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Biological and Clinical Relevance of K_d Values for Estradiol Binding in Primary Breast Cancer Tumors

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Abstract—*The question of whether the dissociation constant (K_d) observed for the binding between eastradiol and the [^{3}H]estrogen receptor (ER) indicates the existence of a single class of estrogen binding proteins in breast cancer tissue has been examined among a population of* 3020 *ER-positive* (≥ 10 fmol/mg *cytosol protein) primary breast cancer patients. The median value for* K_d*s was found to be* 0.9×10^{-10} M. K_d *values were only weakly correlated to ER concentrations in the respective biopsies. Nevertheless, high* K_d *values were associated with lower measured ER concentrations among pre/perimenopausal patients. Meanwhile, the frequencies of PgR-positivity are consistently high among pre/perimenopausal patients irrespective of* K_d *value. In contrast, the frequency of PgR-positivity is significantly lower among postmenopausal patients with high* K_d *values. Furthermore, postmenopausal patients with high* K_d *values* ($>1.4 \times 10^{-10}$ M) *tend to have shorter recurrence-free survivals than other ER-positive patients.*

A possible interpretation of these findings is that high K_d *values reflect physiologically normal, cyclically high endogenous concentrations of estradiol in tumor tissue among pre/perimenopausal patients. Among the postmenopausal patients, the presence of high* K_d *values might reflect the presence of an estradiol binding molecule(s) slightly different from normal ER in the tissue of postmenopausal patients.*

INTRODUCTION

USING the multipoint titration method for analysis of receptor binding, the K_d value of the binding between hormone and receptor can be assessed. Although K_d values are continuously estimated in numerous laboratories, little has been published concerning their character or clinical usefulness. Such an evaluation is timely because attractive alternative methods for routine determination of ER concentration in tumor tissue using monoclonal antibody techniques have recently become available [1–3]. The newer methods preclude determination of K_d values.

Since the K_d value reflects the binding affinity between receptor and ligand, the 'true' K_d value could be expected to be identical within the same target tissue in a single species. However, the observed K_d value also depends upon assay methodology [4], and considerable inter- as well as intralaboratory variations are noted [5].

Any or all of the following three methodological points can influence the K_d value: (a) the compositions of the charcoal slurry and conditions used for separation of bound and free hormone, (b) the method used to correct for assay background counts and nonspecific binding, and (c) the concentrations of endogeneous hormone present in the tissue being analyzed. The latter point (c) is elementary in nature, and more than a decade ago it was noted that 'the calculation of equilibrium binding constants for any specific protein–ligand interaction requires the exact determination of the unbound ligand concentration and the specifically bound ligand concentration' [6]. Nevertheless, this criterion is rarely satisfied in routine determinations of receptor concentrations.

The purpose of the present investigation addresses the question of whether the K_d values

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observed for the binding between [^{3}H]estradiol and ER indicate that there is a single class of estrogen binding proteins in breast cancer tissue. Furthermore, biological and clinical implications of deviating K_d values are explored.

PATIENTS AND METHODS

Patients

The 3020 patients in the present study are primary breast cancer patients included in the Danish Breast Cancer Cooperative Group (DBCG) project that have had estrogen receptor analyses performed on their tumor tissue in a single laboratory between September 1979 and March, 1987, and that have been found to be ER positive ($\geq$ 10 fmol ER/mg cytosol protein).

The organization, design and follow-up of the DBCG program and the 77 protocols have been described in detail elsewhere [7]. When menostasia had persisted for at least 5 years, women were defined as being postmenopausal, otherwise women were classified as being pre/perimenopausal. Arbitrarily, we have adapted an age limit of 50 years to distinguish between pre- and perimenopausal women.

The end-point of the recurrence-free survival (RFS) analyses is recurrent disease or death. Cause of death is not noted in the DBCG registry; the frequency of deaths due to causes other than breast cancer is assumed to be equal in different patient subgroups for patients of the same age.

