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INTERRELATIONS BETWEEN CELLULAR DNA CONTENT, S-PHASE FRACTION, HORMONE RECEPTOR STATUS AND AGE IN PRIMARY BREAST CANCER

A series of 1 342 consecutively detected tumors

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Estrogen and progesterone receptors were assessed by an immuno-biochemical method and DNA content was analysed by flow cytometry in a consecutive series of 1 342 frozen breast cancer samples. Forty-six percent of the ER-positive tumors were DNA diploid compared to 23% among ER-negative cases. The proportion of ER−/PR− cases was highest among hypertetraploid tumors (45%) and lowest among DNA diploids (13%). While receptor positivity and DNA ploidy were strongly related, no differences in mean receptor levels were detected when comparing DNA diploid and aneuploid cases of receptor positive tumors. In receptor positive tumors ER content—but not PR content—increased with age. S-phase fraction (SPF) was estimated in 1 165 cases (87%) with an overall mean of 8.6%. Tumors with high S-phase levels and DNA hypodiploid tumors were significantly more often found in younger than in older patients. The frequency of DNA hypodiploidy was less than 1% among women older than 75 years, while it was 8% among those aged 40 years or younger. S-phase fraction was inversely related to ER and PR status. However, while mean SPF gradually decreased with increasing levels of PR, no significant difference in S-phase fraction was seen for ER concentrations just above the cut-off level for receptor positivity. Tumors positive for both receptors showed the same pattern of DNA ploidy as ER+/PR− tumors while differences in S-phase fraction were observed between the groups. These results support that PR status better than ER status reflects hormone dependent growth in breast cancer.

Steroid hormone receptors and variables obtained by DNA flow cytometry are promising prognostic factors in addition to lymph node status and tumor size in human breast cancer (1–16). The associations between these different factors have been investigated in several studies. DNA ploidy, S-phase fraction (SPF) and receptor status have mostly shown to be weakly correlated to nodal status and tumor size (13, 14, 17–19). Both DNA ploidy and receptor status, however, are strongly related to the fraction of S-phase cells (5, 6, 9, 12, 17–21). The results of studies comparing receptor status and DNA ploidy have been less consistent. Some investigators have found that receptor negative tumors more frequently are DNA aneuploid than diploid (12, 17–19, 22), while statistical significance was not reached in other studies (9, 20, 23–25). In addition, receptor content is associated with age (26–29).

Large series are required if the associations between several variables should be successfully analysed simultaneously. One question is whether the concentrations of

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estrogen receptors (ER) and progesterone receptors (PR), which are significantly correlated to each other, show similar or different associations with DNA-ploidy, S-phase fraction and age. In the last years, ligand binding assays for determination of cytosolic receptor concentrations have been replaced with sensitive enzyme-immunochemical analysis (EIA). A feature of the former technique is that receptors blocked by endogenous estrogens or tamoxifens are not detected. These circumstances may motivate further investigations of the interrelations between DNA ploidy, SPF, receptor status and age. The aim of the present study was to compare flow cytometric results obtained with a well-established technique using frozen samples with hormone receptor content analysed by the immuno-biochemical method in a large series of 1 342 breast cancers detected during the last years.

Material and Methods

Patients. Frozen specimens from newly operated breast cancers detected within the South-East Sweden Health Care Region, or at a few hospitals outside this region, were delivered to the reference laboratory for steroid receptor assays in Linköping. The patients underwent radical or partial mastectomy and had not received therapy for their disease before surgery. Specimens were stored below −70°C before analysis. In 85%, adequate material was sufficient for further analysis with DNA flow cytometry. The present series comprises 1 342 such cases diagnosed from the beginning of 1988 to March 1990.

Hormone receptor analysis. Tumor tissue was homogenized at 0°C in phosphate buffer (5 mmol/l, pH 7.4) using a micro-dismembrator, M2 Braun. After centrifugation of the homogenate at 20 000 × g for 20 min, the supernatant was frozen at −70°C until analysed for receptor content. From the pellet the DNA content was measured using the Burton method. ER and PR concentrations were analysed in the same way using prepared kits for EIA. The assays were performed according to the instructions given by the manufacturer (Abbott laboratories, USA). Briefly, the primary monoclonal antibodies are bound to polysterene beads, which, in a second step, are separated from the supernatant. The intensity of colour developed after addition of a second antibody is then registered by spectrophotometry and the receptor content is obtained using a standard curve. In order to obtain values within the EIA standard curves some of the samples had to be diluted 2 or 3 times. After the assay the concentration value was corrected by the dilution factor to obtain the receptor content in the original sample. Receptor concentrations were expressed as fmol receptor per μg DNA. A cut-off value of 0.1 fmol/μg DNA was used for receptor positivity.

