· Eur J Cancer Clin Oncol, Vol. 25, No. 11, pp. 1595–1602, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

Immunocytochemical Detection of Progesterone Receptor by Monoclonal KD-68 Antibody in Operable Breast Cancer: Correlations with Biochemical Assay, Pathological Features and Cell Proliferative Rate

PIERANTONIO BEVILACQUA, MAURIZIO PEA and GIAMPIETRO GASPARINI
St. Bortolo Hospital USSL 8, Vicenza, Italy

Abstract—*A new immunocytochemical assay (ICA) for progesterone receptor (PgR), employing the rat monoclonal KD-68 antibody and a sensitive peroxidase–anti-peroxidase (PAP) technique as the displaying system, was performed in 129 human breast cancer specimens. PgR-ICA staining was almost all electively located in neoplastic cell nuclei with a substantial heterogeneity in distribution and intensity. To study the basic relationship of the results of the ICA method with the biochemical dextran-coated charcoal (DCC) assay we compared, in all the same specimens, the antibody nuclear staining with the PgR positivity by DCC (cut-off value of 10 fmol/mg of protein). We found an overall agreement of 77% between the two methods and a PgR-ICA sensitivity of 83% and a specificity of 72%, assuming that biochemical PgR is truth. PgR-ICA false-negative results were only nine out of 53 (17%); and false-positive were 21 out of 76 (28%). Using both methods no significant association was observed between PgR positivity with menopausal status, histological type, tumor size and lymph node status. The correlations between PgR expression and cell kinetics were assessed by an immunocytochemical method employing the monoclonal Ki-67 antibody. While a significant negative relationship was found between high Ki-67 score and PgR-ICA positivity ($P < 0.01$) no correlation was found with DCC positivity. The present results demonstrate that ICA is a practical, reliable and inexpensive method with a good correlation to the conventional biochemical assay to determine the PgR status. Moreover, ICA recognizes PgR expression at the single cell level, thus providing additional information to the quantitative DCC assay that should improve the prognostic evaluation and the prediction of responsiveness to endocrine therapy in breast cancer.*

INTRODUCTION

HORWITZ *et al.* [1] suggested in 1975 that certain estrogen receptor (ER)-positive tumors are not responsive to endocrine treatment because of a defect in the estrogen response pathway distal to the binding step, leading to autonomous growth. Based on the observation that progesterone receptor (PgR) expression is an estrogen-dependent phenomenon [2], it was hypothesized that PgR might be a better marker than ER to evaluate the functional activity of the steroid hormonal receptor system in human breast cancer.

Accepted 5 July 1989.
Author to whom correspondence and reprint requests should be sent: Giampietro Gasparini M.D., St. Bortolo Hospital, 36100 Vicenza, Italy.

Several studies have repeatedly demonstrated that the response rate of ER-positive patients to endocrine therapies ranges between 55 and 60% [3, 4]. The addition of the PgR assay allows an improvement of the predictive accuracy by an additional 20%, often as high as 75–80% in ER+/PgR+ patients [5, 6]. In women with operable breast cancer the expression of the steroid hormonal receptors also has an important prognostic value. In stage I disease, the lack of ER seems to be one of the powerful factors for predicting earlier recurrence and poorer survival [7–9]. In stage II breast cancer, PgR content appears to be better than ER content in predicting disease-free and overall survival [10, 11].

It is therefore necessary for the clinician to know the receptor status of breast tumors in as many cases as possible. At present, the biochemical method

(dextran-coated charcoal, DCC) is considered the most useful for routine use. However, its application has some technical limitations because it requires relatively large tumor samples (at least 200–300 mg of tissue), use of radioisotopes, *in vivo* it detects unoccupied sites only which may lead to inaccurate estimates of receptor content in premenopausal women or during hormonal therapy and, finally, this assay does not permit the assessment of receptor status in individual tumor cells and does not address the problem of cellular heterogeneity [12, 13]. In an attempt to avoid these limitations several histochemical or immunohistochemical assays have been developed over the past 10 years, however low sensitivity and specificity have limited the use of methods based on immunofluorescence or radiolabeling of estrogens [14, 15]. In recent years a major advance in receptor measurement was made by the discovery of monoclonal antibodies [16–21]. Recently Abbott Laboratories produced a PgR-ICA kit based on Greene's [22] monoclonal IgG-KD-68 antibody which recognizes both the A and B forms of PgR and detects, by an intense nuclear staining, both frozen specimens and paraffin-embedded sections with no pretreatment.