Estrogen and progesterone receptor analyses

Tissue from primary breast cancer tumors was analyzed for ER and PgR concentrations using the dextran-coated charcoal (DCC) method using multipoint titration analysis and Scatchard analysis of the binding data [8] as recommended by the EORTC with the minor modifications published elsewhere [9]. In brief, 2,4,6,7-[^{3}H]estradiol (TRK.322, 85–110 Ci/mmol, Amersham) in seven different concentrations ranging from 0.3 to 5×10^{-9} M was incubated with 50 µl aliquots of cytosol. Diethylstibestrol (DES) at a concentration of 5×10^{-7} M was used to estimate nonspecific binding of the radioactive ligand and the correction for non-specific binding was performed using the method described by Chamness and McGuire [10]. A straight line is drawn through as many points of the specific binding data as possible using 'the eyeball' method rather than linear regression. Obvious outlying points were ignored—as was the horizontal plateau typically found for the lowest hormone concentrations in samples with high concentrations of ER and the 'hook' observed in some samples at the lowest concentrations of hormone incubated. Continuous quality control studies of the ER and PgR assays are performed in collaboration with other European laboratories in the EORTC receptor group [11].

The sole criterion used to classify patients as receptor positive or receptor negative was the receptor concentration itself. A cut-off level of 10 fmol/mg cytosol protein was employed to make this distinction.

The unit used for the given K_d values is 10^{-10} M. Subgrouping of K_d values into classes of low, intermediate and high values is based upon the distribution curve of K_d values (using values for the median, first and last quartiles).

Statistical analyses

The nonparametric Kruskal–Wallis test has been employed to detect differences in distributions. Regression analysis was employed to fit a parabola to the data. Recurrence-free survival (RFS) was calculated using the Kaplan–Meier method and tests for comparison were done using the log rank test. Otherwise, conventional statistical tests have been applied to the data. A two-sided P-value of 0.05 is considered to be significant.

RESULTS

The inter-assay variability for determination of K_d values in a single sample was estimated by analyzing a standard, lyophilized rabbit uterine cytosol during weekly, routine analyses for 22 consecutive weeks. As can be seen in Table 1, the coefficient of variation for determination of K_d for this sample is 35%. The comparative figure for analyses of 3020 ER positive primary breast cancer biopsies is 123%. This great variability observed in breast cancer biopsies might be due in part to the fact that a wide spectrum of ER values [median, range: 112 (39–2888)] have been determined, while one specific ER concentration was analyzed in the

Table 1. Reproducibility of estimates of K_d values in lyophilized rabbit uterine cytosol and estrogen receptor positive primary breast cancer cytosols

Tissue	*n*	Mean	S.D.	C.V.
Lyophilized rabbit uterine cytosol	22	1.44	0.5	35%
Primary breast cancer cytosols	3020	1.31	1.6	123%

lyophilized standard cytosol (390 fmol/mg). Other possible sources of the observed greater variation in K_d values measured among tumor biopsies may be either differences in the handling and transportation of the individual biopsies to the receptor laboratory and/or differences in non-specific binding among the individual cytosols.

Dependence of K_d *on ER concentrations*

To investigate whether the measured K_d value is associated with ER concentration, a plot of the K_d values for the spectrum of ER concentrations in breast cancer cytosols has been constructed. As can be seen in Fig. 1, the curve appears to be parabolic [the fitted curve: $K_d = 6.5 - 2.2 \log (ER) + 0.22 (\log (ER))^2$] with highest K_d values and greatest variation occurring at the extremes of ER concentrations. Although significant ($P \leq 0.0001$), the overall association between ER concentration and observed K_d is weak.

Dependence of K_d *on menopausal status*

The distribution of K_d values for all 3020 patients subgrouped according to menopausal status is shown as a frequency diagram in Fig. 2 and expressed in terms of median values and quartiles in Table 2. Although the numeric difference in values among the three menopausal groups is slight, the distribution of values for postmenopausal patients was found to be significantly lower (Kruskal–Wallis Test, $P = 0.0001$) than that of both pre- and perimenopausal patients.

Dependence of ER on K_d *value*

While the K_d value was found to be only weakly associated with the concentration of ER measured, the question of whether ER concentrations differ depending on the magnitude of the K_d value has also been investigated. Frequency diagrams of the distributions of the logarithmically transformed ER concentrations for patients with low, intermediate and high K_d values are shown in Fig. 3 A and B. While the shapes of the distribution curves of ER values are approximately the same for all three groups of K_d among pre/perimenopausal patients, progressive shifts to lower ER concentrations are observed as the K_d value increases. In contrast, while no such shifts are observed among postmenopausal patients, the distribution curve of ER concentrations for patients with the highest K_d values differs markedly from those of the other two groups. Among postmenopausal patients with high K_d values, a bimodal distribution curve is observed with ER concentrations tending to be either low (~ 30 fmol/mg cytosol protein) or high (~ 400 fmol/mg cytosol protein).

