

## EVALUATION OF A DISPLACEMENT ASSAY WITH TAMOXIFEN AS PROGNOSTIC INDICATOR IN BREAST-CANCER PATIENTS WITH ESTROGEN-RECEPTOR-POSITIVE TUMORS

Emanuel LEVIN<sup>1\*</sup>, Andrea M. ACTIS<sup>1</sup>, Silvana CARUSO<sup>1</sup>, Hugo GASS<sup>2</sup>, Raquel ROMERO<sup>3</sup>, Nicolás QUALETA<sup>4</sup> and Rosa W. LEVIN<sup>5</sup>

<sup>1</sup>Departamento Bioquímica Humana, Facultad de Medicina, UBA, Buenos Aires, Argentina

<sup>2</sup>Departamento de Ginecología, Hospital de Tigre, Buenos Aires, Argentina

<sup>3</sup>Departamento de Ginecología, Hospital Velez Sarsfield, Buenos Aires, Argentina

<sup>4</sup>Departamento de Ginecología, Hospital Teodoro Alvarez, Buenos Aires, Argentina

<sup>5</sup>Sección de Oncología, Hospital Teodoro Alvarez, Buenos Aires, Argentina

**A displacement assay with tamoxifen, based on the relative binding affinity of tamoxifen and estradiol for the estrogen receptor (ER), was proposed in 1990 as prognostic indicator for breast-cancer patients. Validation of its predictive results in relation to the outcome of 73 patients with ER<sup>+</sup> tumors is analyzed. ER, progesterone receptor (PgR) determinations and other conventional prognostic factors in relation to the displacement assay, were considered. Displacement assay results allowed ER<sup>+</sup> tumors to be grouped as displaceable (D) or weakly displaceable (WD), with the implication that D tumors should respond better to tamoxifen (Tam) administration. Survival and disease-free interval curves showed highly significant differences between patients with ER<sup>+</sup> D and ER<sup>+</sup> WD tumors. For survival, including all tumor stages, 73.9% of patients were alive at 9 years after surgery in the group with D tumors and 37.0% in the group with WD tumors ( $p < 0.005$ ); relative contribution of the different stages is analyzed. Addition of axillary-node number increased the prognostic significance of displacement categories for survival and disease-free interval. PgR determination as another ER functional expression failed to show significant differences for survival and disease-free interval between ER<sup>+</sup> PgR<sup>+</sup> and ER<sup>+</sup> PgR<sup>-</sup> tumors. Thus, results from the displacement assay and from PgR determinations reflect 2 independent ER functional expressions. Displacement assay data appear as reliable prognostic indicators of breast-cancer outcome, and contribute to more appropriate treatment decisions in this pathology. Int. J. Cancer 73:486–491, 1997.**

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The complexity of mammary tumorigenesis has become more evident with the knowledge of the involvement of growth factors, oncogene products and other biochemical messengers from several transduction pathways which, in conjunction with steroid hormones and their receptors, participate in the genesis and progression of the malignant process (Clark *et al.*, 1993). Each of these factors may be expressed differently in each tumor, so that their interdependence affects their individual contribution. Determination of any of the intervening factors *per se* is insufficient to evaluate the tumoral process, and it is becoming mandatory to apply functional assays to disclose some of the interrelations between effector molecules and their clinical significance in each case. In the field of steroid hormones and their receptors, subtle changes induced by ligands and associated proteins on genomic transcription, are able to modify physiological and pharmacological responses related to the cellular proliferation/differentiation processes. In this context, the use of tamoxifen (Tam) as an anti-tumoral agent over-rides its value as an anti-estrogen. In addition to several effects not mediated by estrogen-receptor(ER) mechanisms (Cabot *et al.*, 1995; Greenberg *et al.*, 1987; Kiss, 1994), Tam may evoke agonist/antagonist estrogenic actions simultaneously and/or successively through changes in ER conformation and reactivity with related molecules (Metzger *et al.*, 1995). Tam binding to ER in the presence of endogenous estradiol (E<sub>2</sub>) is governed by the relative binding affinity (RBA) of both ligands for ER (Levin *et al.*, 1995). With Tam administration, there is a continuous dynamic equilibrium between Tam-ER and E<sub>2</sub>-ER complexes according to the fluctuating concentrations of both

compounds at a given time. The co-existence of both complexes and their respective interactions with the DNA, with associated proteins and with members of other related pathways, markedly affects the transcription message and subsequent cellular proliferation. This pleiotropic setting is peculiar to each tumor, and starts with the RBA of Tam and E<sub>2</sub> for the ER, which also differs from one tumor to another.

