TPS resulted in 74% adequacy of predicting lymph node metastasis which means that this combination did not improve the accuracy of predicting lymph node metastasis

achieved by aromatase activity alone. Therefore, to screen for breast cancer, either serum TPS or aromatase activity can be used, but in case of screening for lymph node metastasis in breast cancer patients, serum aromatase activity can be assayed with no need for assaying TPS in order to reduce the patient cost. This can meet the demands of different populations of different sizes.

In conclusion, serum TPS or aromatase activity can be used for detection of breast cancer, while only serum aromatase activity can be used as a predictive marker for lymph node metastasis.

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