Association between K_d *and frequency of PgR positivity*

Since PgR is synthesized in the presence of a functional ER and estradiol, the frequency of PgR positivity in relation to K_d value was investigated in patients with low, intermediate, and high K_d values. While there were no significant differences in the frequency of PgR positivity and K_d value for pre- or perimenopausal patients (Table 3), post-

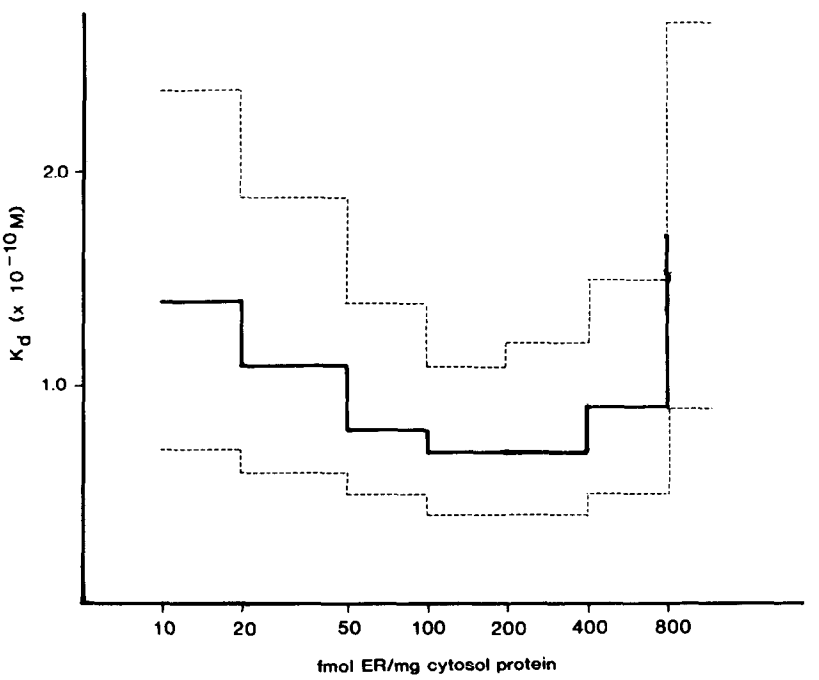

Fig. 1. Median values for the K_d *(solid line) and first and last quartiles (dashed lines) are indicated for ER positive, primary breast cancer patients grouped into the following arbitrary groups of ER concentrations:* 10–20 (n = 333), 20–50 (n = 588), 50–100 (n = 533), 100–200 (n = 573), 200–400 (n = 533), 400–800 (n = 389), *and* >800 (n = 121) fmol *ER/mg cytosol protein. The equation fitting the data is* $K_d = 6.5 - 2.2 \log (ER) + 0.22 (\log (ER))^2$, r = 0.26.