We have developed an assay based on the RBA of Tam and E<sub>2</sub> for ER (Levin *et al.*, 1990), which allows 2 categories of estrogen-receptor-positive (ER<sup>+</sup>) breast tumors to be discerned: displaceable and weakly displaceable, according to the D<sub>50</sub> ratio of each ligand to the receptor. D<sub>50</sub> is the ligand (Tam or E<sub>2</sub>) concentration for 50% inhibition of <sup>3</sup>HE<sub>2</sub>-ER binding. Having used this assay since 1987, we here evaluate 9 years' history of patients who had their surgery 5 to 9 years ago, in order to confirm the predictive value of the displacement assay with Tam. Since so many factors participate in tumor growth, one or even several functional determinations cannot account for all the biological processes modulating malignant development in each case. However, since Tam is a widely used agent in mammary oncology, the proposed assay is directly relevant to the anti-tumoral action of the compound based on its ability to displace E<sub>2</sub> from ER in each particular tumor. Among proteins related to cellular proliferation and regulated by estrogens via ER, in clinical practice, progesterone-receptor(PgR) expression is considered indicative of ER functionality (McGuire and Clark, 1985). As such, it improves the prognostic significance of ER<sup>+</sup> breast tumors, though the integrity of a given route cannot be freely extrapolated to others. Even within ER<sup>+</sup> PgR<sup>+</sup> tumors, there is a sub-set that behaves as estrogen-independent, as judged by its evolution and response to hormonal treatments. Determination of PgR was included in our study as a normal ER functional parameter.

### MATERIAL AND METHODS

#### Patients

This study relates to 73 patients from the oncology and gynecology services at the Teodoro Alvarez, Velez Sarfield and Tigre hospitals, who had breast surgery between 1987 and 1991, that is, with at least 5 years before the study. Patients operated on after 1991 who died during the observation period, until July 1996, were also included. Of the patients, 62 (85%) were post-menopausal. Tumoral staging was adopted according to the 4 conventional categories (Fisher *et al.*, 1993), without subdivisions

Contract grant sponsors: CONICET, University of Buenos Aires; Fundación Oncológica Encuentro.

\*Correspondence to: Departamento de Bioquímica Humana, Facultad de Medicina, Paraguay 2155, (1121) Buenos Aires, Argentina. Fax: (54) (1) 962-5108 & 962-5341. E-mail: levin@fmuba.sld.ar

Received 17 March 1997; Revised 24 June 1997

within stages II and III. Histologic type, nuclear grade, mitotic index, desmoplasia, infiltration and necrosis were recorded. For post-operative treatment, each hospital service followed the conventional treatment guides according to its usual practice. Since all tumors in this study were ER<sup>+</sup>, pre- and post-menopausal patients received 20 mg Tam daily during 5 years irrespective of tumor staging, discontinued if recurrence or metastasis appeared. Chemo- and radiotherapy were included when considered necessary. For evaluation, survival time and disease-free interval (DFI) were taken into account.

### Methods

Immediately after breast surgery, tissue samples were sent to the laboratory in dry ice and stored at -80°C for ER, PgR and displacement assay determinations, within 30 days of their arrival. For ER and PgR dosage, the method of McGuire (1975), with minor modifications, was followed. The analog R5020 was used for PgR measurements. Cut-off value for considering steroid receptors positive or negative was 10 fm E<sub>2</sub> or R5020/mg cytosol protein. The displacement assay for ER has been described (Levin *et al.*, 1990). Briefly, the cytosolic extract containing 10 nM <sup>3</sup>H-E<sub>2</sub> was incubated with increasing displacer concentrations: 5, 10, 30, 50 and 2000 nM for unlabeled E<sub>2</sub> and 1, 3, 5, 10, 30 and 50 μM for Tam, in duplicate aliquots. Incubation conditions, separation by charcoal-dextran and radioactivity measurements were as for ER determinations. D<sub>50</sub> for each ligand, that is, the concentration necessary for 50% inhibition of <sup>3</sup>H-E<sub>2</sub>-specific binding was determined by the EBDA software program. A displacement index (DI) was calculated as: (D<sub>50</sub> E<sub>2</sub>/D<sub>50</sub>Tam) × 100. Tumors were classified as displaceable (D) for a DI equal to or higher than 0.10 and as weakly displaceable (WD) for a DI lower than 0.10.