In the present study, immunocytochemical detection of PgR was performed in 129 breast cancer specimens and compared with the DCC assay, to menopausal status and several histopathologic features and to cell kinetics (Ki-67 antibody), to evaluate in years to come, the possibility of a more accurate definition of prognosis and of the prediction of hormonal responsiveness.

MATERIALS

One hundred and twenty-nine consecutive cases of stage I and II breast carcinoma (T_1–T_{3a}; N_{0-2}; M_0) were analyzed. The age of the patients ranged from 31 to 70 years (median, 53). Menopausal status was defined according to the following criteria: patients were considered in premenopause when at the time of surgery they were actively menstruating or when less than 1 year had elapsed from spontaneous menopause; patients were considered in perimenopause when 1–5 years had elapsed from spontaneous menopause or oophorectomy and patients were considered in menopause when more than 5 years had elapsed from spontaneous menopause or oophorectomy.

METHODS

Histologic procedure

For histologic determination the surgical specimens were evaluated first cryostatically and then fixed in buffered formalin for 24 h at 20°C, dehydrated through graded ethanol and paraffin embedded at 56°C for 30 min. Sections were cut at 4–6 μm and stained with hematoxylin and eosin for light microscopic analysis. Tumors were classified by histologic type according to the criteria of the National Surgical Adjuvant Breast Project [23].

All identifiable lymph nodes in the axillary specimens were examined by light microscopy.

Histopathology

Surgically obtained breast cancer specimens were stripped of blood, fatty tissue and necrotic areas and were immersed for 2 min in a beaker of isopentane, suspended in liquid nitrogen and then removed from the isopentane and immersed directly in the liquid nitrogen. Cutting and staining were done immediately or after freezing at −70°C for a maximum of 1 month. Sections were air-dried for 30 min at room temperature, immersed in acetone at 4°C for 10 min and then dried for another 30 min.

Serial sections used for cryostatic intraoperative diagnosis were used both for Ki-67 and for PgR-ICA immunocytochemistry determinations.

Receptor immunocytochemical assay

Specimens were analyzed for PgR using the rat monoclonal antireceptor antibody IgG KD-68 with the PgR-ICA kit assay developed by Abbott Diagnostics (Abbott Laboratories, North Chicago, IL). The assay uses a peroxidase–anti-peroxidase (PAP) technique for the visualization of PgR in frozen tissue sections. Frozen samples were cut into 4–6 μm sections at −20°C, thaw-mounted onto glass slides and fixed in 4% formaldehyde-phosphate buffer saline solution (PBS) for 10 min. The slides were transferred to cold methanol at −10°C for 5 min and then to cold acetone at −10°C for 3 min. The slides were then rinsed in PBS for 5 mins and stored in specimen storage medium (0.25 M sucrose and 6.9 mM magnesium chloride) in 250 ml PBS plus 250 ml glycerol at −10°C for up to 1 month before assay. Stored sections were washed in PBS prior to the PgR-ICA and then incubated for 15 min with normal goat serum (blocking agent). The primary antibody (anti-human PgR rat monoclonal antibody KD-68) was added dropwise to one section of each specimen, control antibody (rat IgG) was added to the second section from the same specimen and sections were incubated with the bridging antibody (goat anti-rat IgG) for 30 min. Slides were washed again in PBS for 5 min before and after a further 30 min incubation with rat PAP complex. Sections were immersed in distilled water before counterstaining with Mayer's modified hematoxylin for 5 min. Sections were rinsed in water for 5 min and mounted in Kaiser's gelatine.