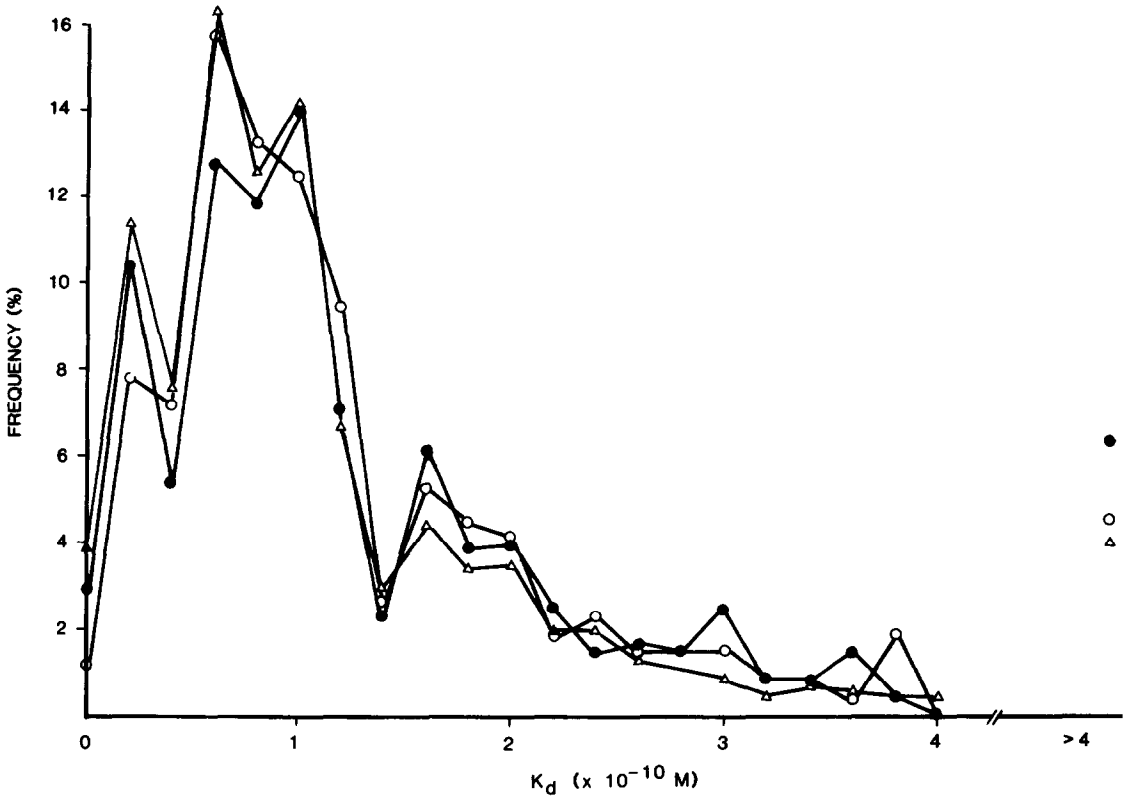

Fig. 2. Frequency diagram of the distribution of K_d values for ER in biopsies from pre-, peri- and postmenopausal patients with primary breast cancer. The total number of patients within each menopausal group represents 100% [*premenopausal (closed circles)*: n = 601; *perimenopausal (open circles)*: n = 267; *postmenopausal (open triangles)*: n = 2152].

Table 2. Distribution of K_d for patients with ER positive primary breast cancer (n = 3020)

Patient group	*n*	Median (quartiles)	*P* value for univariate analysis of RFS
All patients	3020	0.9 (0.5–1.5)	0.332
Menopausal status*			
pre-	601	1.0 (0.6–1.8)	0.624
peri-	267	1.0 (0.6–1.7)	0.739
post-	2152	0.8 (0.5–1.4)	0.180

*Kruskal–Wallis test for difference in distribution of K_d values according to menopausal status: $P = 0.0001$.

Table 3. Frequency of PgR positivity using quartiles for the overall distribution of K_d values

K_d value	Frequency of PgR positivity Menopausal status		
	Pre- ($n = 520$)	Peri- ($n = 228$)	Post- ($n = 1772$)
Low			
$0 \leq K_d < 0.5$	94%	84%	79%
Intermediate			
$0.5 \leq K_d \leq 1.5$	93%	79%	81%
High			
> 1.5	93%	87%	75%
P-value*	0.93	0.35	0.024

*The χ^2-test was applied to the data.