### Statistical procedures

We based our procedures on Selvin (1991). Multivariate analysis for the prognostic value of determinant variables (age, stage, nodes, nuclear grade, mitotic index, ER, PgR, displacement assay) was performed by the Cox proportional-hazards regression technique, also applied to obtain predictive values. By backward stepwise process the final variable significant model was obtained. The dependent dichotomic variables were survival, death and DFI, metastasis. As continuous variable, the Mann-Whitney U-test or the Student *t*-test was employed. Probability curves for survival and DFI were computed according to the Kaplan-Meier method and compared using the log-rank test. Significance for 2-sided contrasting comparisons were considered at 95% confidence interval (CI) (*p* < 0.05). CSS Statistica 3.1 (StafSoft, Tulsa, OK) and EPIINFO 6.0 (OMS-CDC, Atlanta, GA) computer programs were used for processing the data.

## RESULTS

The main concept introduced in this study, as reported earlier (Levin *et al.*, 1990), is the significance of Tam "potency" to displace E<sub>2</sub> from the E<sub>2</sub>-ER, quantitated by the displacement index as expression of the relative binding affinity of both ligands for the same receptor: (D<sub>50</sub> E<sub>2</sub>/D<sub>50</sub> Tam) × 100 = DI. A wide concentration range, from 5.4 to 35.0 nM, was obtained for the D<sub>50</sub> E<sub>2</sub>, and the D<sub>50</sub> Tam range was 1.4 to 45.3 μM (data not shown), reflecting the diverse affinities of E<sub>2</sub> and Tam for ER in each tumor. The cut-off value between D and WD tumors was tentatively set at 0.10. Values higher than 0.10 should indicate greater Tam potency for displacing E<sub>2</sub> from ER and values lower than 0.10, weaker Tam *in vitro* displacement potency.

Out of the 73 tumors, 46 (63%) were D and 27 were WD. In the D group of tumors, 35 (76%) were PgR<sup>+</sup> and in the WD, 24 (89%) were PgR<sup>+</sup>. ER values, sub-divided into 3 categories, 10 to 49, 50 to 99 and 100 or more fm/mg cytosol protein, as expression of low, medium or high ER content, failed to affect DI values. ER range for D was 11 to 480 and for WD tumors was 10 to 504 fm E<sub>2</sub>/mg cytosol protein. For PgR, corresponding values for the 35 PgR<sup>+</sup> D tumors ranged from 10 to 507 and, for the PgR<sup>+</sup> WD tumors, from

TABLE I - STAGE AND DISPLACEMENT CATEGORIES IN 73 ER<sup>+</sup> BREAST TUMORS

| Stage | Displaceable |            | Weakly displaceable |            |
|-------|--------------|------------|---------------------|------------|
|       | n            | DI (range) | n                   | DI (range) |
| I     | 15           | 0.11-1.68  | 7                   | 0.02-0.09  |
| II    | 21           | 0.10-1.60  | 12                  | 0.03-0.09  |
| III   | 7            | 0.13-0.68  | 6                   | 0.04-0.09  |
| IV    | 3            | 0.12-0.33  | 2                   | 0.07-0.09  |

DI, displacement index.

