

# Epidermal Growth Factor Receptor in Human Breast Cancer: Correlation with Cytosolic and Nuclear ER Receptors and with Biological and Histological Tumor Characteristics

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**Abstract**—Epidermal growth factor receptor (EGFr) and cytosolic (cER) and nuclear (nER) estradiol receptors were quantified in 220 primary breast cancers. The EGFr was significantly more frequent ( $\chi^2 = 5.9$ ;  $P < 0.025$ ) and its concentration was significantly higher ( $P < 0.001$ ) among ER- tumors than in ER+ tumors. There was a significantly greater proportion ( $\chi^2 = 6.4$ ;  $P < 0.05$ ) of node involvement in EGFr+/ER+ tumors than in EGFr-/ER+. Increases in the proportion of EGFr+ in ER- tumors are parallel to Scarff-Bloom scores ( $\chi^2 = 6.1$ ;  $P < 0.05$ ) and there is a significant trend (Spearman  $r_s = 0.25$ ;  $P < 0.05$ ) towards increased EGFr concentrations with histologic dedifferentiation.

In ER+ tumors the median concentrations of EGFr in the different age groups show a linear correlation ( $LCC = 0.89$ ;  $P < 0.05$ ) and follow a parallel profile with the medians of nER. These findings support the hypothesis that EGFr is a bad prognosis factor and suggest that EGFr expression and concentration in ER+ tumors might be considered an estrogenic action mediated through the binding of ER to their nuclear acceptors.

## INTRODUCTION

ALTHOUGH there is a great deal of knowledge about the role of estrogen (ER) and progesterone receptors (PR) in human breast cancer, an increasing number of publications in recent years have pointed out the importance of the epidermal growth factor (EGF) or its related peptide tumoral growth factor  $\alpha$  (TGF $\alpha$ ) in tumor growth and spread [1, 2]. EGF or TGF $\alpha$ , probably secreted by the stromal or tumor cells [1, 2], acts by binding to a 170 kD membrane receptor (EGFr) and provoking mitosis. The EGFr have sequential homology with the product of c-erb B protooncogene and the HER-2/neu oncogene [3, 4] found in rat neuroblastoma cell lines.

EGFr has been found in human breast cancer and is more prevalent among ER- than ER+ tumors [5-8]. In recent publications EGFr has been related to factors indicating a poor prognosis such as

node involvement [8, 9], histologic dedifferentiation [10], lack of response to endocrine therapy [11, 12], and shorter overall survival and disease-free interval [13]. So far, few studies have been made in human breast cancer and most of them based their conclusions more on qualitative rather than on quantitative data.

In the present study we try to assess the reliability of EGFr in human breast cancer, both qualitatively and quantitatively, by comparing EGFr results obtained in 220 primary breast cancers with ER measured in cytosol (cER) and nucleus (nER), with histologic dedifferentiation TNM stage, and with other prognostic factors.

## MATERIAL AND METHODS

### Patients

Two hundred and twenty primary breast cancers (188 ductal, 18 lobular, 4 medular, 2 tubular, 8 other carcinomas) were examined. One hundred and eighteen patients had nodal involvement at the time of mastectomy and nine patients developed distal metastasis during the follow-up period. The

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patients had a median age of 55 years (27–83 years), with a median follow-up period of 11 months (1–72 months).

Tumor samples were obtained at the time of mastectomy or tumorectomy and brought to the laboratory, where, after being trimmed of fat and blood, they were stored in liquid nitrogen until analyzed.

In each sample cER and nER were measured and EGFr were quantified when enough sample was available.

### Histology

The tissue sections were stained by the hematoxylin and eosin method and the degree of dedifferentiation was assessed by the Scarff–Bloom index scored from 3 to 9, or grouped in three grades: grade I comprising scores 3–5; grade II, 6–7; and grade III, scores 8–9 [10, 14, 15].

### EGFr quantification

Due to the small sample size, EGFr were quantified following a two-point [<sup>125</sup>I]EGF radioreceptor assay using [<sup>125</sup>I]EGF (sp.act. 164–178 µCi/µg, NE) as ligand and EGF (Biomedical Technologies Ltd.) as cold competitor.