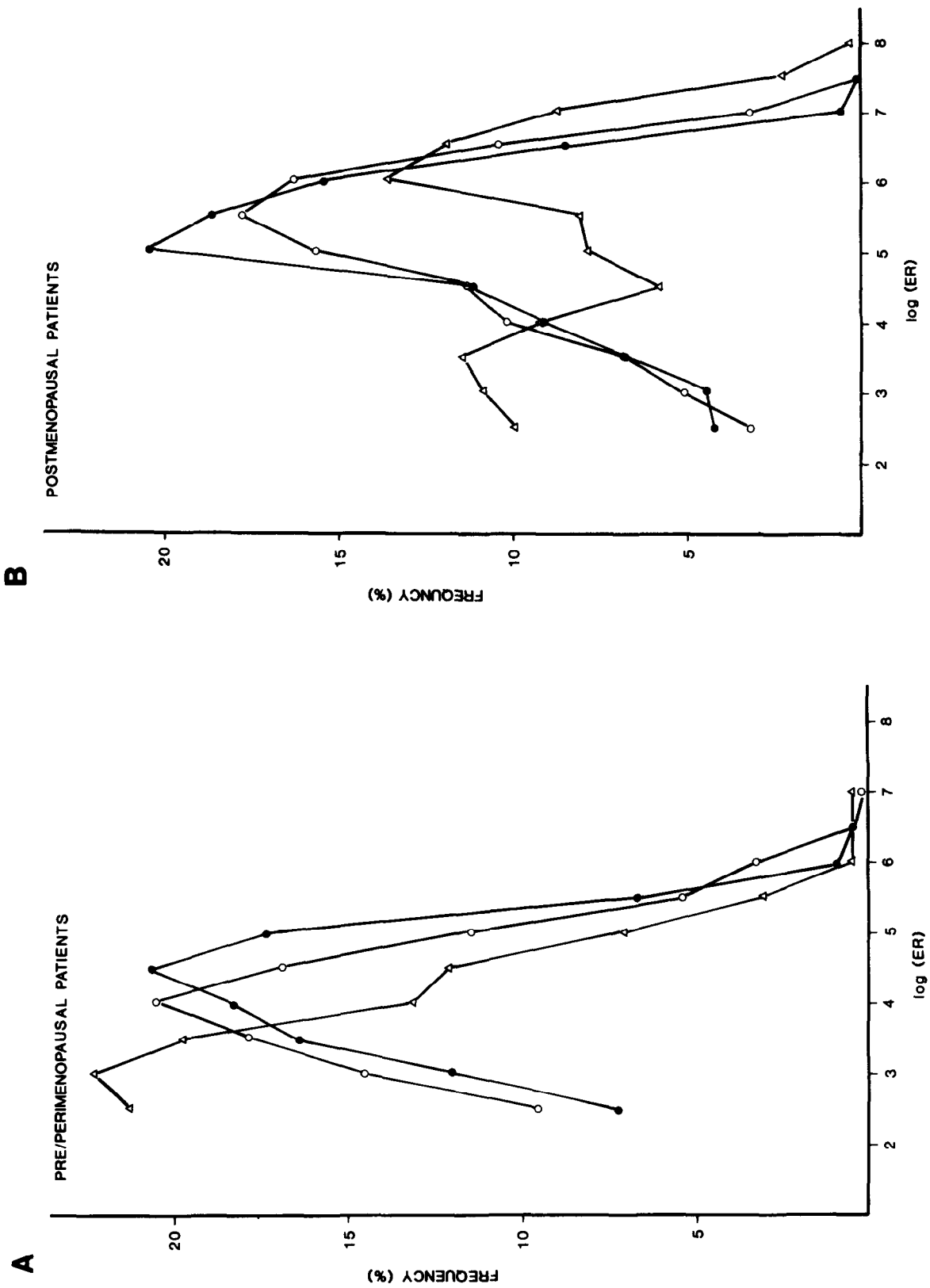

Fig. 3. A. Pre/perimenopausal patients (n = 868): *frequency diagram of ER concentrations (logarithmically transformed values) among patients with low (solid circles,* $K_d < 0.6$), *intermediate (open circles,* $0.6 \leq K_d \leq 1.8$) *and high (open triangles,* $K_d > 1.8$) K_d *values. The distributions differ significantly among the three groups (Kruskal–Wallis test,* P = 0.0001). *B. Postmenopausal patients* (n = 2152): *frequency diagram of ER concentrations (logarithmically transformed values) among patients with low (solid circles,* $K_d < 0.5$), *intermediate (open circles,* $0.5 \leq K_d \leq 1.4$) *and high (open triangles,* $K_d > 1.4$). *The distributions do not differ significantly among the three groups (Kruskal–Wallis test,* P = 0.065).

menopausal patients with high K_d values have a significantly lower frequency of PgR positivity. Referring to the bimodal distribution of ER concentrations observed for postmenopausal patients with high K_d values (Fig. 3 B), it can be noted that this low frequency of PgR positivity is observed among those patients with low to intermediate ER concentrations (< 240 fmol/mg cytosol protein) where the frequency of PgR positivity is only 67%. In contrast, patients with both high K_d values and high ER concentrations have a high frequency of PgR positivity (88%).