Controls consisted of PgR-positive cells provided with the kit and treated with primary or control

antibody. In addition, a series of tumor specimens were treated with primary or control antibody. For each tumor a total of 30 fields were counted at random and a mean percentage of stained nuclei was calculated. The same tumors were evaluated independently by two observers (P.B. and M.P.). Tumors were classified as PgR-ICA-positive with two independent methods of evaluation: the first adopting only a quantitative expression with an arbitrary single cut-off point (>5% of nuclei stained) and the second adopting a modified semiquantitative approach as described by McCarty *et al.* [24] which incorporates both the intensity and the distribution of staining in a single numerical score. To simplify the evaluation method the score was calculated with the formula $= \Sigma(i+1) \times Pi$ and consists of the sum of only three staining intensities i [−(0); +(1); ++(2)] multiplied by the percentage of nuclei cells stained (Pi) in each category. Heterogeneity was defined as the proportion of positively stained tumor cells. The immunocytochemical slides were scored independently, without knowledge of the results of the biochemical assay.

Biochemical receptor assay

Biochemical steroid receptor analysis was performed in tumor samples snap-frozen in liquid nitrogen and stored at −70°C. The DCC assay was employed according to the EORTC method [25]. The evaluation of the apparent affinity constant (K_a) and the free receptor concentration was carried out according to Scatchard [26]. Samples with K_a higher than 0.56×10^9 M^{-1} with more than 10 fmol of cytosol were considered positive.

Immunocytochemical assay of growth fraction (Ki-67 antibody)

Sections were first washed briefly in 0.01 M PBS pH 7.2 and then incubated at room temperature for 20 min in normal blocking serum and successively without washing, with Ki-67 monoclonal antibody (Dakopatts Ltd, U.K.) diluted 1:50 for 60 min, biotinylated horse anti-mouse Ig for 30 min (Vector Lab., Burlingham, CA, U.S.A.) and avidin–biotinylated horseradish peroxidase complex for 30 min (Vector Lab., Burlingham, CA, U.S.A.). Between incubations the slides were washed with PBS. A detailed description of the method has been reported elsewhere [27].

Statistical analyses

The association between positive and negative results in receptor assay with ICA versus DCC methods was assessed by the kappa (κ) statistic [28]. This test determines the probability of obtaining κ in more discordant couplings of a type on a total of n discordant couplings. The total number of concordant cases was 99 and the total number of discordant numbers was 30. Kappa is interpreted as the degree of agreement between two methods beyond change agreement. The chi-square (χ^2) test for contingency tables was employed to test for association between receptor expression with menopausal status, cell kinetics and histopathological features. The type I-α value < 0.05 was considered for statistical significance.

The relationship between PgR-ICA and PgR-DCC and histologic type, tumor size and lymph node status was examined by Fisher's exact test.

RESULTS

Correlation between immunocytochemical and biochemical methods

KD-68 antibody produced staining to the nucleus of breast cancer cells and showed a variable intensity. The percentage of stained cells also varied from case to case. There were patchy positive and negative areas (focal positivity) and areas in which the neoplastic cells showed different staining intensities or an alternation of positive and negative nuclei (heterogeneity). Very rare cells had weak cytoplasmatic staining. No binding was ever observed in stromal or myoepithelial cells or in blood vessels or in infiltrating lymphocytes in the carcinomas. There were 65 (50%) immunoreactive tumors (PgR-ICA positive) of the 129 examined. The proportion of KD-68 positive nuclei ranged from 1 to over 90%. Tumors with more than 5% nuclei stained were arbitrarily considered PgR-ICA positive. In fact, with this cut-off we obtained comparable results and with a reduced possibility of subjective error with respect to those using the more complex histoscore method. Three histochemical classes were defined (0; 1; 2) so that each tumor could be assigned to one of these, on the basis of the estimated proportion of the entire tumor cell population showing specific staining. Using the above semiquantitative method there were: 64 (50%) PgR-ICA-negative tumors (0); and 65 (50%) PgR-ICA-positive tumors, 37(57%) of which had weak or intermediate levels of PgR-ICA expression (1 = from 5 to 30%) and 28/65 (43%) tumors had high levels of PgR-ICA (2 = >30%). All slides were evaluated independently by two observers. Intraobserver variation was <5%. Interobserver variation was present in 25% of cases, however concerning only a disagreement regarding the assignment to contiguous classes of positivity (1 or 2) while interobserver variation was present in <10% of cases between negativity (0) or weak positivity (1).

