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Philip J. Migliore, M.D.
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The Moran Foundation
Department of Pathology
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Dear Dr. Migliore:

I am writing this letter to report to you, and the Scientific Advisory Committee of the Moran Foundation, on the status of my projects supported by The Foundation.

I- "Quantitation of Estrogen and Progesterone Receptors in Breast Cancer by Immunofluorescence-based Image Analysis" (1-92-0063):
This project was completed , and the results were presented in poster format at the IAP meeting in San Francisco, in March of this year. A manuscript was submitted for publication a few weeks ago. A copy of the manuscript is enclosed.

II- "Specific Diagnosis of Celiac Sprue by Immunohistochemistry and/or Immunofluorescence" (4-93-0068):
This project is still active. After trying several methods and materials, enzymatic digestion products were successfully labeled with biotin. The labeled preparation reacted by ELISA with a commercial anti-gliadin antibody up to 8ng antibody per well, and with labeled preparation dilution up to 1:1,000,000,000.
The next step will be to test the biotinylated preparation on formalin-fixed and paraffin-embedded tissues, and if it does not work, then on frozen sections after approval from the human investigations committee. I will report to the Committee on these results later.

Thank you for your support

Sincerely,

Mamoun Younes, M.D.
Assistant Professor

# THE PERCENT OF ESTROGEN RECEPTOR-POSITIVE CELLS IS A SIGNIFICANT PROGNOSTIC MARKER IN BREAST CANCER: COMPARISON WITH OTHER METHODS.

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Running title: ER in breast cancer.

**ABSTRACT:**

Frozen tumor samples from 64 patients with breast cancer were assayed for ER by the biochemical assay (BA) or ER immunocytochemistry (ERICA) using either immunofluorescence-based computer-assisted image analysis (CAIA) or immunoperoxidase. In the immunoperoxidase part of the study, ER were evaluated by the HSCORE semi-quantitative method and by calculating the percent of positive cancer cells (%PC). The actuarial survival curves and the log rank test were used for statistical analysis. Patients with ER positive cancers had a significantly better survival than those with ER negative cancers, regardless of the method used for ER assay. The difference in survival, however, was more significant when ER was determined by BA or %PC ($p<0.01$) than by CAIA or HSCORE ($p<0.05$). We conclude that ER status as determined by ERICA and the %PC method is a significant prognostic indicator that is superior to HSCORE and CAIA. Given the tumor size limitation and sampling errors attributed to BA and the simplicity and significant predictive value of %PC, %PC will probably become the method of choice for ER assay. Studies are needed to explore the value of ER determined by %PC in predicting response to hormonal therapy.

**Key words: breast cancer, estrogen receptor, survival, HSCORE, ERICA, biochemical assay, image analysis.**

## INTRODUCTION:

Estrogen receptor (ER) status has been found to be a significant predictor of survival (1-3) and response to hormonal therapy (4,5) in women with breast cancer. Traditionally, ER has been measured biochemically using a radiolabeled ligand and separation by dextran-coated charcoal and sucrose density gradient centrifugation (6). Although the biochemical assay (BA) is a well established quantitative and objective test, it has many disadvantages (6), and its value as a significant predictor of outcome in women with breast cancer is still debatable (7-9). One of the serious limitations of BA is that it requires a minimum of 200 mg of tumor tissue for adequate determination of ER (6). Since the most notable increase in the incidence of breast cancer in recent years has been attributed to an increase in the incidence of in situ, localized, and small cancers (10), an ER immunocytochemical assay (ERICA) that can be applied to small tumors, using sections of breast cancer and specific anti-ER monoclonal antibodies, has been developed (11-19). ERICA has the added advantage of allowing the pathologist and other investigators to directly visualize and assess ER in cancer cells, rather than in a homogenate that may contain variable proportions of cancer cells, normal breast epithelium, and stroma. Because the quantity of ER in breast cancer is important (4), semiquantitative manual and computer-assisted methods have been developed for ERICA (16-18). Although most studies have focused on the correlation between ERICA and BA (11-18), only a few studies addressed the value of ERICA as a prognostic indicator (7,8,19,20), and there is no study in which the three methods of ER evaluation, computer-assisted image analysis (CAIA) of

ERICA, manual evaluation of ERICA, and BA, are compared.

The aim of this study is to compare the prognostic value of ER as determined by BA, CAIA, and manual evaluation of ERICA in frozen human breast cancer tissues.

## MATERIALS AND METHODS:

**Patients:** 64 women with breast cancer with a mean age of 66 years ( range, 37-91 years) were entered in this study. The criteria for entry in this study were: Patients should have breast cancer treated at The Methodist Hospital in Houston, had biochemical assay for estrogen receptors done on the cancer , had frozen tumor tissue available, and had a minimum of one year follow-up (for the alive patients). Perioperative deaths, and patients with more than one breast cancer (including bilateral) were excluded from the study.