DNA flow cytometry. A small peice of the tumor specimen was minced in citrate buffer (0.2 ml) and afterwards chicken and trout red blood cells were added as internal marker cells. A suspension of isolated nuclei was prepared as described by Vindelöv et al. (30). This procedure was washless and included treatment with a detergent (0.1% NP40), trypsin and RNA:se followed by filtering through a 41 μm nylon mesh. The suspension was finally stained with propidium iodide and measured within 1 h. In addition, an imprint from the tissue was routinely stained and examined to ascertain the presence of tumor cells in the sample. Cell suspensions were analyzed with a Leitz MPV FLOW flow cytometer (Leitz GmbH, Wetzlar, F.R.G.) interfaced to a Monroe OC8888 personal computer system (Litton Business, U.S.A.). The software used for data acquisition and analysis was developed in our laboratory. Illumination from a high-pressure mercury lamp was used with light filtered through an AL interference filter with peak transmission at 546 nm and with a 20 nm bandwidth. Emitted fluorescence was recorded after passing a dicroic mirror TK 580 and a 590 nm long-pass filter.

Histogram evaluation. Usually, 20 000 cells were measured. DNA indices (DI) were calculated after zero point adjustment by using the chicken and trout red blood cells as internal controls. These showed 35% and 80% respectively of the fluorescence of human (female) diploid cells stained with propidium iodide. The coefficient of variation (CV) of tumor $G_{0/1}$ peaks was estimated from the width of the peak at half-maximum peak height. Median CV was 3.8%. Calculations of DNA index, peak CV and the percentages of cells in $G_{0/1}$, S and G_2/M phase were performed by the software after selection of adequate peaks and S-phase interval by the user. The S-phase interval was chosen in such a way that the influence of debris or disturbing peaks should be as small as possible. Considering a rectangular S-phase distribution, the number of cells in S-phase was estimated by the software by multiplying the number of channels between the $G_{0/1}$ and G_2/M peaks by the mean number of registrations per channel in the selected interval. Furthermore, additional peaks interfering with the population of interest could be labeled by the user and was then subtracted from the histogram before the calculation of cell cycle parameters was performed. The majority of the histograms were clean from background debris to the right of the G_2/M peak and peaks generated by cell clumps were generally small. Therefore, background correction was not performed. SPF was estimated in 1 165 cases or 87%. It was less often evaluated in hypodiploid and multiploid tumors and in aneuploid populations having a small cell number compared to the total number of cells registered. Tumors were classified into 6 categories of DNA ploidy in the following ways. A single peak in the DI range 0.90–1.10 was classified as DNA diploid. This category showed a mean DI of 1.006, a median at 1.00 and 92% had a DI between 0.95 and 1.05. If an additional peak was present, the tumor was classified

Table 1

The distribution of different receptor status and the relations to DNA ploidy and S-phase fraction

n = 1 342	Frequency (%)			DNA ploidy non-diploid (%)	SPF	
	All ages	< 50 y	⩾ 50 y		No.	mean (%)
ER+PR+	61	61	61	54	723	7.2
ER+PR−	15	5	17	53	172	9.0
ER−PR+	3	6	2	74	30	10.4
ER−PR−	22	29	21	77	240	12.4

into one of the 5 non-diploid categories depending on DI. Thus, tumors were considered DNA hypodiploid for DI < 1.00, hyperdiploid for DI in the range 1.01–1.90, tetraploid for DI ranged 1.91–2.10 and hypertetraploid for a DI greater than 2.10. If more than one non-diploid peak was observed the tumor was classified as multiploid. Small aneuploid or tetraploid populations were separated from artefacts or diploid G_2/M cells by looking for a corresponding G_2/M peak.

Statistical methods. Differences in S-phase fraction between various categories were tested using linear regression analysis. Relationships between grouped variables were tested by means of χ^2-tests for contingency tables with ordered categories (31). Multivariate analyses were performed by means of multiple linear regression analysis. All p-values cited were two-sided, and p-values less than 5% were judged as statistically significant.

Results

Hormone receptor status. Tumors positive for ER and PR were found in 75% and 63% respectively (Table 1). While the proportion of tumors positive for both receptors was the same in the group of patients younger than 50 years and in the group of older women, the occurrence of ER+/PR− tumors was highest among older women and ER−/PR+ tumors mostly observed in the younger. ER+/PR− tumors were most frequent in the ages around 60 years and formed 22% of all tumors in this age group. Except for this period, positivity for PR was equally frequent at different ages. The proportion of ER-positive tumors, however, increased with age ($p < 0.001$).