Assuming a cut-off value of ≥10 fmol/mg of proteins for PgR positivity, there were 53 (41%) DCC-positive tumors of the 129 analyzed. The range of positivity ranged from 10 to 350 fmol/mg

of protein. Within DCC-positive tumors there were 32 (60%) expressing weak or intermediate levels of PgR (from 10 to 99 fmol/mg) and there were 21 (40%) tumors expressing very high levels of receptor (≥100 fmol/mg).

The degree of correlation was measured between the immunocytochemical assay of receptor and the cytosol receptor concentration of each specimen determined by DCC. A good overall relationship between semiquantified PgR-ICA results and the biochemical values was observed (Table 1). There was an overall agreement of 77%, $\kappa = 0.510$ ($P = 0.03$). The sensitivity and specificity of the immunocytochemical method were respectively 83% and 72%; assuming that DCC method is truth. Eighty-three per cent (44/65) of the PgR-ICA-positive tumors were also positive by the DCC method, and only 27.5% (9/64) of the PgR-ICA-negative tumors contained biochemical values above 10 fmol/mg of cytosol protein (five of which with DCC values comprised between 10 and 13 fmol/mg; the other four: 32; 56; 58 and 249 fmol/mg).

Receptor expression and clinico-pathological features

Thirty-six women were pre- or perimenopausal and 93 were in postmenopausal. As shown in Table 2 no correlation was observed between the distribution of the receptor and the menopausal status ($\chi^2_1 = 0.71$). The degree of the association between PgR-ICA and PgR-DCC methods was better in premenopause ($\chi^2_1 = 0.05$) than in postmenopause ($\chi^2_1 = 2.61$). Histologic types were: 101 (78%) invasive ductal carcinomas; 24 (19%) invasive lobular carcinomas; three medullary and one papillary carcinomas (3%). No correlation was observed between histologic type and receptor positivity ($\chi^2_2 = 0.2480$). Fisher's exact test revealed a similar distribution of positivity for both methods ($P = 0.1990$ for ductal; $P = 0.3850$ for lobular and $P = 0.5$ for other types).

In our series there were 78 pT_1, 49 pT_2 and 2 pT_3 tumors and no different distribution of receptor positivity was found with tumor size classification ($\chi^2_2 = 1.2657$). A similar distribution between the two methods was observed with Fisher's exact test ($P = 0.1299$ for pT_1; $P = 0.2720$ for pT_2 and $P = 0.8333$ for pT_3).

There were 57 (44%) lymph node (N)-positive and 72 (56%) N-negative cases. Fifty-nine per cent (38/65) of PgR-ICA-positive and 55% (29/53) of PgR-DCC-positive tumors were N-negative, thus a positive trend between receptor expression and N-negative status was found ($\chi^2_1 = 3.7539$). A similar distribution of the two methods was revealed with the Fisher's exact test ($P = 0.3533$ for N+ and $P = 1910$ for N−).

Receptor expression and cell kinetics

The data presented in Table 3 demonstrate the relationship between PgR distribution and Ki-67 rate.

Eighty-four (65%) carcinomas had 19% of nuclei stained with the Ki-67 antibody (low proliferating) and 45 (35%) had ≥ 20% of nuclei labeled (high proliferating).

Adopting the immunocytochemical assay for PgR, a significant negative relationship was observed between PgR-ICA-positive tumors with a high Ki-67 antibody rate ($\chi^2_1 = 10.27$; $P < 0.01$).

In fact, 31% (14/45) of the PgR-ICA-positive were fast proliferating vs. 69% (31/45) of the PgR-ICA-negative cases.

With the biochemical method no significant correlation was found between the receptor expression and the growth fraction ($\chi^2_1 = 2.86$).