TABLE II - PREDICTIVE VALUE OF THE DISPLACEMENT ASSAY FOR SURVIVAL AT 9 YEARS IN THE 73 PATIENTS' ER<sup>+</sup> BREAST TUMORS (INCLUDING ALL TUMOR STAGES)

| Displacement     | Survived | Dead | Total |
|------------------|----------|------|-------|
| D<br>(DI ≥ 0.1)  | 34       | 12   | 46    |
| WD<br>(DI < 0.1) | 10       | 17   | 27    |
| Total            | 44       | 29   | 73    |

D, displaceable; WD, weakly displaceable; DI, displacement index. Predictive values: D, positive prediction 73.9% (34/46); false negative 26.1% (12/46). WD, negative prediction 63.0% (17/27); false positive 37.0% (10/27). Chi<sup>2</sup> *p* < 0.005.

11 to 350 fm R5020/mg cytosol protein. According to tumor stage, D/WD ratios are shown in Table I.

Considering survival as a dependent variable in the ER<sup>+</sup> D group, including all tumor stages, 34 out of 46 cases survived at the end of the 9-year observation period: 73.9% predictive value (12 patients died: 26.1% false negative). For the WD group, 17 out of 29 cases died during the observation period: predictive value 63.0%; 10 were alive (37.0% false positive) (Table II).

The limited number of stage-III and stage-IV tumors weakened their significance for survival differences between patients with D and WD tumors. Mean survival time for the 10 RE<sup>+</sup> D tumors, stages III and IV, was 3.9 years; for the 8 ER<sup>+</sup> WD tumors, stages III and IV, it was 2.4 years, but due to their dispersion this trend difference was not significant (*p* = 0.10). For the 36 patients with ER<sup>+</sup> D tumors, stages I and II, the mean survival time was 6.0 ± 2.1 years, while for the 19 ER<sup>+</sup> WD tumors it was 4.1 ± 1.5 years (*p* < 0.001). Relative risk for death and metastasis was higher for descending DI units (0.01) and for ascending node number, as estimated by the Cox proportional-hazards regression method (Table III).

Kaplan-Meier survival and DFI probability curves for patients with ER<sup>+</sup> tumors of each category are presented (Fig. 1). A highly significant difference between patients with D and WD tumors was apparent: 66.0% survival probability at 9 years for those with D and 22.3% for those with WD tumors (Fig. 1a; *p* < 0.0001). Corresponding figures for DFI probability were 69.0% and 36.0% (Fig. 1b; *p* < 0.006). From the Kaplan-Meier plots, 5-year survival and DFI rates were obtained (Table IV).

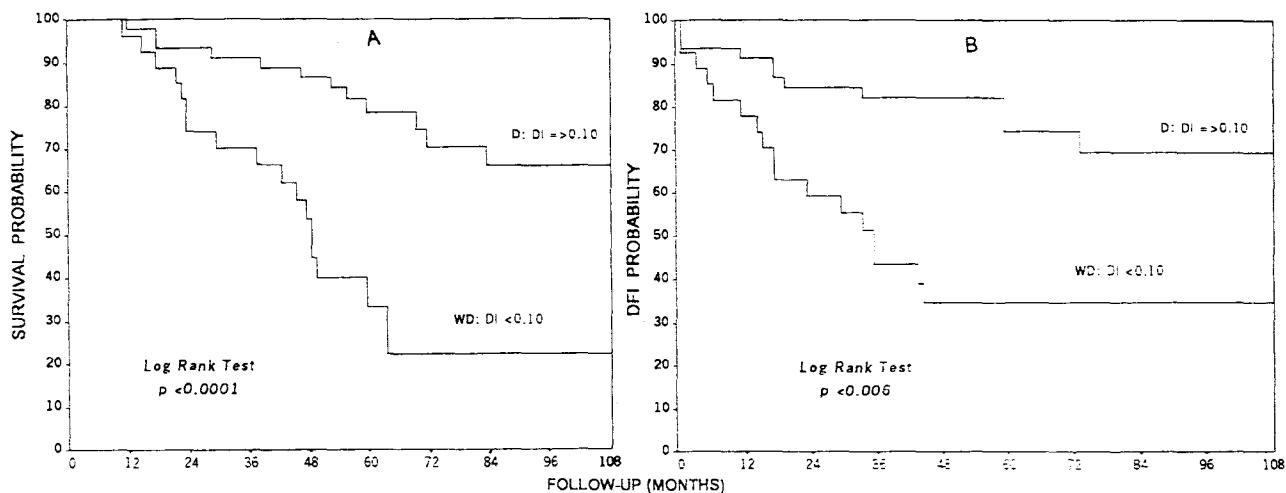
By correlating axillary-node number plus displacement-assay categories, with survival and disease-free interval, probability curves were constructed (Fig. 2) showing the predictive value of both indicators when considered together. Complementary data for DFI from Figure 2 provided the probability values for metastasis or relapse in the 9-year follow-up period, as shown in Table V. Among the prognostic histologic indicators, nuclear grade and mitotic index were recorded in 61 of the 73 tumor samples, and their D vs. WD ratio was not significantly different (data not shown; *p* > 0.2). Remaining histologic parameters were apparently not different for each category.