DNA ploidy and S-phase fraction. DNA diploid and hyperdiploid tumors constituted the two main groups of DNA ploidy (Table 2). The minor groups of hypodiploid, multiploid and hypertetraploid tumors represented together 15% of the cases. DNA index ranged between 0.6 and 4.5. The majority of non-diploid cases showed a DNA index in the range 1.6–1.9. The distribution of S-phase fraction is shown in Fig. 1 for DNA diploid and non-diploid tumors separately. The distributions were considerably different as were the corresponding mean values of 5.1 and 11.6% respectively ($p < 0.001$). Mean SPF for all cases

Table 2

The distribution of different DNA ploidy subgroups and corresponding S-phase levels

DNA ploidy n = 1 342	Frequency %	S-phase fraction (n = 1 165)		
		n	mean (%)	SD (%)
Diploid	40.6	529	5.1	3.3
Tetraploid	7.2	72	8.0	4.3
Hyperdiploid	37.8	435	11.8	4.3
Multiploid	6.3	41	11.0	6.7
Hypodiploid	2.5	22	9.7	5.2
Hypertetraploid	5.7	66	14.7	7.1

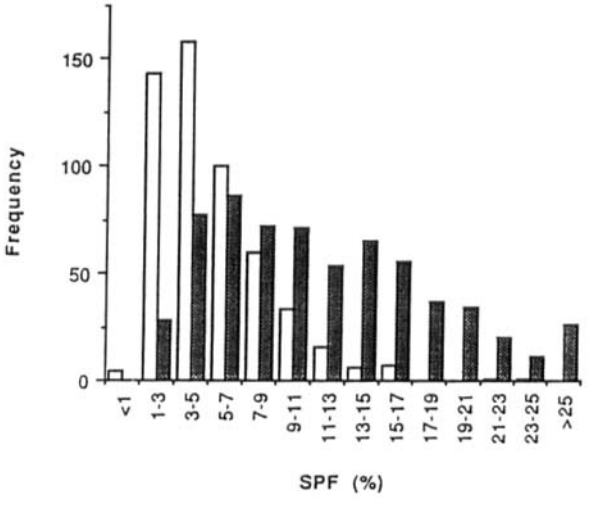

Fig. 1. The distribution of S-phase fraction in the subgroups of DNA diploid and non-diploid tumors. Corresponding median values were 4.2% and 10.3% respectively. SPF was estimated in 1 165 cases. □ diploid. ■ non-diploid.

was 8.6% and the median was 6.7%. Among the non-diploid tumors the DNA tetraploid showed a moderate mean percentage S-phase cells while the hypertetraploid exhibited the highest (Table 2).

Receptor status related to DNA ploidy and DI. The proportion of DNA diploid tumors was roughly twice as high in ER-positive compared to ER-negative cases

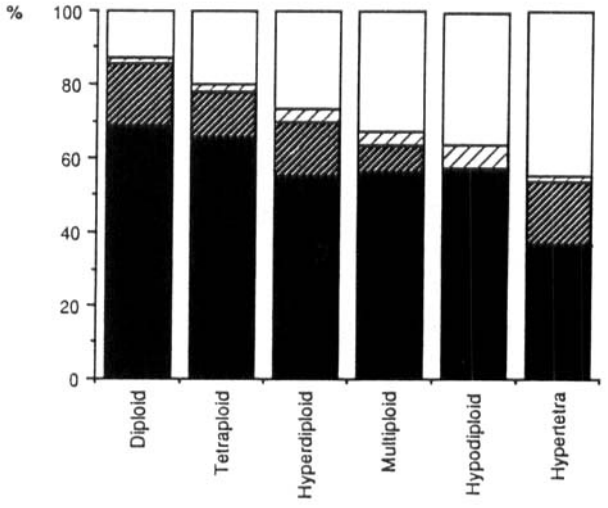

Fig. 2. The distribution of different receptor status related to DNA ploidy. The relationship was shown to be significant χ^2-test ($p < 0.001$). □ ER−PR−; ▨ ER−PR+; ▨ ER+PR−; ■ ER+PR+.

($p < 0.001$, Table 1). While the distribution of ER/PR− groups was almost the same in DNA diploid and tetraploid populations, marked differences could be seen between these categories and different aneuploid groups of tumors (Fig. 2). The greatest difference was observed between DNA diploid tumors among which 86% were ER-positive and DNA hypertetraploid showing a proportion of 45% of tumors negative for both receptors. Classified into six categories, DNA ploidy was related to ER status independent of all the other variables ($p < 0.01$). While 17% of both DNA diploid and hypertetraploid tumors were ER+/PR−, none of the 33 DNA hypodiploid cases showed this status. A high proportion receptor positive tumors was not only found for DNA diploid and tetraploid tumors but also for tumors having a DNA index between 1.1 and 1.2 and in a wide range around 2.0 (Fig. 3). In contrast, the proportion of receptor positive cases was less for tumors with a DNA index less than 0.9. For S-phase fraction versus DNA index a mirror image of those for receptor status was obtained (Fig. 3c). Thus, DNA aneuploid tumors with a DI in the range 0.91 to 1.20 showed a mean SPF close to that of DNA diploid cases.