Unless otherwise stated, all the steps were carried out in an ice bath or at 4°C. 150–300 mg of breast tumor were homogenized in assay buffer (1 mM hepes, 5 mM NaCl pH = 7.4, containing 1 g BSA/l) with a 2–3 × 20-s burst from an Ultra-Turrax. An aliquot of approx. 200 µl was kept to quantify total proteins (TP) by the Bradford method [16] and the remaining homogenate was centrifuged at 1000 g × 5 min at 4°C. The supernatant was centrifuged again at 25,000 g × 30 min at 4°C. The supernatant was discarded and the pellet, containing plasma membranes, was resuspended in its original volume with assay buffer.

The assay procedure consists of incubating duplicate samples of 100 µl of plasma membranes plus 100 µl [<sup>125</sup>I]EGF (40,000 cpm ≈ 200–300 fmol/ml) and 200 µl of assay buffer, to evaluate the total binding; or with 200 µl of a solution of EGF in assay buffer containing 8333 fmol of cold EGF, to evaluate the non-specific binding (NSB). The tubes were incubated for 2 h at room temperature and then centrifuged at 20,000 g × 10 min at 4°C. The specific binding was obtained by subtracting the mean cpm of total binding from that of non-specific binding tubes. Final results were expressed in terms of fmol [<sup>125</sup>I]EGF bound to plasma membranes/g homogenate TP.

To check the performance of the method, some large samples were quantified simultaneously by the two-point method and the multipoint Scatchard method [17]. The latter was carried out by the procedure described previously for the two-point

assay, but using [<sup>125</sup>I]EGF in seven increasing concentrations ranging from  $1.5 \times 10^{-10}$  to  $1.5 \times 10^{-11}$  M.

Homogenates of placental tissue (rich in EGFr) were used in each assay as positive control.

### Criteria of positivity

We have taken as criteria of positivity (EGFr+) an EGFr concentration greater than 0.5 fmol/ml homogenate and NSB lower than 70%.

### ER quantification

Except for 18 samples in which ER were quantified following an immunochemical procedure using monoclonal antibodies with final colorimetric quantification [18], the ER of the rest of samples were assessed according to the following procedure.

Breast cancers were first screened for the presence of ER using a two-point assay [19] which consists of incubating duplicates of the cytosolic fraction with [<sup>3</sup>H]estradiol and diethylstilbestrol (DES) using the concentrations of the last point of the multipoint assay (see below). A tumor was considered ER- when the percentage of NSB was >30%; otherwise the sample was assayed following the multipoint Scatchard analysis.

### Scatchard analysis

cER and nER were quantified following Leake's method [20]. A minimum of 150 mg of tissue was homogenized in hepes–EDTA–dithiothreitol buffer (HED: 20 mM hepes, 1.5 mM EDTA, 0.125 mM dithiothreitol, pH = 7.4), 1 ml for each 50 mg tissue. After the tissue was homogenized (Ultra-Turrax followed by further homogenization in glass tissue grinder), it was centrifuged at 5000 g × 15 min at 4°C to yield a cytosolic supernatant and a nuclear pellet. 50 µl of [<sup>3</sup>H]estradiol with or without 100 times DES in seven increasing concentrations ranging from  $10^{-10}$  to  $10^{-8}$  M were added to 150 µl of cytosolic and nuclear resuspension. All the tubes were incubated at 4°C for 18 h. The [<sup>3</sup>H]estradiol–receptor complex was separated from the free [<sup>3</sup>H]estradiol using the dextran-coated charcoal method for the cytosolic fraction and by filtering for the nuclear fraction.

The results, calculated following the Scatchard plot [17], were expressed in fmol cER/mg of total protein for the cytoplasmic fraction and fmol nER/mg DNA for the nuclear pellet. The DNA was determined using a modification of Burton's method [21, 22].

According to the results obtained from the Scatchard plot, a tumor was considered ER+, either for the cytosolic or nuclear fraction, when the linear correlation coefficient obtained on the Scatchard plot was greater than 0.75,  $K_d$  between 0.05 and

$0.5 \times 10^{-8}$  M and the non-specific binding was less than 30% of the total binding.