DISCUSSION

The search for new techniques for a more accurate measurement of receptor status represents a major area of research in breast cancer because of the relationship between ER and PgR expression with prognosis and responsiveness to endocrine thera-

Table 1. Correlation of results of semiquantified PgR-ICA and biochemical DCC*

	PgR-ICA		
PgR-DCC	Positive	Negative	Total
Positive	44 (83%)	9 (17%)	53
Negative	21 (27.5%)	55 (72.5%)	76
Total	65	64	

*Overall agreement = 77%; sensitivity = 83%; specificity = 72%.

Table 2. Comparison of distribution of PgR-ICA and PgR-DCC with menopausal status

	PgR-ICA		PgR-DCC	
Menopausal status	Positive	Negative	Positive	Negative
Pre- perimenopausal ($n = 36$)	16 (44%)	20 (56%)	15 (42%)	21 (58%)
Postmenopausal ($n = 93$)	49 (53%)	44 (47%)	38 (41%)	55 (59%)

Table 3. Comparison of the distribution for PgR-ICA and PgR-DCC with Ki-67 antibody

	PgR-ICA		PgR-DCC	
Ki-67*	Positive	Negative	Positive	Negative
Low (n = 84)	51 (61%)	33 (39%)	39 (46%)	45 (54%)
High (n = 45)	14 (31%)	31 (69%)	15 (33%)	30 (67%)

*Low = Ki-67 < 19%; high = Ki-67 ≥ 20%.

pies. The development of monoclonal antibodies has permitted the assessment, by a practical immunocytochemical assay, of hormonal receptor antigenic sites in human tumors and to investigate the heterogeneity of ER and PgR distribution which could not be assessed by the biochemical method [17, 22]. Our study with the PgR-ICA method results in nuclear staining of breast PgR-positive cancer cells. This nuclear localization is in agreement with previous immunocytochemical determinations, suggesting that ER and PgR are predominantly nuclear proteins [16, 29].

Breast cancer is a disease characterized by a substantial biological heterogeneity, being composed of various cell populations, with different metastatic potential [30]. Our experience demonstrates the heterogeneous distribution of nuclear PgR-ICA staining in the majority of sections examined. In the present study the overall frequency of PgR-ICA positivity is 50% and the proportion of positive nuclei ranged from 1 to over 90%. These results are comparable with the range of PgR-ICA values reported by other authors [31–34] using different monoclonal antibodies.

We found a significant correlation between results of the PgR-ICA and the DCC methods based on the assumption that the second is truth. In our series of 129 patients we obtained an overall agreement of 77% between the two methods and a PgR-ICA sensitivity of 83% and a specificity of 72%. A total of 30 out of 129 cases (23%) were classified either as 'false positive' or 'false negative' relative to the biochemical assay. This percentage of discordance is similar to that reported by other authors [32, 34], but does not confirm the excellent correlations shown by Perrot-Applanat *et al.* [35] and Charpin *et al.* [33] (respectively 92% and 91.5%), the latter using a multiparametric computerized analysis. The rates of discordance between PgR-ICA and DCC by the above authors are not due to different DCC cut-off points (10 fmol/mg for all the laboratories).

In general, the discrepancy observed in some instances between the two methods may be explained by a variety of reasons. The two assays were performed for technical reasons on different portions of the same specimen. The PgR-ICA method has the intrinsic possibility of identifying the degree of cellularity, while this is not possible in the DCC assay. Consequently, a low biochemical positivity could result from a low density of neoplastic cells or from an abundant necrosis, fibrosis or fat. The immunocytochemically positive but biochemically negative results could be also due to the fact that, *in vivo*, the latter method detects unoccupied sites only. On the contrary it is possible that antigenic PgR determinants are destroyed during the storage or fixation, causing false-negative ICA results. Therefore the main explanations for discordant results could be the heterogeneity of the receptor content in the same carcinoma and the choice of the method of evaluation of the immunohistochemical staining.

Regarding menopausal status we found no correlations with PgR-ICA or DCC positive. These data are in accordance with those previously reported with the ICA assay [32, 34] and with the biochemical method [36, 37].