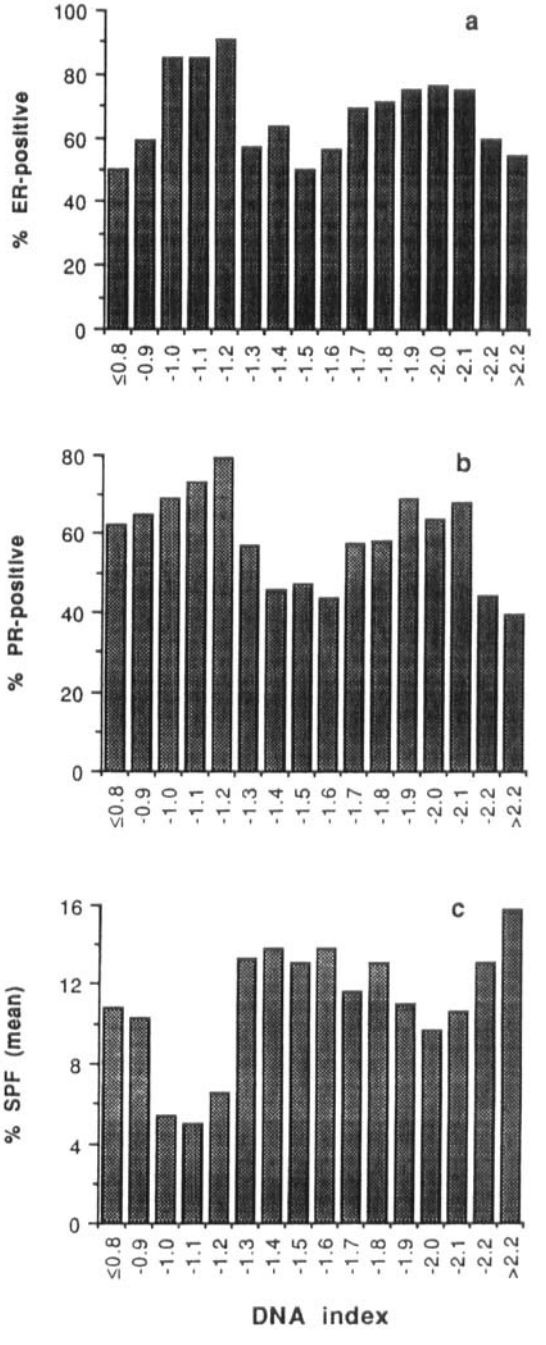

Fig. 3. The proportions of a) ER-positive, b) PR-positive and c) mean S-phase fraction among tumors with different DNA index.

Receptor status related to S-phase fraction. Both ER-positive and PR-positive tumors showed lower mean S-phase levels than receptor negative tumors (Table 1). Moreover, this was significant within the subgroups of DNA diploid and non-diploid cases (Fig. 4). However, ER and PR content exhibited dissimilar patterns when mean SPF was compared at different receptor concentrations. ER-positive tumors showed approximately the same mean S-phase fraction for different concentrations just above the cut-off level for positivity. In contrast, a gradual decrease in S-phase levels was found with increasing concentrations of progesterone receptors ($p < 0.001$). This decrease in mean values reflected more narrow frequency distributions of SPF with the accumulation of low SPF values (Fig. 5). Similarly, a difference between ER+/PR+ and ER+/PR− tumors was noted by comparing the S-phase distributions for these groups. Tumors positive for both receptors had S-phase levels more clustered to lower values compared with the ER+/PR− group, especially within the DNA aneuploid subset. The mean S-phase values