A tumor was considered ER positive (ER+) when ER was simultaneously present in the cytosolic (cER+) and nuclear fractions (nER+), and ER negative (ER-) when cER was not found by screening, or, when after the multipoint assay, it was not present in either cellular fraction (-/-) or was present in only one of them (+/- or -/+). When the immunochemical method was used a tumor was considered ER+ when the concentration of ER was greater than 5 fmol/ml homogenate.

Following these procedures 40–45% tumors were ER+. However, the fact that in the present study we analysed EGFr in samples remaining after ER determination accounts for a greater abundance of ER- tumors, most of which were screened by the two-point assay.

#### Statistics

Since the EGFr results follow a log-normal distribution [6, 7], mostly non-parametrical statistical tests were used. To compare the results of two groups the Mann-Whitney *U* test (Mann *W U*) was

used and the Kruskal-Wallis *H* (KW *H*) test was used when more than two groups were being compared. The proportions were compared with  $\chi^2$ . The correlation studies were carried out using the Spearman rank correlation coefficient ( $r_s$ ) or the linear correlation coefficient (LCC).

## RESULTS

#### Distribution of results and comparison of methods

The results of 92 EGFr+ follow a log-normal distribution (Fig. 1A). The tumors containing large amounts of EGFr showed only one dissociation constant ( $K_d = 2.5 \times 10^{-10}$  M; Fig. 1B), whereas in those with EGFr < 4 fmol/ml two dissociation constants could be distinguished ( $K_d = 2.4 \times 10^{-11}$  M and  $4.2 \times 10^{-10}$  M; Fig. 1C). A good linear correlation between the EGFr measured by the two-point assay and the Scatchard method was found ( $r = 0.968$ ;  $N = 13$ ;  $P < 0.001$ ) and similar results (paired *t* test = 1.51; NS) were obtained for values of EGFr less than/equal 10 fmol/ml (approx. 84% of values).