We compared PgR-ICA positivity with some histopathological features. Regarding histologic type, we did not observe any association with PgR-ICA or DCC positivity, however 78% of carcinomas tested were invasive ductals while the other histotypes analyzed represented a small proportion. No correlations were found between both methods for assessing PgR expression and pathologic tumor size and, to date, to our knowledge, no other author has compared PgR-ICA with tumor size. Using the DCC method, Howat *et al.* [38] found no differences regarding tumor size while Clark *et al.* [36] revealed a significantly higher PgR-positivity in pT_1 vs. pT_3 tumors (57% vs. 44%). In our study, the comparison between PgR expression and pathological tumor size was performed however mainly within pT_1 and pT_2 cases. Contrary to Charpin *et al.*'s [33] data which show a significantly higher PgR-ICA positivity in stage I (lymph node negative) disease, our results found no difference in PgR distribution regarding lymph node status and are in agreement with the results of Clark *et al.* [36] and Fisher *et al.* [37] with the DCC method.

The finding that PgR, tumor size and lymph node status are independent variables suggests that these factors might be a powerful combination of prognostic indicators that should be used for a better

stratification of patients in adjuvant therapy clinical trials.

Further, we have also determined the tumor cell kinetics with the monoclonal Ki-67 antibody using a rapid, practical and easily performed immunocytochemical method, as just reported [27, 39, 40]. The mean value of Ki-67 distribution was chosen as cut-off value (20%). The Ki-67 rate was correlated with the PgR expression using both PgR-ICA and biochemical assays. While only a negative trend was observed between high proliferative rate and PgR with the DCC method, a significant inverse correlation was found with PgR-ICA positivity ($P < 0.01$).

An inverse correlation between PgR determined by DCC and proliferative rate determined by the thymidine labeling index was found [41, 42]. In our series the different correlations observed between the two methods for assessing receptor status and tumor proliferative activity need to be studied further.

In conclusion, immunocytochemical detection of PgR is of potential clinical value since this assay has the advantage of a low cost and not requiring large tissue samples. It also permits a cell by cell analysis of the receptor expression and enables hormonal receptors to be detected in fine needle aspirates of primary breast cancer, bone marrow or other metastatic sites [43, 44]. The ICA method can also explore the dynamics of intracellular PgR in response to changes in the hormonal environment and can provide information on cellular heterogeneity. Since PgR-ICA is a very practical method with a wider potential application, it may be employed in routine pathological laboratories in association with other ICA determinations to better select high risk patients. These considerations suggest that PgR-ICA (as well as ER-ICA) could improve the precision and reliability of hormonal receptor detection and should be considered as a qualitative method complementary to the quantitative biochemical assay. The association of both methods should enhance the possibility of evaluating the prognosis and predicting the responsiveness to hormonal treatment in breast cancer patients.

Acknowledgements—The authors thank M. Gion M.D., Radiotherapy and Oncology Center, General Hospital, Venice, Italy for performing the PgR assay by DCC, G. Pellizzari for statistical assistance and D. Mazzocco for help in preparing the manuscript.