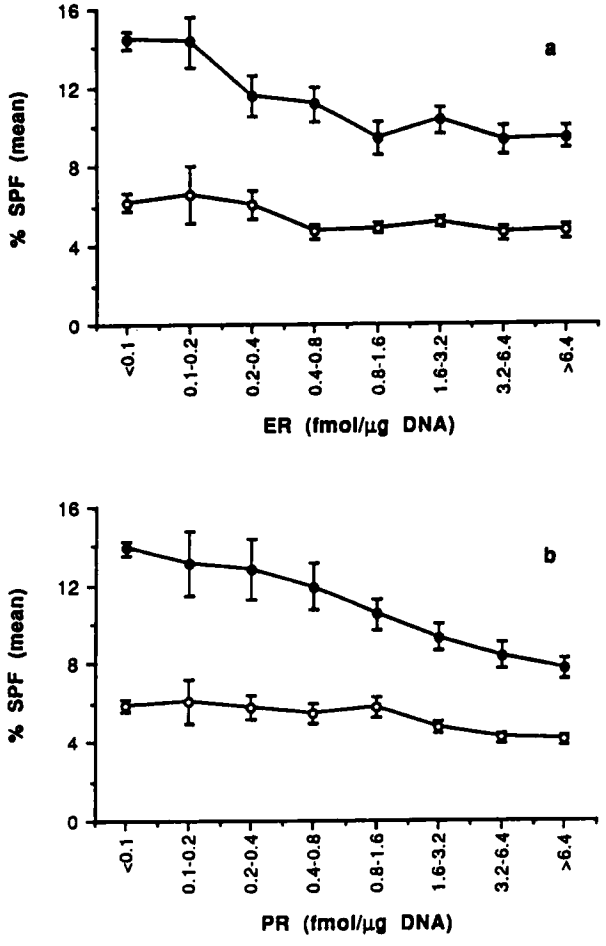

Fig. 4. Mean SPF in subgroups of tumors with different concentrations of a) ER and b) PR. The bars indicate standard errors. For both DNA diploid (open circles) and non-diploid tumors (filled circles), receptor negative cases (ER < 0.1 or PR < 0.1) showed significantly higher S-phase values than receptor positive cases ($p < 0.001$). For concentrations higher than 0.2 no significant trends were seen for ER while negative trends were observed for PR in both diploid and non-diploid cases ($p < 0.001$).

(Table 1) differed significantly ($p < 0.001$). On the other hand, the two receptor groups could not be distinguished based on differences of DNA ploidy (Table 1). Of the ER-positive tumors, approximately 80% expressed progesterone receptors in both the DNA diploid and non-diploid subgroups.

Hormone receptors, DNA ploidy and S-phase fraction related to age. The occurrence of DNA aneuploid tumors decreased in frequency with increasing age, as did tumors with high S-phase fractions (Table 3). DNA hypodiploid tumors were significantly more common among younger women, also when adjusting for receptor status and SPF ($p < 0.01$). Among women aged 40 years or less, 8% of the tumors were hypodiploid compared to 0.7% among those older than 75 years. On the other hand, the association of other DNA ploidy types with age was not independent of S-phase fraction. Independent of DNA ploidy, the proportion of ER-positive tumors increased with age (Fig. 6a). Tumors expressing progesterone receptors were less often found in the ages around 60 years while they were approximately equally common in younger and older patients (Fig. 6b). In receptor positive tumors, mean ER concentration increased strongly with age, but no such trend could be seen for the concentration of PR. Furthermore, no significant differences in receptor concentrations were found due to DNA ploidy, taking into account receptor status (Fig. 6c–d). Similarly, the relationship between ER levels and age was almost the same in tumors with low and high S-phase fractions respectively (Fig. 6e). By this separation the PR levels were still shown to be independent of age, while it could be seen that the inverse relationship between PR levels and SPF was valid for all ages (Fig. 6f).

Discussion

The proportion of ER-positive tumors of 75% in the present study was close to those obtained in two of our previous series (12, 20) and in some large ones (26, 28, 29). Around 50% of the cases have shown positivity for progesterone receptors in several studies (26, 27, 29), while proportions of 63% and 69% were found in the present, and a Danish study (28) respectively. This discrepancy may depend on differences in assay techniques between the centers and different cut-off levels for receptor positivity. While the fraction of ER-positive tumors significantly increased with age, the proportion of PR-positive cases was approximately the same for different ages except for those around 60 years, of which more tumors were negative for PR. This in line with a high frequency of ER+/PR− tumors in this age group. Other studies have found an accumulation of ER+/PR− tumors in older patients (27–29), and one may speculate whether the low concentration of estrogens during the menopause makes it less likely for tumors with hormone dependent growth compared to those with autonomous growth to present shortly after the menopause. The fact that the subgroups of ER+/PR+ and ER+/PR− showed different distributions of S-phase fraction, although the patterns of DNA ploidy were similar, is in line with this idea. It has been shown that ER+/PR− tumors can be converted into ER+/PR+ in the presence of exogenous estrogen (32).