Association between K_d values and recurrence-free survival (RFS)

Finally, to investigate whether deviant K_d values might be important clinically, lifetable analysis of the RFS was performed for all patients protocolled in the DBCG project irrespective of post-operative treatment. Low, intermediate, and high K_d values were defined using the distribution of K_d values relevant within each menopausal group. As can be seen in Table 2 post- but not pre- or perimenopausal patients tend to have different RFSs according to K_d value. For further investigation, postmenopausal patients not receiving post-operative systemic adjuvant therapy were considered separately from those who had received such therapy in order to distinguish between potential prognostic versus predictive value of K_d values. While no difference in RFS was observed for patients who received systemic adjuvant therapy (Tamoxifen with or without chemotherapy) ($n = 435$, $P = 0.61$), a nearly significant difference is observed among patients who have not received systemic post-operative treatment ($n = 647$, $P = 0.054$). Patients with low or intermediate K_d values have a longer RFS than patients with high K_d values (Fig. 4). Furthermore, if the lifetable analysis includes the corresponding 160 postmenopausal patients who have not received systemic adjuvant therapy and are ER-negative as well as the 647 patients shown in Fig. 4, the ER-positive patients with high K_d values are nearly superimposed on the curve for the ER-negative patients ($P = 0.03$, curve not shown). The frequency of PgR positivity is 14%, 72% and 81% among the ER−, ER+ with high K_d, and ER+ with low K_d groups of patients, respectively. However, it is unlikely that the distinction between patients with short versus long RFS is founded on a classification of patients into PgR− and PgR+ groups. Using PgR status as the prognostic criterion, lifetable analysis of the same group of patients yielded a P-value of 0.27.

DISCUSSION

Estimation of K_d values based on the incubation of patient samples with only eight hormone concentrations is at its very best associated with a high degree of uncertainty. Nevertheless, with this reservation in mind, and since there has been no conscious bias in constructing lines in the Scatchard plots of the data, we have examined whether routine analysis of K_d values in breast cancer tissue might yield information concerning the biology of primary breast cancer. Of the 3020 analyses that form the foundation of this paper, approx. 85% have been performed by two individual laboratory technicians, which ensures built-in consistency both in assay technique and in the interpretation of binding data.

While the inter-assay coefficient of variation found for the standard lyophilized preparation of ER from rabbit uterine tissue is high, the 3-fold greater coefficient of variation as well as the fact that there is only a weak association between ER level and K_d value suggests that differences in K_d in patient biopsies might indeed reflect biological differences rather than mere assay variability. While the majority of biopsies have a low, relatively well-defined K_d value ($\sim 0.9 \times 10^{-10}$ M), a 2–4-fold higher figure is found in a significant proportion of the biopsies.

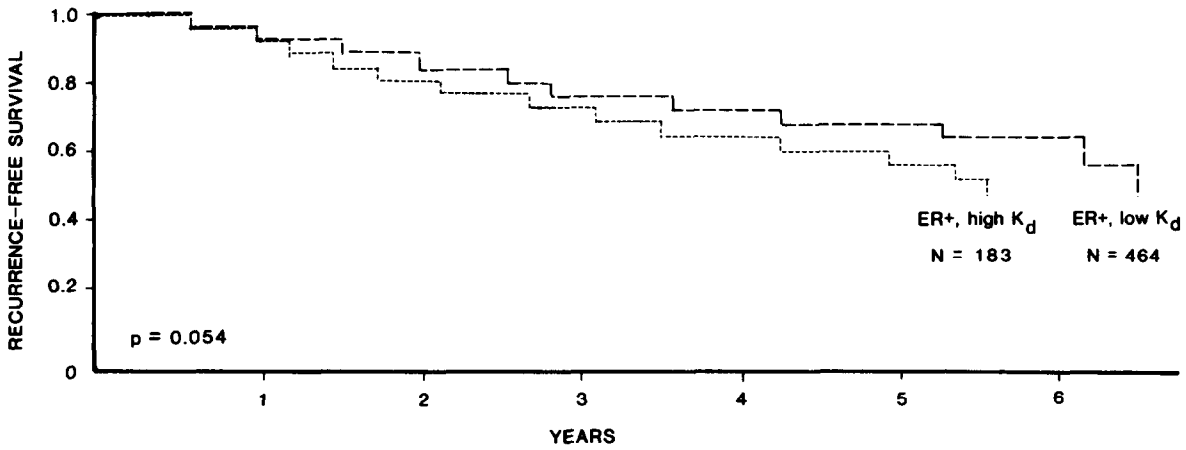

Fig. 4. Lifetable analysis of postmenopausal, primary breast cancer patients who have not received adjuvant systemic therapy. Two groups of ER-positive patients are shown: those with high K_d values ($K_d > 1.4$) and those with low K_d values ($K_d \leq 1.4$) (P = 0.054).