Finally, by comparing the significance of the 2 studied functional ER expressions (displacement assay and PgR) in relation to survival and DFI (Fig. 3), it can be appreciated that for the 73-patient cohort, displacement categories were predictive of better or worse survival and DFI performances for D or WD tumors

**TABLE III** – COX PROPORTIONAL-HAZARDS REGRESSION VALUES FOR NODES AND DISPLACEMENT<sup>1</sup> IN RELATION TO THE DICHOTOMIC VARIABLES SURVIVAL-DEATH AND DFI-METASTASIS

| Co-variate                  | Survival-death   |                  | DFI-Metastasis   |                  |
|-----------------------------|------------------|------------------|------------------|------------------|
|                             | Nodes            | Displacement     | Nodes            | Displacement     |
| Regression coefficient ± SE | 0.38 ± 0.08      | -2.24 ± 0.92     | 0.50 ± 0.09      | -1.49 ± 0.39     |
| Relative risk (95% CI)      | 1.46 (1.23–1.73) | 9.36 (1.54–56.7) | 1.64 (1.38–1.97) | 4.43 (2.05–9.58) |
| p value                     | <0.0001          | <0.02            | <0.0001          | <0.001           |

<sup>1</sup>Displacement index: cut-off value: 0.10 (D; ≥0.10; WD; <0.10). Age, stage, ER and PgR content: p > 0.05 (not significant).

**FIGURE 1** – Survival (a) and DFI (b) probability curves according to displacement-assay tumor categories (n = 73). D, displaceable; WD, weakly displaceable; DI, displacement index.**TABLE IV** – 5-YEAR RATES FOR SURVIVAL AND DFI IN 73 PATIENTS WITH ER<sup>+</sup> MAMMARY TUMORS

| Tumors                                  | 5-year rates  |               |
|---|---------------|---------------|
|   | Survival      | DFI           |
| ER <sup>+</sup> D (46)                  | 80.4% (37/46) | 82.5% (38/46) |
| ER <sup>+</sup> WD (27)                 | 40.7% (11/27) | 37.0% (10/27) |
| Total ER <sup>+</sup> (73) <sup>1</sup> | 65.8% (48/73) | 65.8% (48/73) |

<sup>1</sup>In the absence of categorization.

respectively. PgR expression failed to correlate survival and DFI either for PgR<sup>+</sup> or for PgR<sup>-</sup> tumors. That is, the displacement assay should predict the response to Tam (favorable or unfavorable) irrespective of PgR expression, also indicating that displacement by Tam and PgR expression are independent ER functional variables.

#### DISCUSSION

Determination of ER has been a hallmark for establishing the hormone dependence of mammary neoplasms (Jensen *et al.*, 1982). However, a significant proportion of ER<sup>+</sup> tumors behave as if they were estrogen-independent. Growth factors, oncogenes and interactions between steroid receptors and members of other signaling pathways are some of the multiple parameters that can modify ER behavior and give unexpected responses to pharmacological agents (Aronica *et al.*, 1994; Actis *et al.*, 1994). Functional assays can help to predict receptor response according to the function or to the gene expression under study (Levin *et al.*, 1995; Landers and Spelsberg, 1992). PgR determination is one of such assays, evidencing ER participation in the expression of this protein (McGuire and Clark, 1985). Many other proteins involved in cellular proliferation are also regulated by estrogens, but individual results cannot be generalized. This is one of the limitations of using a single test to predict clinical outcome in patients with mammary tumors. At present, by immunohistochemistry, flow cytometry and other

molecular techniques several prognostic markers are in use (Dowsett *et al.*, 1996) and contribute to making more appropriate treatment decisions.