**ER biochemical assay (BA):** We used the sucrose gradient centrifugation method (21,6) on frozen breast cancer tissues which were snap frozen within less than 30 min of removal. We used 16a-[125] I-iodo-3,17ß-Estradiol (NEN DuPont, Wilmington, DE) as the ligand.

**Tissue sections:** The unused portion of the breast cancer tissue submitted for biochemical assay of ER was kept frozen in liquid nitrogen. The frozen tissue was embedded in OCT compound (Miles, Elkhart, IN) and snap frozen, then 6um-thick cryostat sections were cut on Fisher Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and immediately fixed in Zamboni's fixative for 10 minutes at room temperature (RT), then washed in 20% sucrose in PBS and frozen at -80C until used for staining.

**Immunofluorescence staining of ER:** All procedures were carried out at RT, and all washings were with PBS 5 min x 3 (unless otherwise specified). Sections were washed, incubated with 5% normal goat serum in 1% BSA in PBS for 30 min, washed, incubated with 1:40 anti-estrogen antibody in 0.1% BSA in PBS (Novo Castra, distributed by Vector Laboratories, Burlingame, CA) or 1% BSA in PBS (negative control) for 120 min, washed, incubated with 1:50 FITC-goat anti-mouse IgG (Boehringer-Mannheim, Indianapolis, IN) for 60 min, washed x 4, and coverslipped using Aqua Mount as mounting medium ( Lerner Laboratories, Pittsburgh, PA). The system was calibrated with fluorescent beads, and a section of a recent case known to be ER-positive by the BA was used as positive control.

**ER immunocytochemical staining (ERICA):** All procedures were carried out at RT, and all washings were with PBS 5 min x 3 . Sections were washed, incubated with 3% normal goat serum in PBS for 30 min, washed, incubated with 1:40 anti-estrogen antibody in 0.1% BSA in PBS (Novo Castra) or 1% BSA in PBS (negative control) for 60 min, washed, then the bound antibody was detected using a pre-diluted StrAviGen Multilink HRP kit (Bio Genex, San Ramon, CA) and DAB for color development. The sections were then counterstained with methyl green (Sigma Chemical Company, St Louis, MO), dehydrated through graded alcohols, cleared in xylene, and mounted and coverslipped using Accu Mount mounting medium (Baxter Scientific, McGaw Park, IL). Sections of recent cases known to be ER-positive by the BA were used as positive control.

**Evaluation of ERICA:** The immunocytochemically-stained sections were evaluated by one pathologist (RB) without prior knowledge of the patient outcome or the results of the BA or CAIA. Two separate parameters were evaluated for each tumor, the percent of positive tumor cells, and HSCORE (18). Up to 612 cancer cells were evaluated in each case (depending on the size of the tumor in the section, the minimum was 120 cells).

**Computer-assisted image analysis (CAIA) of fluorescently stained ER:** The fluorescently labeled sections were evaluated for ER by CAIA by one pathologist (MY) without prior knowledge of the patient outcome or the results of the BA or ERICA. Images were captured on a computer hard drive using a Nikon microscope and a 40X Nikon Fluor objective, and an Optronics LX-450 camera. The images were then evaluated using the Optimas image analysis software (BioScan, Inc., Edmonds, WA). The average fluorescence intensity in nuclei of up to 507 cancer cells was calculated in each case ( number of cells evaluated depended on size of cancer in the tissue section, minimum number was 53). The fluorescence intensity was estimated in arbitrary units.

**Statistical analysis:** We used the actuarial survival curves and the log rank test for statistical significance.

## RESULTS:

The ER values in the BA assay ranged from 0.9-449 fmol/mg protein. Using a cut off value of 7.5 fmol/mg protein, patients with ER positive tumors had a better overall survival than patients with ER negative tumors ($p<0.01$) (Figure 1).

The values for CAIA ranged from 1-99 arbitrary units. With a cut off value of 17.5, patients with ER positive tumors had a better overall survival than those with ER negative tumors ($p<0.05$) (Figure 2).

The value of HSCORE ranged from 0-225. With a cut off value of 85.5, women with ER positive tumors had better overall survival than women with ER negative tumors ($p<0.05$) (Figure 3).

Finally, the percent of ER positive cancer cells in each tumor ranged from 0-99%. With a cut off value of 82%, women with ER positive tumors had better overall survival than those with ER negative tumors ($p<0.01$) (Figure 4).

## DISCUSSION:

Breast cancer is the most common cancer and second most common cause of cancer deaths in women in the United States (22,23). It has been estimated that in 1983, 182,000 new cases of breast cancer will be diagnosed, and 46,000 women will die of it (23). Decreasing the mortality from this disease will require a combination of prevention and appropriate therapy. For therapy to be appropriate, an analysis of the risk to benefit ratio should be done before the therapeutic decision is made. Risk assessment depends largely on the analysis of several prognostic markers of which ER status is one of the established and clinically used ones (24). Most clinical trials and survival analysis using ER as a prognostic marker or predictor of response to hormonal therapy have largely depended on BA.