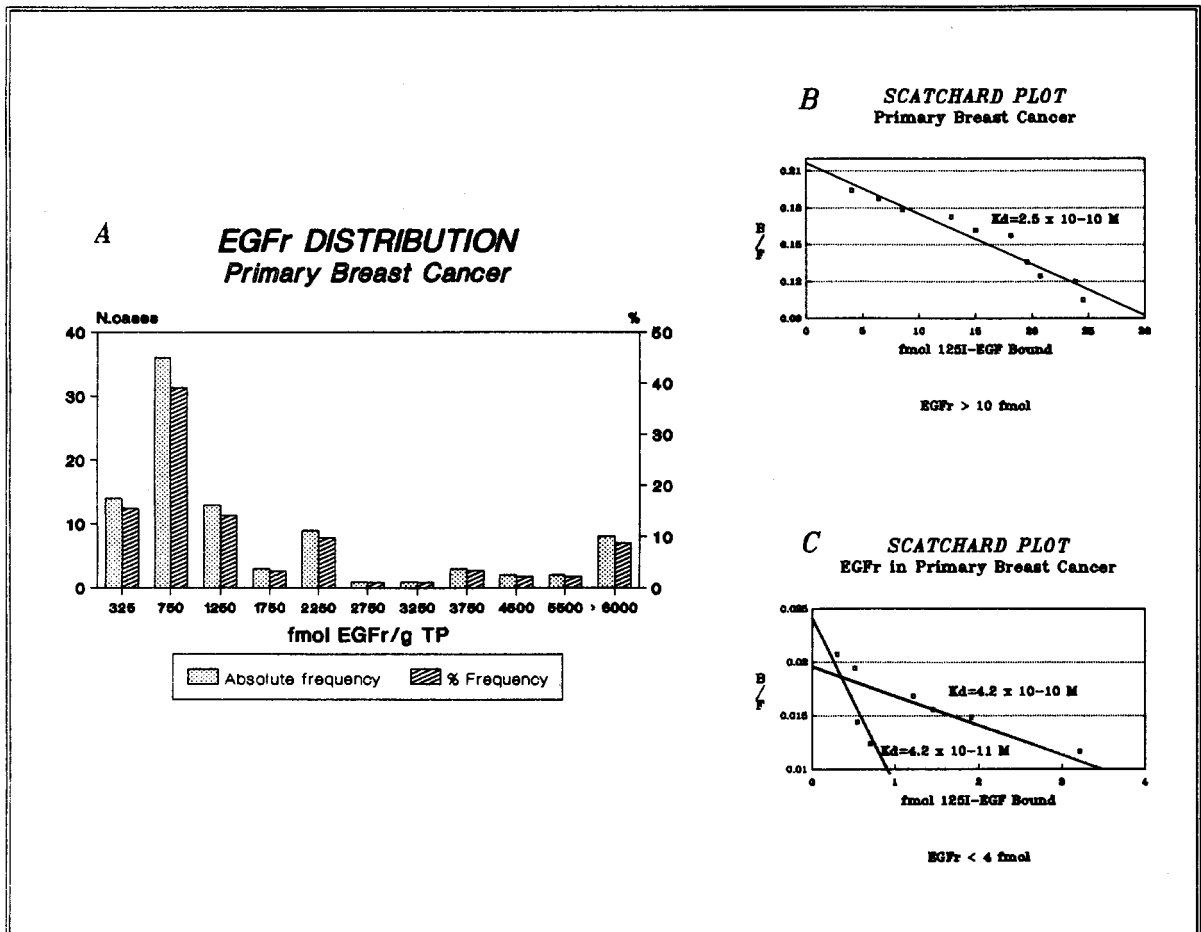


Fig. 1. EGFr distribution in primary breast cancers (A) and Scatchard plots obtained in samples with high (B) and low (C) amounts of EGFr.

Table 1. EGFr and ER in primary breast cancer

ER Status	Age years median (n) (range)	EGFr/TP fmol/g median (n) (range)	cER/TP fmol/mg median (n) (range)	nER/DNA fmol/mg median (n) (range)
-/+				
-	55 (145) (27-83)	1167 (69) (161-25078)	— —	— —
+	60 (75) (29-81)	668 (23) (260-4663)	65 (71) (5-475)	562 (53) (73-3099)
Mann W U $\chi^2$ ; P	2.3; <0.02	3.2; <0.001 5.9; <0.025	—	—

### EGFr and ER

EGFr+ tumors were significantly more frequent among ER- than among ER+ tumors ( $\chi^2 = 5.9$ ;  $P < 0.025$ ; Table 1). Thus, there were 69 EGFr+ tumors in the 145 ER- breast cancers (48%) vs. only 23 EGFr- among the 75 ER+ (31%) primary breast cancers. Likewise, ER- tumors had also significantly greater concentration of EGFr than their ER+ counterparts (Table 1).

### EGFr and tumor TNM

**EGFr and tumor size (T).** There were no statistically significant differences among the proportions of EGFr+ tumors found in the different groups of ER+ and ER- tumors grouped according to their size (Table 2). No statistical differences were found in the EGFr concentrations related to tumor size, regardless of their ER status (Table 2).

Table 2. EGFr and tumor size in primary breast cancer

T	ER-	ER+
	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)
1	2094 (9) 22 (716-13471)	867 (2) 13 (693-1041)
2	1044 (36) 78 (249-25078)	681 (12) 34 (442-2199)
3	867 (13) 25 (243-9068)	403 (5) 11 (260-4663)
4	1645 (6) 11 (162-6119)	778 (3) 10 (594-870)
KW H†; P $\chi^2$ ; P	5.4; ns 0.8; ns	2.9; ns 2.8; ns

\*T = Total number of tumors for each size and ER status.  
†Kruskal-Wallis.

### EGFr and nodal involvement

Although the EGFr status did not influence the incidence of nodal involvement in ER- tumors (Table 3), nodal involvement in the ER+ group varied significantly depending on EGFr status ( $\chi^2 = 6.4$ ;  $P < 0.01$ ; Table 3). Thus, nodal involvement was present in 17 of the 23 EGFr+/ER+ tumors (74%), vs. only 22 of the 52 (42%) EGFr-/ER+ tumors (Fig. 2C). Nevertheless, these findings were only qualitative (Table 3).