REFERENCES

1. Horwitz KB, McGuire WL, Pearson OH, Segaloff A. Predicting response to endocrine therapy in human breast cancer: a hypothesis. *Science* 1975, **189**, 726–727.
2. Rao BR, Wiest WG, Allen WH. Progesterone receptor in rabbit uterus. Characterization and 17-β-estradiol augmentation. *Endocrinology* 1973, **92**, 1229–1240.
3. Kiang DT, Frenning DH, Goldman AI, Ascensao VF, Kennedy BJ. Estrogen receptors and responses to chemotherapy and hormonal therapy in advanced breast cancer. *N Engl J Med* 1978, **299**, 1330–1334.
4. Gapinski PV, Donegan WL. Estrogen receptors and breast cancer: prognostic and therapeutic implications. *Surgery* 1980, **88**, 386–393.
5. McGuire WL, Clark GM. Role of progesterone receptors in breast cancer. *Semin Oncol* 1985, **12**, 12–16 (Suppl 1).
6. Horwitz KB. The central role of progesterone receptors and progestational agents in the management and treatment of breast cancer. *Semin Oncol* 1988, **15**, 14–19 (Suppl 2).
7. Knight WA III, Livingston RB, Gregory EJ *et al.* Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res* 1977, **37**, 4669–4671.
8. Godolphin W, Elwood JM, Spinelli JJ. Estrogen receptor quantification and staging as complementary prognostic indicators in breast cancer: a study of 583 patients. *Eur J Cancer Clin Oncol* 1981, **28**, 677–683.
9. Crowe JP, Hubay CA, Pearson OH *et al.* Estrogen receptor status as a prognostic indicator for stage I breast cancer patients. *Breast Cancer Res Treat* 1982, **2**, 171–176.
10. Clark GM, McGuire WL, Hubay CA, Pearson OH, Marshall JS. Progesterone receptors as a prognostic factor in stage II breast cancer. *N Engl J Med* 1983, **309**, 1343–1347.
11. Pichon MF, Pallud C, Brunet M, Milgrom E. Relationship of presence of progesterone receptors to prognosis in early breast cancer. *Cancer Res* 1980, **40**, 3357–3360.
12. Osborne CK. Heterogeneity in hormone receptor status in primary and metastatic breast cancer. *Semin Oncol* 1985, **12**, 317–326.
13. Paulsen SM, Pedersen KO. Comparison of histo- and biochemical methods for estrogen and progesterone receptor analysis in primary breast carcinoma. *Eur J Cancer Clin Oncol* 1985, **21**, 1331–1336.
14. Pertschuk LP, Tobin EM, Carter AC *et al.* Immunohistologic and histochemical methods for detection of steroid binding in breast cancer: a reappraisal. *Breast Cancer Res Treat* 1982, **1**, 297–314.
15. Underwood JC. Oestrogen receptors in human breast cancer: a review of histopathological correlations and critique of histochemical methods. *Diagn Histopathol* 1983, **6**, 1–22.
16. King WJ, Greene GL. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* 1984, **307**, 745–747.