Only 3% of the tumors were of type ER−/PR+. In an older series, using a ligand binding assay with iso-electric focusing, we classified 7% to be of this category (unpublished data). In the old series, as well as in the present, the frequency was three to four times higher in women under the age of 50 than in older women. These data support the general opinion that ER−/PR+ tumors actually may be positive for both receptors but have a high fraction of

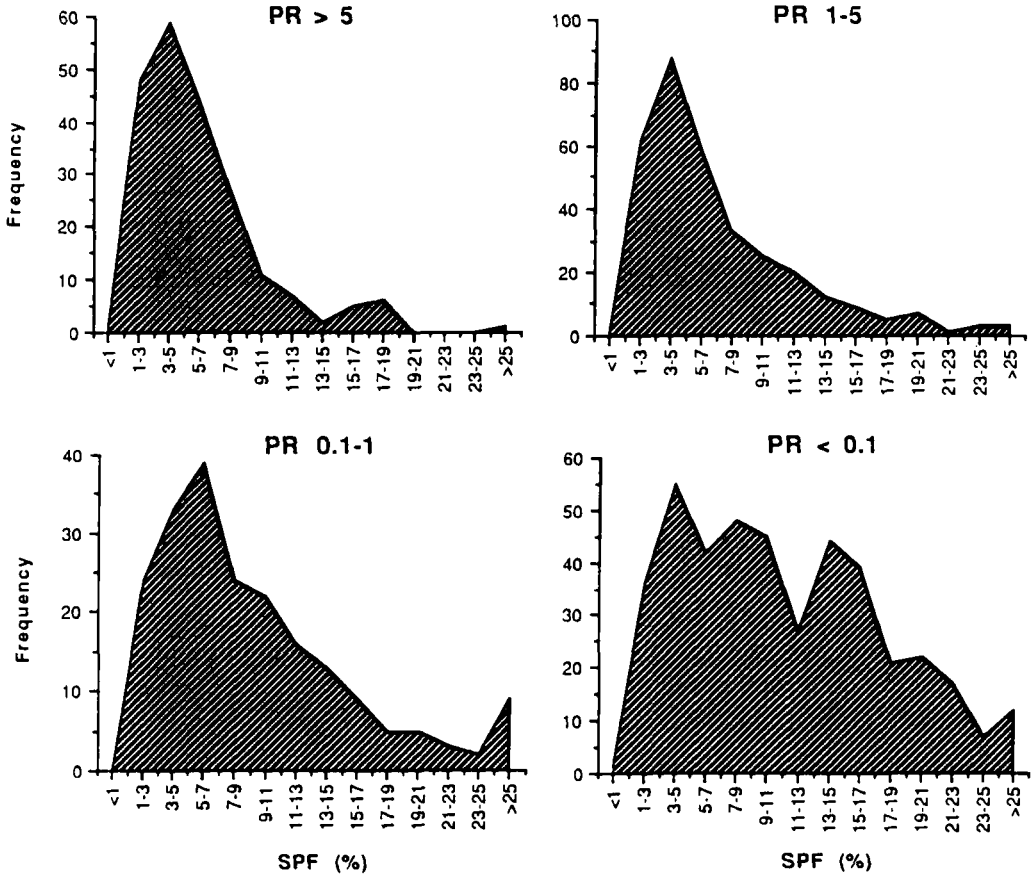

Fig. 5. The frequency distribution of S-phase fraction in subgroups of tumors with different concentrations of progesterone receptors.

Table 3

Associations of DNA ploidy and S-phase fraction with age

Age (years)	No. of patients	DNA ploidy (%)		SPF	
		Aneuploid[a]	Hypodiploid	n	Mean (%)
<50	259	56[b]	5[c]	217	9.4[b]
50–65	449	56	3	385	8.9
>65	634	47	1	568	8.1

[a] DNA tetraploids not included.
[b] $p < 0.01$.
[c] $p < 0.001$. p-values refer to tests of trend.

occupied ER (33). Estrogen receptors have been detected in high salt extracts from nuclear pellets obtained with tumors primarily classified as ER−/PR+ (33, 34).

From Table I and Fig. 2 it is evident that DNA ploidy and receptor status were strongly related. In several studies, no significant difference in ER-status has been found comparing DNA diploid with non-diploid tumors (9, 20, 23–25). However, our data indicate high discrepancies between different non-diploid subtypes. Kute et al. (19) demonstrated differences in receptor status related to DNA ploidy. In contrast to the present study, tetraploid and hypertetraploid showed a high and low frequency of ER−/PR− respectively. However, this comparison was based on 21 cases only. Whether wide or narrow DI limits are used for the definition of DNA diploidy should have little influence on the proportions of receptor positive cases (Fig. 3). However, underestimation of the number of ER-positive tumors or too small series may imply that only a weak correlation with DNA ploidy is obtained. It is noteworthy that none of the 33 DNA hypodiploid tumors