### EGFr and metastases (M)

No statistically significant variation in either the proportion of metastases or EGFr median concentrations could be related to the EGFr status of ER- or ER+ tumors (Table 4); however, due to the scarcity of metastases no definitive conclusions can be drawn.

In spite of the small number of metastases in this study, it seems that visceral metastases (lung and liver) are more frequent in EGFr+ tumors. Thus, four of the five visceral metastases came from EGFr+ tumors, while only two out of three skeletal metastases arose from EGFr- breast cancers.

Table 3. EGFr and node involvement in primary breast cancer

N +/-	ER-	ER+
	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)
-	958 (32) 66 (162-25078)	706 (6) 36 (419-2115)
+	1298 (38) 79 (123-13248)	638 (17) 39 (260-4663)
Mann W U; P $\chi^2$ ; P	1.1; ns 0.1; ns	MW = 64; ns 6.4; <0.05

\*T = Total number of tumors in each node status.

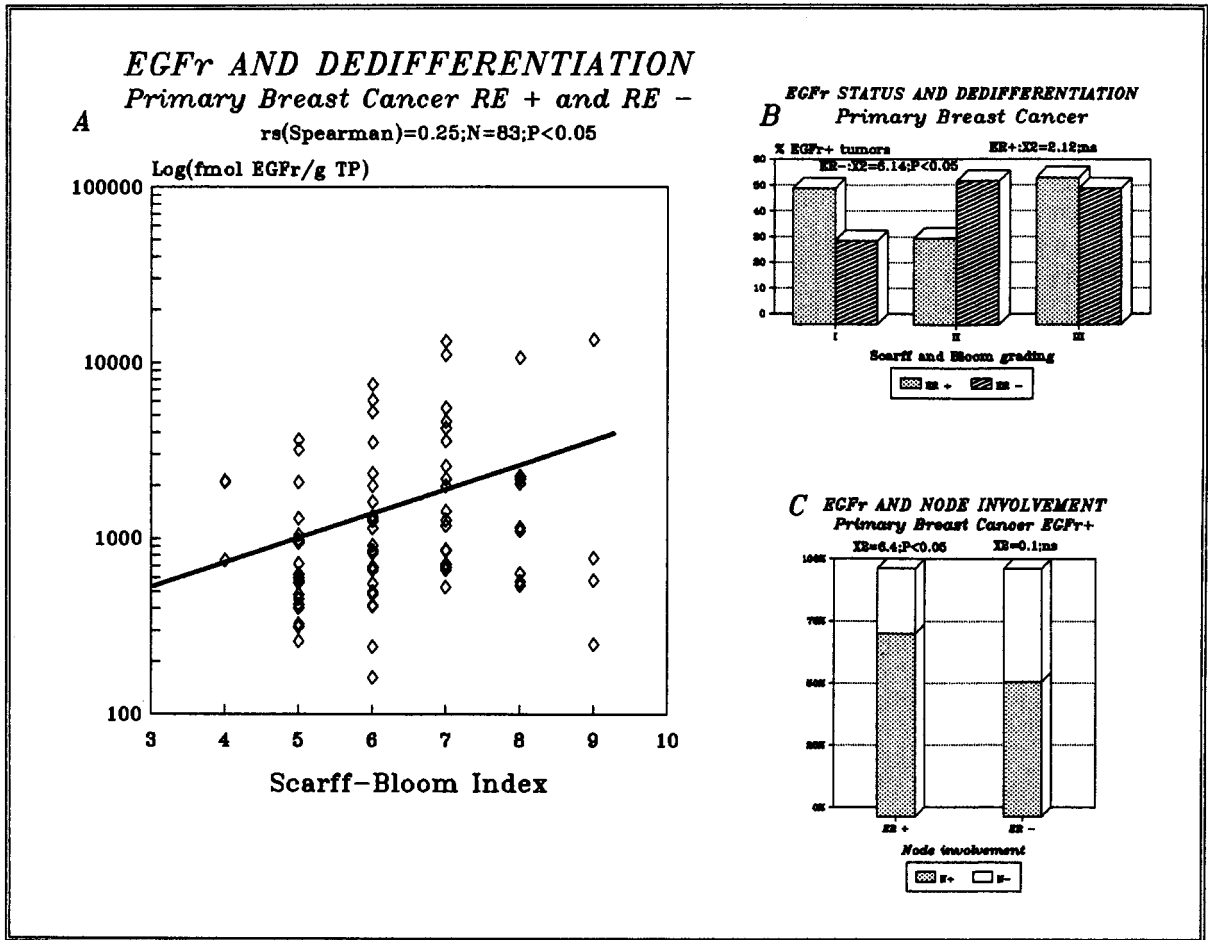


Fig. 2. Correlation between EGFr concentrations (A) or EGFr status (B) and histologic grade (Scarff-Bloom index). Nodal involvement in EGFr+ tumors with regard to ER status (C).

**EGFr and histologic dedifferentiation**

In ER- tumors there was a significant increase in the proportion of EGFr+ with histologic dedifferentiation of tumors grouped according to their Scarff-Bloom scores ( $\chi^2 = 6.14$ ;  $P < 0.05$ , Table 5 and Fig. 2B). This increase, however, could not be found in the ER+ group.

Table 4. EGFr and metastases in primary breast cancer

Status	ER-		ER+	
	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)
No metastases	1167 (67) 140 (123-13471)	668 (21) 71 (260-4663)		
Metastases	954 (3) 5 (562-25078)	678 (2) 4 (578-778)		
Mann W U; P	0.2; ns	0.1; ns		

\*T = Total number of tumors.

Although there was an increase in the median levels of EGFr for both ER- and ER+ tumors, this was not statistically significant (Table 5). However, when both ER+ and ER- tumors were considered

Table 5. EGFr and Scarff-Bloom index in primary breast cancer

Scarff-Bloom index	ER-		ER+	