We propose a functional ER assay based on relative binding affinities of E<sub>2</sub> and Tam for ER, directly related with displacer Tam potency of E<sub>2</sub>/ER binding. To set the separation between tumors more displaceable by Tam from those less displaceable, we started from the accepted paradigm that around 40% of ER<sup>+</sup> breast tumors fail to respond to hormone therapy (Jensen *et al.*, 1982). By applying this 40% to the initial 125 cases (none of which had more than 2 years follow-up), we ranked the DI values according to their increasing order. Tumor number 50 (40% of 125) had a DI value of 0.102; tumor 49 had a value of 0.094, and tumor 51, a DI value of 0.107. The rationale of the proposed 0.10 threshold value, would be that ER<sup>+</sup> tumors with DI below 0.10 should correspond to poor responders or non-responders to hormone therapy. The outcome of the 73 patients in this study, all of whom were evaluated at least 5 years after initial surgery, is in agreement with the prediction derived from their DI values, considering 0.10 as the cut-off between D and WD categories. Further studies on larger populations should validate or modify this proposed cut-off threshold.

Diverse mechanisms besides Tam/ER binding have been advanced as additional or alternative routes for the Tam anti-tumoral effect: inhibition of calcium channels (Greenberg *et al.*, 1987), phospholipase activity (Kiss, 1994), lipid peroxidation (Custodio *et al.*, 1994), protein kinase C (Baltuch and Yong, 1996), as well as changes in plasma membrane fluidity (Cabot *et al.*, 1995) or regulation of growth factors (Stewart *et al.*, 1992) are among the mechanisms adduced in this context. However, in oncological practice Tam has proven to be active mostly in cancers where ER has been found, whereas in its absence, Tam can hardly be effective in tissues where the above-mentioned metabolic routes are operative. On the other hand, cellular proliferative effects of Tam are induced in the uterus (Uziely *et al.*, 1993), a tissue rich in ER, meaning that Tam mechanisms, whether estrogen antagonist or

