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Original Paper

The Clinical Significance of Androgen Receptors in Breast Cancer and Their Relation to Histological and Cell Biological Parameters

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To analyse the clinical significance of the presence of androgen receptors (AR) in breast carcinomas, clinical and histological parameters of 153 primary breast carcinomas (median follow-up 46 months) were examined. Oestrogen (ER) and progesterone receptor (PR) levels were determined in cytosol preparations using enzyme immunoassay assays and in cryostat sections by immunohistochemistry. AR and Ki-67 levels were only determined immunohistochemically. Data were analysed by uni- and multivariate models. 94/153 (61%) breast carcinomas were ER+ PR+ AR+, while 14 cases were only positive for AR. All grade III tumours ($n = 17$) were steroid receptor negative and 14 (76%) of these cases demonstrated high Ki-67 values suggestive of more aggressive behaviour. Strikingly, 14 ductal carcinomas negative for ER and PR were positive for AR. In univariate analysis, AR as well as ER, tumour size, lymph node status, grade and Ki-67 proved to be significant prognostic factors for disease-free survival (DFS). Multivariate analysis, however, showed lymph node status, tumour size and ER status to be the only independent prognostic factors for DFS within this model. We conclude that simple histological and cell biological parameters, including AR, can be used to select high- and low-risk patients at the time of primary surgery and can provide valuable information on treatment options.

Key words: breast cancer, androgen receptor, immunohistochemistry, prognosis, multivariate analysis
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INTRODUCTION

THE IMPORTANCE of the histological subclassification of breast cancer as one of the indicators for tumour aggressiveness is well recognised. Histological subtypes, such as tubular, cribriform and colloid cancers, are known to have a good prognosis, while not otherwise specified (NOS) ductal carcinomas have a relatively poor prognosis; the lobular, medullary and mixed carcinomas are considered to represent an intermediate prognostic group [1–5]. Other tumour characteristics, such as lymph node status and histological grading, are considered to be of major importance [1, 2, 5–9]. The prognostic impact of additional parameters, such as proliferative activity [10–12] and the expression of oestrogen (ER) and progesterone receptors (PR), is also well established [13, 14]. A relationship between subtypes of breast cancer and proliferative activity has been demonstrated in both histological [12, 15] and cytological material [15].

The clinical significance and functional role of androgen receptor (AR) expression in breast cancer is less well defined. In a previous study [16] on human breast cancer, we found a close relationship between AR, ER and PR expression. AR was detectable in 76% of the 100 cases of breast cancer investigated. Nine per cent of the tumours expressed AR as the only sex steroid receptor. As various studies [17, 18] have reported that the combined use of androgen and anti-oestrogen therapy has therapeutic advantages over anti-oestrogen treatment alone, AR determination may give additional information regarding the response to different endocrine treatment modalities.

In this study, we report on the prognostic impact of the aforementioned tumour characteristics, with special emphasis on the relationship between AR and these factors.

Using these parameters, we performed uni- and multivariate

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analyses. In addition, we correlated ER and PR detected immunohistochemically with ER and PR levels measured by a biochemical method based on the enzyme immunoassay (EIA).

PATIENTS AND METHODS

Patients

This study was performed on a group of 153 patients (mean age 55 years, range 29–88 years) with primary breast cancer who underwent either breast conserving surgery ($n = 71$) or modified mastectomy ($n = 82$) with axillary lymph node dissection, from 1988 to 1991 in the Dr Daniel den Hoed Cancer Centre (median follow-up 46 months, range 12–73 months). Patients' age and menopausal status were recorded at the time of primary surgery. The tumours were graded according to Bloom and Richardson, with minor modifications as described by Elston in Page and Anderson [19]. Histological typing was performed following the WHO classification [20], modified according to Page [19]. Only patients without signs of distant metastasis at the time of surgery, with known immunohistochemically determined ER, PR, and AR status and known follow-up were included in this study. All patients were routinely examined every 3–6 months during the first 5 years. In the follow-up period, 46 patients (30%) showed evidence of recurrent disease, and 25 of these women died. 3 patients died without recurrent disease.

Methods

Biochemistry. In 133/153 cases, material was also available for cytosolic ER and PR assays. As described previously [14], tumour tissue (0.4–0.8 g) was pulverised and homogenised as recommended by the EORTC for processing breast tumour tissue for cytosolic ER and PR determinations. The homogenate was centrifuged for 30 min at 100000 ***g*** at 4°C, and the supernatant fraction (cytosolic extract) was used for ER and PR determination by enzyme immunoassays (ER-EIA and PGR-EIA kits, Abbott, Chicago, Illinois) (cut-off values 10 fmol/mg protein).