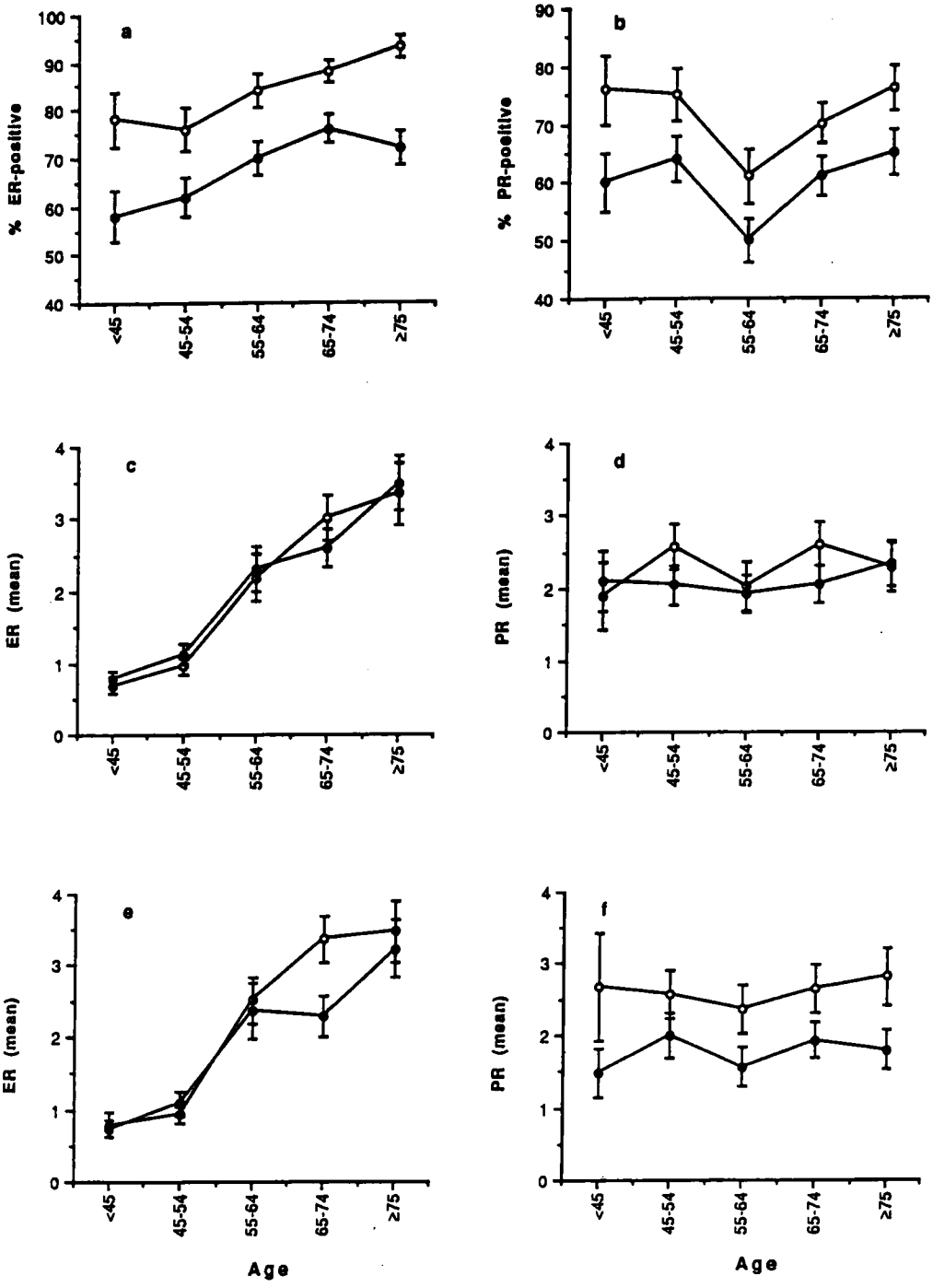

Fig. 6. The proportion of a) ER-positive and b) PR-positive cases related to age in DNA diploid (open circles) and non-diploid tumors (filled circles). In the other panels (c–f), receptor concentrations in ER+/PR+ tumors are plotted against age for various subgrups. The subgroups in c) and d) comprise DNA diploid (open circles) and non-diploid (filled circles) tumors, while subgroups of tumors with below median SPF (open circles) and above median SPF (filled circles) are presented in e) and f). The concentration values plotted are geometrical means and the bars in all panels represent standard errors.

was ER+/PR− opposed to 15% among all cases. This may in part be explained by the fact that the proportion of ER+/PR− was higher among older women and that the proportion of hypodiploid tumors decreased with age. DNA ploidy was more strongly related to ER status than to the presence of PR. This can be seen as a consequence of the similar patterns of DNA ploidy for ER+/PR+ and ER+/PR− tumors.