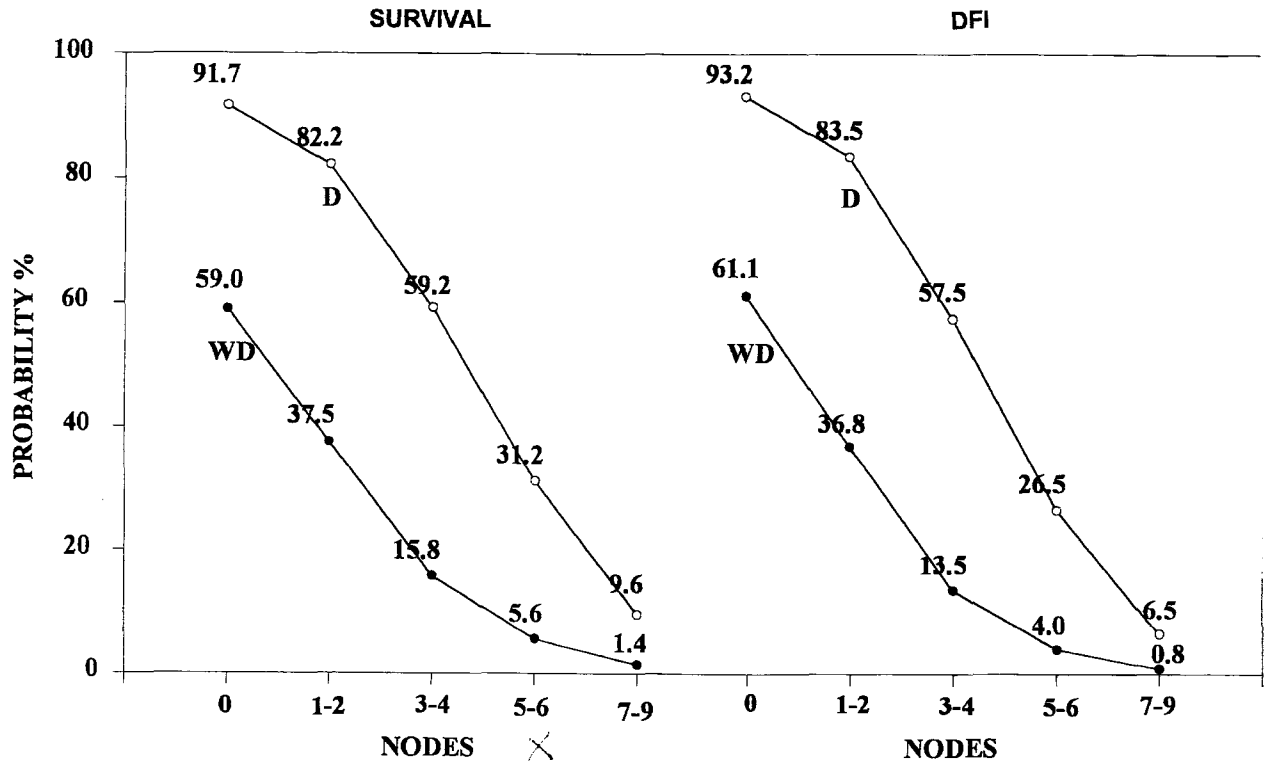


FIGURE 2 – Survival and DFI probability in breast-cancer patients with ER<sup>+</sup> tumors according to displacement-assay categories and axillary-node number. Logistic regression for survival: displacement,  $p < 0.002$ , nodes,  $p < 0.0006$ ; for DFI: displacement,  $p < 0.002$ , nodes,  $p < 0.0003$ .

TABLE V – PROBABILITY OF METASTASIS OR RELAPSE AT 9 YEARS ACCORDING TO DISPLACEMENT-TUMOR CATEGORIES AND AXILLARY-NODE NUMBER

| Nodes | Displaceable | Weakly displaceable |
|-------|--------------|---------------------|
| 0     | 6.8%         | 38.9%               |
| 1-2   | 16.5%        | 63.2%               |
| 3-4   | 42.5%        | 86.5%               |
| 5-6   | 73.5%        | 96.0%               |
| 7-9   | 93.5%        | 99.2%               |

agonist, are expressed mainly via ER or other pathways impinging upon these receptors.

A functional assay such as the one proposed in this report, based on the competition of a compound with the natural ER ligand in each tumor, should reflect the main anti-neoplastic capability of the drug under study in the particular tissue. However, resistance to hormonal therapy develops in most cases, especially after prolonged treatment, even when associated with chemo- and/or radiotherapy (Wiebe *et al.*, 1993). In this respect, treatments applied in the current study, though not following a unified protocol, used Tam for D and for WD tumors, showing that the response was more favorable and prolonged in the D category. The WD group, even under Tam administration, had a poorer response, as if the tumors were ER<sup>-</sup>, as stated in earlier reports (Levin *et al.*, 1990, 1995). Table I shows that DI values in D tumors are higher for lower tumoral stages, as an indication of a greater relative E<sub>2</sub>/Tam affinity when the neoplastic process is milder. This is corroborated by data from Figure 2, where it can be appreciated that as node number increases, differences in survival and DFI probability for patients with D or WD tumors are less evident, and disappear for node number  $\geq 7$ . No single prognostic indicator by itself is able to account fully for the different biological variables in mammary tumorigenesis. This is also apparent for the displacement assay, where false positive and negative predictions are recorded (Table II), and indicate the convenience of associating prognostic

markers other than those corresponding to the steroid-receptor pathways. However, survival and DFI rates at 5 and 9 years (Tables II, and IV) show highly significant differences for patients with D and WD tumors and validate the prognostic value of the displacement-assay results for ER<sup>+</sup> mammary neoplasias.