There are two main problems with the BA: The contradicting reports about its utility as a prognostic indicator, (7-9), and the required sample size of more than 200 mg (6), which renders a good proportion of breast cancer in today's practice unassayable for ER .

The contradicting reports on the utility of ER determined by BA as a prognostic indicator may be largely attributed to the well known sampling problem. This problem is unavoidable, mainly because the biochemical assay is based on measuring the cytosolic ER in fmol/mg protein in tissue homogenate. The ER value, measured as fmol/mg protein, could be influenced by contaminating normal non-malignant epithelial cells which may express ER ( 25,26) which in turn may vary depending on

menstrual cycle, and exogenous hormones (27,28) . The value could also be influenced by the amount of protein in the homogenate contributed by non-cancer cells, including normal epithelial cells, fibroblasts, endothelial cells and inflammatory cells.

In this study, women with ER positive tumors determined by any of the methods used had a significantly better overall survival than those with ER negative tumors (Figures 1-4). Moreover, ERICA, as determined by three different methods, showed comparable predictive value to that obtained by BA. Because ERICA can be performed on small tumors, and has consistently proven to be of significant prognostic value in published studies, (probably because direct visualization of cancer cells avoids sampling errors), it should be considered as an alternative to BA. Of the 3 methods we used to evaluate ER in breast cancer sections, we think that the percent of ER positive cells (%positive) is the best method. The results obtained with this method were more significant ($p<0.01$), it is less subjective than the popular HSCORE since there is no need to subjectively evaluate the staining intensity, and it can be done in any laboratory performing immunohistochemistry without the need for expensive image analysis equipment. Others have previously shown that the behavior of breast cancer can be determined by the percent of ER positive cells (7,8), and with cut off values similar to ours, even using different antibodies. It is unknown, however, whether response to hormonal therapy can be predicted by the percent of ER positive cells.

Finally, since the report that women with breast cancer who were treated with

tamoxifen developed significantly fewer cancers in the other breast ( 29), there has been an increased interest in chemoprevention for breast cancer (30-35). Since tamoxifen safety has been questioned (36,37), it is important that this approach be limited, at least until the risk of long-term tamoxifen therapy is well known, to women at increased risk for the development of breast cancer. Recently, it has been reported that ER positivity by ERICA in normal breast tissue may be a risk factor for breast cancers (38). Since this positivity has been reported earlier in all benign biopsies and normal tissues (25-28), it may be important to determine whether a higher percent of positive cells is associated with a higher risk. ERICA will have a definite advantage over BA in such studies because of the abundance and variability of the stromal component in normal breast tissue.

**Acknowledgement:**

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## FIGURE LEGENDS:

**Figure 1.** Survival according to estrogen receptor status as determined by the biochemical assay. ER positive, O ER negative.

**Figure 2.** Survival according to estrogen receptor status determined by immunofluorescence staining and computer-assisted image analysis (CAIA). ER positive, O ER negative.

**Figure 3.** Survival according to estrogen receptor status determined by ERICA and the HSCORE method. ER positive, O ER negative.

**Figure 4.** Survival according to estrogen receptor status determined by the percent of estrogen receptor positive cancer cells. ER positive ($\geq$ 82% ER positive) , O ER negative (< 82% ER positive).