The importance of high resolution in flow cytometric DNA analysis for the detection of aneuploid populations

with a DNA index close to 1 has been discussed (35, 36). Our results indicate that tumors with different DNA indices in the range 0.91 to 1.20 show biological similarities with regards to receptor status and mean S-phase fraction. In this view, it is not plausible that the prognostic value of DNA ploidy in human breast cancer should increase with increasing resolution. However, to evaluate SPF accurately, high resolution is important and helps to separate a non-diploid population from a peak of diploid cells which otherwise might be mixed with the population of interest. As tumors with a DNA index less than 0.9 showed lower frequency of receptor positivity and higher mean SPF, the ability of detecting DNA hypodiploid populations seems to be important. These populations are in general misclassified when using paraffin-embedded material.

The fact that a high S-phase fraction is more often found in DNA aneuploid and receptor negative tumors is well documented both in studies using flow cytometry (9, 12, 17, 18) and thymidine labeling (21, 24). In the present material the variable most strongly linked to SPF was DNA ploidy. Multiple regression analysis showed that, in addition to DNA ploidy, ER status and PR content were independently related to S-phase fraction. A thymidine labeling study (21) has previously indicated that ER content correlates with SPF only at low concentrations as opposed to PR showing decreasing S-phase values with increasing concentrations. This is verified in the present study for both DNA diploid and non-diploid tumors in a wide range of receptor concentrations (Fig. 4). The changes seen in the frequency distribution of SPF at different PR concentrations may reflect different grades of hormone dependency. Heterogeneity of receptor expression has been demonstrated by multiple sampling (37) as well as at the microscopical level (38-40). One might expect that tumors homogeneously controlled by ER activity should more often show high levels of receptor content and low or moderate S-phase levels. In contrast, low PR concentrations and a broad SPF distribution may reflect a greater role of autonomous growth regulation. This is supported by the fact that hormone independent breast cancer cells have been shown to secrete the same collection of growth factors as estrogen dependent tumor cells, but in increased amounts (41, 42). Furthermore, it has been shown that amplification of the proto-oncogene c-erbB-2 is significantly more frequent in receptor negative and rapidly proliferating breast cancers (43) as is the expression of EGF receptors (44–47).

As in other studies, we found associations between mean receptor concentrations and age. Since these differences may be due in part to varying proportions of receptor positive tumors at different ages, we also examined the relation between concentration and age for the subgroup of receptor positive tumors. Then we still observed increasing ER levels with increasing age while no significant differences in PR means were obtained (Fig. 6). We cannot conclude, however, that no correlation exists between PR and age since in some studies a more pronounced association was found by separating patients according to the menopausal status (26, 28, 29). In these and other studies, possible explanations for the increase in ER concentrations with age have been discussed. One suggestion is that lower levels of ER are detected in younger women due to a higher amount of receptors occupied by circulating estrogens. It is not likely that this is the main reason because the association between ER and age has not only been found in studies using ligand binding techniques but also in the present series and in immunocytochemical studies using image cytometry (38). Furthermore, no difference in ER between premenopausal and postmenopausal women of the same age was observed in two large series (26, 28). Data presented by Thorpe (28) suggests that PR positivity requires higher concentrations of ER in older women compared with younger. This may reflect that decreasing levels of estrogens are compensated by increasing levels of receptors. Accordingly, the increase of ER with age has been observed as an increase per cell rather than an increase of the proportion of ER-positive cells in the tumor (38). Our results show that the relationships between receptor content and age are independent of DNA ploidy and S-phase fraction.

There is so far not much evidence for significant associations of DNA ploidy or SPF with age. Some reports have presented an inverse relationship for DNA ploidy (9, 17, 48) and others an inverse relationship for S-phase fraction (9, 17, 21). In the present series SPF was related to age, but DNA ploidy was not, if both factors were analysed simultaneously. However, the trend of decreasing proportions of DNA hypodiploid tumors with increasing age was highly significant. A few percent of DNA hypodiploid cases have been reported in some studies (24, 49) while Coulson et al. (35) found 8%, primairly among young patients. The correlation between this ploidy type and age needs further elucidation from other kinds of investigations. It has been suggested that the growth characteristics of the tumor are related to the physiology of the glandular tissue at the time of initiation (50). The higher mean SPF found in younger patients might then reflect that a tumor initiated at a younger age has a higher proliferation rate since, in the mammary gland, proliferation is highly dependent on age, being higher in younger women. Furthermore, according to the hypothesis that the genesis of breast cancer is in the premenopause (51), SPF would show a decreasing trend with increasing age at diagnosis.

In conclusion, the present large series of breast cancers showed that receptor status, DNA ploidy, S-phase fraction and age were interrelated. ER status, but neither receptor concentration nor PR status among ER-positive tumors, correlated with DNA ploidy. On the other hand, mean SPF and the frequency distribution of S-phase fraction were related to the concentration of PR. The latter relation may reflect different grades of hormone dependency for the regulation of growth in breast cancers.

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