Tam binding to ER introduces changes in the receptor protein reflected in pleiotropic routes that convey and modulate the transcription message: binding to the cognate DNA response elements (Sabbah *et al.*, 1991), dimer formation (Fawell *et al.*, 1990), cross-talk with associated proteins (Church Landel *et al.*, 1994) and members of other transduction pathways (Webb *et al.*, 1995), phosphorylation patterns (Le Goff *et al.*, 1994), isoform profiles and nucleo-cytoplasm shuttling (Actis *et al.*, 1996), as well as others not yet sufficiently explored. Displacement and PgR data in relation to survival and DFI showed that PgR expression was not a reliable prognosis indicator in this 73-patient series. Patients with ER<sup>+</sup>/PgR<sup>+</sup> D tumors should have a better outcome than those with ER<sup>+</sup> WD tumors, irrespective of their PgR expression.

Out of 16 patients with tumors nuclear grade 3 and mitotic index higher than 6, 11 were WD. The other 5 were D, and had a favorable evolution according to survival: one tumor, stage II, 102 months; 3 tumors, stage III, 56, 70 and 84 months; and one tumor, stage IV, 46 months survival. To sum up, the displacement assay with Tam, as an ER functional expression, appears to be a reliable prognostic indicator in ER<sup>+</sup> breast neoplasias by providing further categorization into displaceable and weakly displaceable tumors. Data from the displacement assay, associated with other prognostic features help to make more appropriate treatment decisions, even for some cases where discrepancies with other prognostic factors are apparent.

#### ACKNOWLEDGEMENTS

We are grateful to Ms. A. Pritika and Ms. M. Leale who collaborated with the anatomico-pathology reports, to Mr. U. Questa, who was in charge of the statistical processing of data, and Ms. I. Nievas, who assisted with the laboratory work.

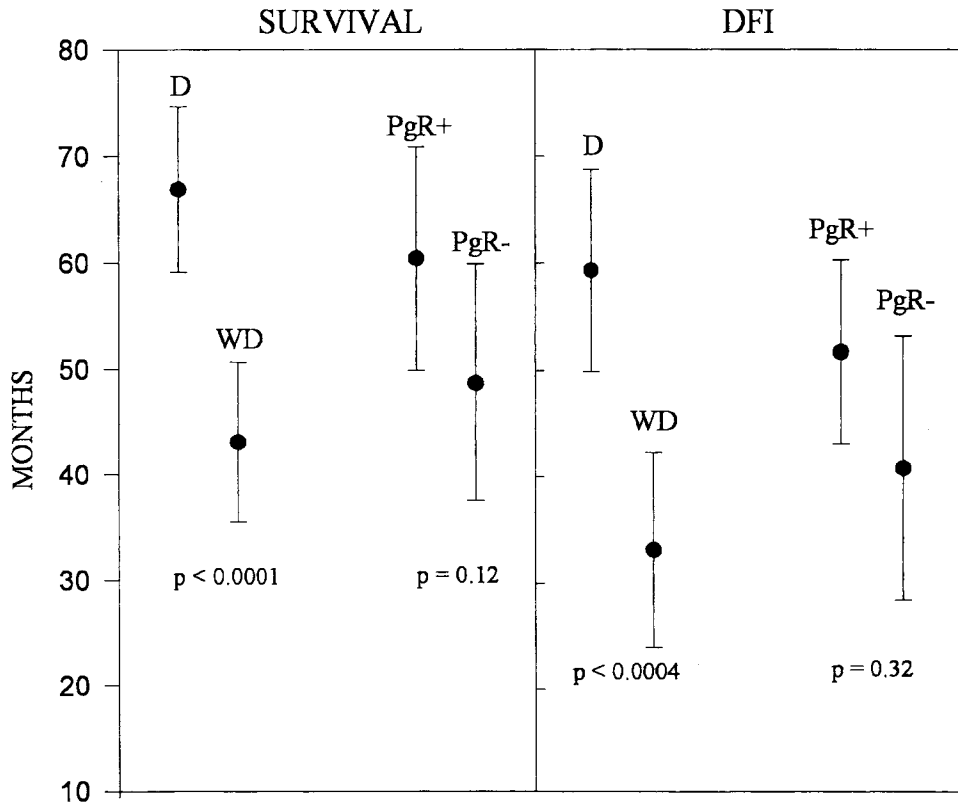


FIGURE 3 – Survival and disease-free interval in patients with ER<sup>+</sup> breast tumors (n = 73). Data for displacement-assay categories and PgR expression. Mean ± 2 SE. (Mann-Whitney U-test).

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