Figure 1 Younes

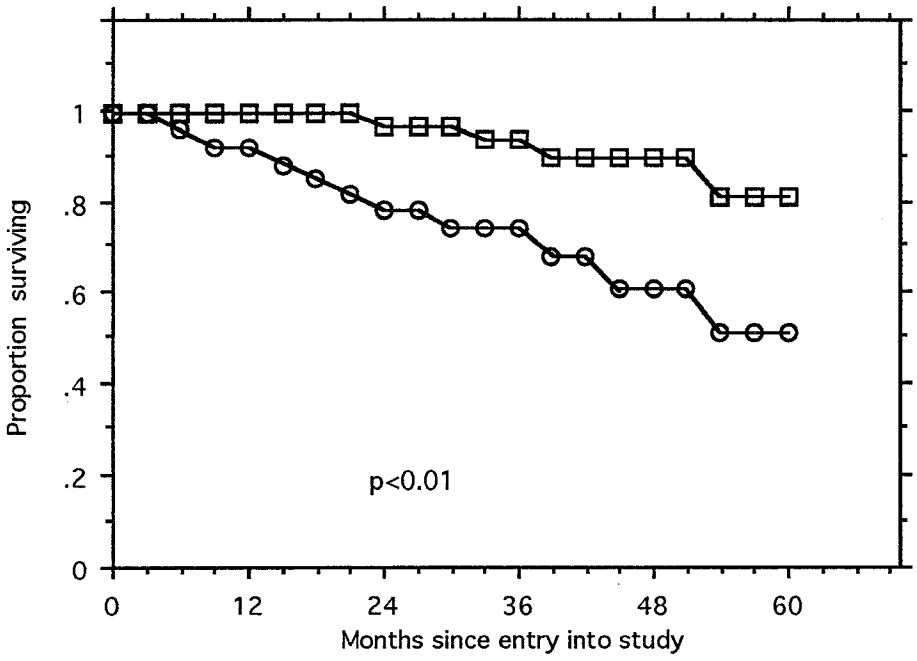

Figure 2 Younes

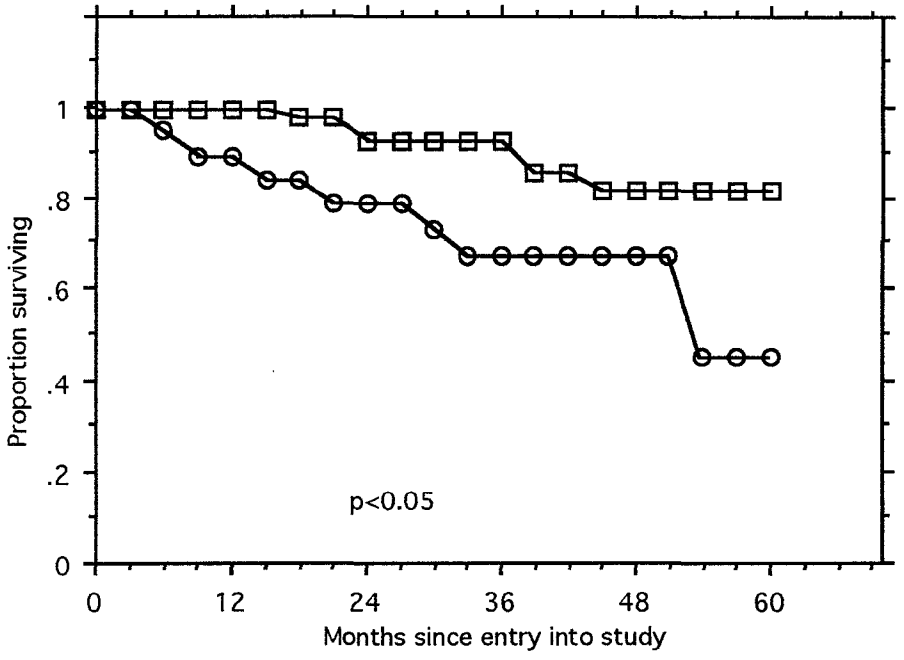

Figure 3 Younes

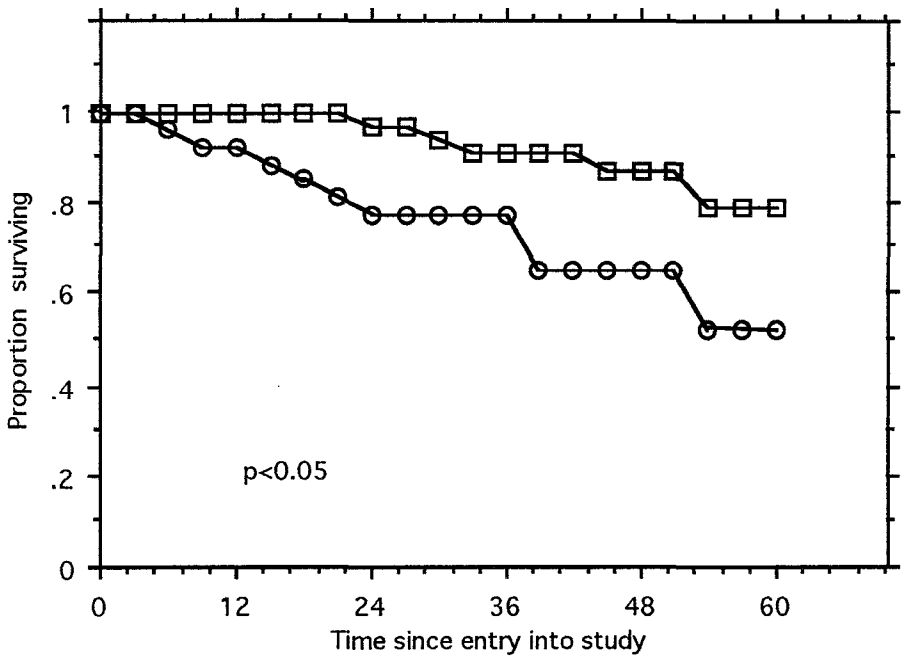

Figure 4 Younes