Two of the possible factors that could lead to unusually high K_d values will be considered here. One is the possibility that the concentrations of endogenous estradiol present in some samples is so high as to significantly influence assay results. Under such circumstances higher K_d values as well as false low receptor concentrations would be registered. Alternatively, the high K_d values observed could reflect a binding of estradiol to a molecule(s) different from the specific ER and that has a lower binding affinity for estradiol. This molecule(s) could be another protein (e.g. 'dysfunctional' ER, a putative Type II site ER, or SHBG) or merely be micelles of lipids that act as 'reservoirs' for lipophilic molecules and thus harbor a significant fraction of total ligand added to the assay incubation.

Plasma levels of estradiol is many fold higher among pre- than postmenopausal patients, while tissue levels of estradiol are the same [12]. Moreover, the plasma concentration of estradiol fluctuates up to a factor of 8-fold during the menstrual cycle [12]. While a broad distribution of K_d values could be expected among pre- and perimenopausal patients under such circumstances opposed to postmenopausal patients, the 'tail' of the distribution curve would reflect physiologically normal, cyclically high concentrations of endogenous hormone levels. A reduction in frequency of PgR-positivity would, therefore, not be expected. Analagously, among postmenopausal patients where both tissue and plasma concentrations of estradiol are constant in time, deviant K_d values might reflect abnormal physiological conditions that could also be reflected by other aspects of tumor biology (e.g. lower frequency of PgR-positivity). In addition, if the unusually high K_d values among postmenopausal patients *are* due to hyper-normal concentrations of estrogens, tumor growth rate could be influenced by such continuous high, local concentrations of estradiol and a shorter RFS might be observed among these patients.

The experimental data for the premenopausal patients (i.e. those patients under 50 years of age in whom menostasia has not occurred) supports the hypothesis that high K_d values reflect high endogenous estradiol levels in the biopsy: high K_d values are associated with lower measured ER concentrations, and the frequency of PgR positivity is the same irrespective of K_d values among premenopausal patients. Moreover, high K_d values do not seem to be associated with a poorer prognosis. This phenomenon of lower ER concentrations being associated with high K_d values among the premenopausal patients can be due to two independent factors: (a) the DCC assay method measures only unoccupied receptor and (b) the number of receptors will be underestimated whenever radioactive ligand is diluted with a nonradioactive ligand without taking the fact into consideration in the calculation of the binding data.

On the basis of the present data, high endogenous concentrations of estradiol cannot be excluded as a cause of high K_d values among postmenopausal patients. However, other observations indicate that high K_d values here may be due to the presence of a molecule different from the specific ER, but that binds estradiol with a lower affinity. The most conclusive evidence in this respect is that the frequency of PgR positivity is low among postmenopausal patients with high K_d values. Moreover, prognosis of the ER-positive, postmenopausal patients who have high K_d values tends to be poorer than that for other ER positive patients. Equally noteworthy, no such tendency is apparent among patients treated with systemic adjuvant therapy (Tamoxifen). In this respect, an observation made while working with the monoclonal technique for quantitation of ER (ER-EIA method, Abbott Laboratories) is of interest: the ligand binding DCC assay method was found to detect a significantly greater number of binding sites than the ER-EIA assay particularly among older patients. One of the possible interpretations of this finding was that the DCC assay may also be detecting a protein other than the specific ER that is not recognized by the monoclonal antibody [3]. In this regard, it may also be of interest that binding of estradiol in breast cancer cytosols has been reported by Poulsen *et al.* [13] to be displaceable by both testosterone and progesterone analogs (R5020 and ORG 5020) in a significant proportion of estrogen receptor positive biopsies.

In conclusion, the present data regarding premenopausal patients indicates that the occurrence of high K_d values might reflect normal, physiological conditions (cyclically high endogenous serum levels of estrogen). Among postmenopausal patients, high K_d values may indicate 'abnormal' physiological conditions. Lower frequencies of PgR positivity as well as a shorter RFS are observed among postmenopausal patients with high K_d values. Whether these latter circumstances are associated with (a) the existence of a different estrogen binding protein, (b) unusually high serum and/or tissue estrogen levels in certain patients or (c) a combination of these two possibilities remains to be determined. Due to the large inter-laboratory variations in assay results that are often observed [5], it is not the intent of this report to encourage routine analysis of K_d values in breast cancer tissue cytosols. Rather, it is our purpose to suggest that there might be molecules other than the specific ER molecules that have a lower affinity for binding of estradiol and that may be of clinical significance. Resolution of this possibility will undoubtedly rely on molecular

biological techniques in addition to the more traditional ligand binding assays.

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