	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)	EGFr/TP fmol/g median (n) T* (range)
I	945 (15) 46 (422-25078)	455 (9) 22 (260-2115)		
II	1262 (39) 70 (162-13248)	694 (8) 33 (412-4663)		
III	1166 (9) 17 (249-13472)	708 (4) 11 (582-2199)		
KW H†; P	0.2; ns	2.7; ns		
$\chi^2$ ; P	6.2; <0.05	1.8; ns		

\*T = Total number of tumors in each score.

†Kruskal-Wallis.

together, a significant positive correlation between the EGFr and the ungrouped Scarff-Bloom index was found ( $r_1 = 0.25$ ;  $P < 0.025$  and Fig. 2A).

*EGFr and the patients' age*

The proportion of EGFr+ tumors remained at about 47% for ER- tumors and 30% for ER+ irrespective of the age group (Table 6). No statistically significant variation in EGFr with age could be demonstrated (Table 6); nevertheless, two distinct median profiles were observed for ER- and ER+ tumors (Fig. 3A). The median levels of the former seem to remain almost steady in the different age groups, except among the elderly, where they showed a slight decrease. In ER+ tumors, however, the medians seemed to follow a profile parallel to the ER, especially to nER (Table 6 and Fig. 3A), with a significant linear correlation between the medians of EGFr and nER (LCC = 0.89;  $P < 0.05$ ; Fig. 3B).

*EGFr, overall survival and disease-free period*

The follow up time was too short (median 11 months) to draw any definitive conclusions. However, five out of the nine relapses in the EGFr+ group had a disease-free interval of less than 1 year,

while six patients of EGFr- group with relapses had longer disease-free intervals. With regard to overall survival, in the group of EGFr- tumors the five patients who died had an overall survival of more than 17 months, whereas only two out of five deaths produced in the EGFr+ group lived longer than 17 months.

**DISCUSSION**

As in other studies [6, 7] our results show that EGFr follows a log-normal distribution and that when the EGFr concentration is low two  $K_d$  could be distinguished in the same tumor [5]. In other studies, however, either the two kinds of  $K_d$  were found in separate tumors [7] or only a single  $K_d$  was found [6].