17. King WJ, Desombre ER, Jensen EV, Greene GL. Comparison of immunocytochemical and steroid-binding assays for estrogen receptor in human breast tumors. *Cancer Res* 1985, **45**, 293–304.
18. Pertschuk LP, Eisenberg KB, Carter AC, Feldman JG. Immunohistologic localization of estrogen receptors in breast cancer with monoclonal antibodies. Correlation with biochemistry and clinical response. *Cancer* 1985, **55**, 1513–1518.
19. Vlatkovic L, Lykkesfeldt AE, Hov-Jenssen K, Briand P. Comparison of the immunocytochemical assay (ER-ICA) and the biochemical assay for estrogen receptor in human breast cancer cell lines. *Eur J Cancer Clin Oncol* 1987, **23**, 755–760.
20. Marchetti E, Querzoli P, Moncharmont B *et al.* Immunocytochemical demonstration of estrogen receptors by monoclonal antibodies in human breast cancer: correlation with estrogen receptor assay by dextran-coated charcoal method. *Cancer Res* 1987, **47**, 2508–2513.
21. Reiner A, Reiner G, Spong J, Schemper M, Holzner JH. Histopathologic characterization of human breast cancer in correlation with estrogen receptor status. A comparison of immunocytochemical and biochemical analysis. *Cancer* 1988, **61**, 1149–1154.
22. Press MF, Greene GL. Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. *Endocrinology* 1988, **122**, 1165–1175.
23. Fisher ER, Gregorio RM, Fisher B. The pathology of invasive breast cancer: a syllabus derived from findings of the National Surgical Adjuvant Breast Project (protocol No. 4). *Cancer* 1975, **36**, 1–85.
24. McCarty KS Jr, Miller LS, Cox EB, Konrath J, McCarty KS Sr. Estrogen receptor analyses: correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch Pathol Lab Med* 1985, **109**, 716–721.
25. EORTC Breast Cancer Cooperative Group. Standards for the assessment of estrogen receptors in human breast cancer. *Eur J Cancer* 1973, **9**, 379–381.
26. Scatchard G. The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 1949, **51**, 609–672.
27. Gasparini G, Dal Fior S, Pozza F, Bevilacqua P. The correlation of growth fraction by immunohistochemistry (monoclonal Ki-67) with histologic factors, hormonal receptors and menopausal status in operable breast carcinoma. *Breast Cancer Res and Treat* 1989, in press.
28. Fleiss JL. *Statistical Methods for Rates and Proportions*, 2nd edn. New York, John Wiley, 1981, 218.
29. Welshous WV, Krummel BM, Gorski J. Nuclear localization of unoccupied receptors for glucocorticoids, estrogens and progesterone in GH_3 cells. *Endocrinology* 1985, **117**, 2147–2170.
30. Dexter DL, Kowalski HM, Blazer BA, Fligiel Z, Vogel R, Heppner GH. Heterogeneity of tumor cells from a single mouse mammary tumor. *Cancer Res* 1978, **38**, 3174–3181.
31. Elashry-Stowers D, Zava DT, Speers WC, Edwards DP. Immunocytochemical localization of progesterone receptors in breast cancer with anti-human receptor monoclonal antibodies. *Cancer Res* 1988, **48**, 6462–6474.
32. Helin HJ, Helle MJ, Helin ML, Isola JJ. Immunocytochemical detection of estrogen and progesterone receptors in 124 human breast cancers. *Am J Clin Pathol* 1988, **89**, 137–142.
33. Charpin C, Jacquemier J, Andrac L *et al.* Multiparametric analysis (SAMBA 200) of the progesterone receptor immunocytochemical assay in non-malignant and malignant breast cancer disorders. *Am J Pathol* 1988, **132**, 199–211.
34. Pertschuk LP, Feldman JG, Eisenberg KB *et al.* Immunocytochemical detection of progesterone receptor in breast cancer with monoclonal antibody. Relation to biochemical assay, disease-free survival and clinical endocrine response. *Cancer* 1988, **62**, 343–349.
35. Perrot-Applanat M, Groyer-Picard MT, Lorenzo F *et al.* Immunocytochemical study with monoclonal antibodies to progesterone receptor in human breast tumors. *Cancer Res* 1987, **47**, 2652–2661.
36. Clark GM, Osborne CK, McGuire WL. Correlations between estrogen receptor, progesterone receptor and patient characteristics in human breast cancer. *J Clin Oncol* 1984, **10**, 1102–1109.
37. Fisher B, Wickerham L, Brown A *et al.* Breast cancer estrogen and progesterone receptor values: their distribution, degree of concordance and relation to number of positive axillary nodes. *J Clin Oncol* 1983, **1**, 349–358.
38. Howat JMT, Barnes DM, Harris M, Swindell R. The association of cytosol oestrogen and progesterone receptors with histological features of breast cancer and early recurrence of disease. *Br J Cancer* 1983, **47**, 629–640.
39. Lellé RJ, Heidenreich W, Stauch G, Gerdes J. The correlation of growth fractions with histologic grading and lymph-node status in human mammary carcinoma. *Cancer* 1987, **59**, 83–88.
40. McGurrin JF, Doria MI Jr, Dawson PJ, Karrison T, Stein H, Franklin WA. Assessment of tumor cell kinetics by immunohistochemistry in carcinoma of the breast. *Cancer* 1987, **59**, 1744–1750.
41. Myers GS, Frey MV, Babcock DS *et al.* Breast carcinoma, cell kinetics, morphology, stage

and host characteristics: a thymidine labeling index study. *Lab Invest* 1986, **54**, 41–46.
42. Cattoretti G, Silvestrini R, Canova S, Valentinis B, Rilke F. Proliferative indexes determined on breast cancer by monoclonal antibody Ki-67 reactivity and ^{3}H thymidine labeling. In: Proceedings of the 16th Meeting of the European Study Group for Cell Proliferation, Milan, 3 May 1989, Abst. No. 44.
43. Coombes RC, Powles TJ, Berger V *et al.* Prediction of endocrine response in breast cancer by immunocytochemical detection of oestrogen receptor in fine-needle aspirates. *Lancet* 1987, **ii**, 701–703.
44. Berger V, Mansi JL, Wilson P, Coombes RC. Detection of estrogen receptor in bone marrow from patients with metastatic breast cancer. *J Clin Oncol* 1987, **5**, 1779–1782.