The 42% of EGFr+ tumors found in our study coincides with the results of some authors [6, 8] but is higher than that reported by other investigators [5, 7, 23]. This may be the result of the cut-off criteria adopted and the method; the Scatchard analysis is the one that yields the lowest percentage of positives [23].

There is also general agreement that the proportion of EGFr+ tends to be significantly higher

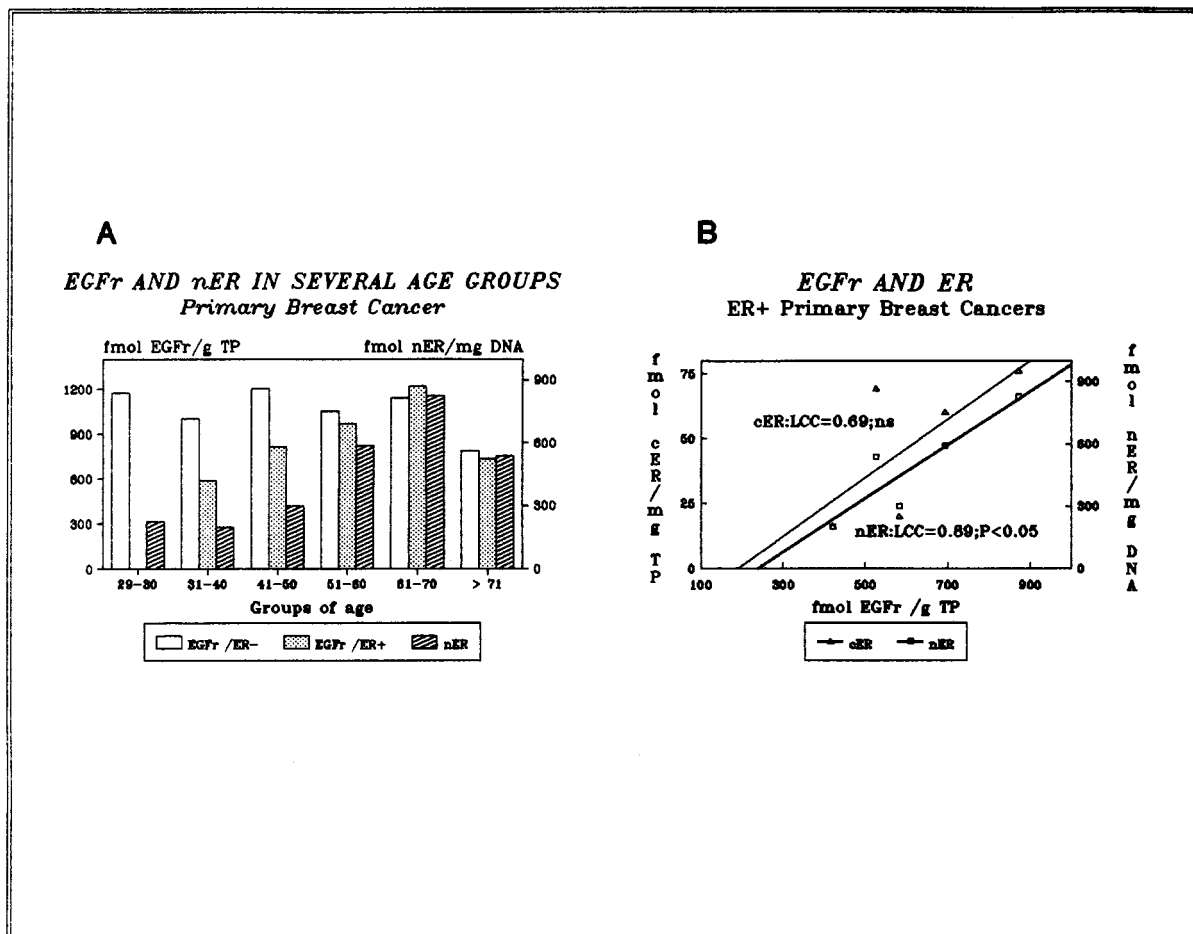


Fig. 3. Profile of median EGFr and nER concentrations in ER+ and ER- tumors in the different age groups (A) and correlation between EGFr with cER or nER in ER+ tumors (B).

Table 6. EGF<sub>r</sub> and age in primary breast cancers

Age years range	ER status		cER/TP fmol/mg median (n) (range)	nER/DNA fmol/mg median (n) (range)
	ER-	ER+		
20-30	1175 (4) 5 (123-5230)	— —	19 (2) (11-27)	224 (2) (141-308)
31-40	1004 (8) 15 (485-7483)	420 (3) 5 (315-2200)	16 (5) (5-77)	198 (4) (146-1687)
41-50	1203 (17) 32 (543-13,248)	582 (6) 15 (260-2115)	20 (15) (7-139)	298 (12) (77-2811)
51-60	1053 (14) 36 (250-11,133)	693 (5) 16 (595-725)	60 (16) (11-417)	588 (12) (73-2720)
61-70	1143 (19) 40 (243-25,078)	870 (5) 18 (455-4663)	76 (18) (18-370)	826 (12) (391-3098)
>71	786 (8) 17 (162-1996)	525 (4) 19 (327-778)	69 (19) (31-475)	536 (10) (252-1560)
KW H†; P χ <sup>2</sup> ; P	3.6; ns 3.6; ns	4.0; ns 3.5; ns	12.8; <0.025	10.0; =0.07

\*T = Total number of tumors in each age group.

†Kruskal-Wallis.

in ER- [5-7, 10] than in ER+ tumors. Moreover, similar to what other studies proved for PR [6], we found here that ER- tumors also have significantly higher levels of EGF<sub>r</sub> than ER+.

We have shown that the proportion of EGF<sub>r</sub>+ was significantly higher in ER+ tumors with node involvement, but although other studies [9, 13] have indicated that the overall proportion of EGF<sub>r</sub>+ is higher in primary tumors with node involvement, we were unable to demonstrate this nor were we able to confirm the results of other studies that show a qualitative or quantitative relationship between EGF<sub>r</sub> and tumor size [9, 13].

Although the number of metastases is limited, it seemed, in accordance with other studies [12, 13], that EGF<sub>r</sub>+ tumors are more likely to metastasize in viscera than EGF<sub>r</sub>- ones.

There is a tendency not only toward a higher proportion of positivities when the scores of histological dedifferentiation are high, as is stated in other studies [9, 10, 23], but also a positive trend toward an increase in EGF<sub>r</sub> concentration with dedifferentiation.

Although we were unable to find any significant variation in the concentration of EGF<sub>r</sub> with age, probably because of the scatter of results, it seems that EGF<sub>r</sub> concentrations in ER- and ER+ tumors follow different patterns. In ER+ the EGF<sub>r</sub> concentrations varies parallel to ER, and especially to nER, whereas the EGF<sub>r</sub> of ER- tumors remains almost steady in the different age groups, except for a fall observed in the elderly. The correlation

between nER and EGF<sub>r</sub> in ER+ tumors could be satisfactorily explained by the Jordan hypothesis [24], which postulates that EGF<sub>r</sub> is induced by the action of E<sub>2</sub> through the binding of ER in the nuclear acceptors.

With regard to overall survival and the disease-free interval, and despite the short follow-up time in our study, it seems that the presence of EGF<sub>r</sub> is bound to shorten both periods, as was indicated in previous studies [13, 23].

The findings of the present study confirm the previous reports [5, 9, 10, 13, 23] in the sense that the presence of EGF<sub>r</sub> is bound to greater tumour aggressiveness regardless of ER status. Thus, when present in ER+ tumors it changes their lesser into greater tumour aggressiveness, characteristic of EGF<sub>r</sub>+, as shown by the higher proportion of nodal involvement. The present study also shows that quantitative as well as qualitative EGF results are related to signs of bad prognosis, such as histological dedifferentiation.

The greater proportion of node involvement in EGF<sub>r</sub>+ / ER+ tumors and the parallelism between the EGF<sub>r</sub> concentrations of ER+ tumors and nER, found in the present study, would suggest that EGF<sub>r</sub> concentration and its expression in ER+ tumors were estrogenic actions, which would permit to attribute separate meanings, to EGF<sub>r</sub> depending on the ER status of primary tumor.

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