Immunohistochemistry. The immunohistochemical methods used have been previously described [15, 16]. In short, representative tissue samples were snap-frozen in liquid nitrogen and stored at −70°C until use. Proliferative activity was assessed with MAb Ki-67 (DAKO, Glostrup, Denmark). Cryostat sections, 4 μm thick, were air-dried and fixed in acetone for 10 min, after which the indirect immunoperoxidase technique was used for visualising Ki-67. For immunostaining of ER, PR and AR, the cryostat sections were fixed with formalin (4%) diluted in phosphate-buffered saline (PBS). Incubation was performed with the ERICA or PRICA kits for ER and PR, respectively (Abbott). For immunostaining of AR, the MAb F39.3 was used, specific to a unique epitope in the *N*-terminal domain of the human AR molecule [21]. Specific binding of ER and PR was visualised using the peroxidase–antiperoxidase (PAP) technique; for AR the s-ABC (streptavidin–biotin–enzyme complex) technique was applied. All immunostained sections were counterstained with Mayer's haematoxylin for 1 min. Control sections consisted of known positive and negative specimens identified by ligand-binding assay. In addition, for negative control sections, the primary antibody was replaced with PBS or non-immune ascites fluid.

Quantification. The percentage of ER-, PR- and AR-positive tumour cells was calculated by counting the number of positive cells in a total of 300 cells in three different areas of the tumour [16]. A staining percentage of less than 10 was regarded as negative.

The Ki-67 score was assessed by counting 300 cells in the areas with the highest proliferative activity, as described previously [15]. Arbitrarily, tumours with a Ki-67 score equal to or over 20% were defined as tumours of high proliferative activity. In 5 cases, no Ki-67 could be assessed, due to inadequate staining results ($n = 4$) and loss of material ($n = 1$).

Statistics. Spearman rank correlations (r_s) were used to study associations between continuous variables. The associations between continuous and grouped variables were tested using the Wilcoxon rank sum or Kruskal–Wallis tests and the associated trend test, when appropriate. When patients died of unknown causes, they counted as failures for disease-free survival (DFS) at time of death ($n = 3$). The Cox proportional hazards model was applied for both univariate and multivariate analyses, using the associated likelihood ratio test to test for differences. Cox regression analyses are summarised in Table 4 by the relative relapse rates.

Relapse-free survival probabilities were calculated by the actuarial method of Kaplan and Meier. The log rank test for trend was used for curves with three ordered groups. For all tests, we considered a two-sided *P*-value of less than 5% as significant. Because of the relatively short follow-up period of the patients and, as a result the low number of events in overall survival, we chose to focus only on DFS.

RESULTS

Patient and tumour characteristics

Patient and tumour characteristics are summarised in Table 1. The histological subtypes included 114 ductal, 14 lobular, five colloid, two tubular, one cribriform, 14 mixed, two medullary and one metaplastic carcinomas. Of the premenopausal women with positive lymph nodes ($n = 36$), 27 received adjuvant chemotherapy, 1 hormonal therapy and 8 received no adjuvant therapy. Postmenopausal women with positive lymph nodes ($n = 38$) received adjuvant hormonal therapy in 10 cases, adjuvant chemotherapy in 13 cases, while 15 received neither chemo- nor hormonal therapy.

Correlation between ER and PR data assessed immunohistochemically and cytosolic ER and PR levels measured with EIA

Immunohistochemically detectable nuclear ER and PR were present in variable percentages of the tumour cells, but not in stromal cells.

The Spearman rank correlation between data obtained immunohistochemically and data obtained by EIA was $r_s = 0.66$ ($P < 0.0001$) for ER, and $r_s = 0.74$ ($P < 0.0001$) for PR. When dichotomised, a discordance was observed in 11 cases for ER and in 20 cases for PR.

Association between expression of three steroid receptors and Ki-67 score

The relationship between the immunohistochemical expression of the three examined steroid receptors is shown in Table 2. In 94 cases (61%), expression of all three receptors was observed. Interestingly, in 14 cases (9%) only AR expression was found.

An inverse relationship between AR, ER and PR expression

Table 1. Patient and tumour characteristics

		n	%
Patients		153	
Premenopausal		70	46
Postmenopausal		83	54
Tumour			
Histology			
T	T1	77	50
	T2	55	36
	T3	12	8
	T4	4	3
	Tx	5	3
N	N0	78	51
	N1–3	49	32
	N > 3	25	16
	Unknown	1	1
Type	Ductal	114	75
	Lobular	14	9
	Others	25	16
Grade	I	20	13
	II	79	52
	III	54	35
Biochemistry			
ER	<10	31	20
	⩾10	102	67
	Unknown	20	13
PR	<10	45	29
	⩾10	88	58
	Unknown	20	13
Immunohistochemistry			
ER	<10	32	21
	⩾10	121	79
PR	<10	54	35
	⩾10	99	65
AR	<10	25	16
	⩾10	128	84
Ki-67	<20	97	63
	⩾20	51	33
	Unknown	5	3

AR, androgen receptors; ER, oestrogen receptors; PR, progesterone receptors.

Table 2. Relationship between the immunohistochemical expression of the three steroid receptors

	Positivity for AR	
	<10(%)	⩾10 (%)
ER– PR– (*n* = 31)	17 (11)	14 (9)
ER+ PR– (*n* = 23)	4 (3)	19 (12)
ER– PR+ (*n* = 1)	0 (0)	1 (0.7)
ER+ PR+ (*n* = 98)	4 (3)	94 (61)
Total (*n* = 153)	25 (16)	128 (84)

AR, androgen receptors; ER, oestrogen receptors; PR, progesterone receptors.

and the Ki-67 score was demonstrated; a high Ki-67 score was significantly ($P < 0.01$) associated with low receptor values (data not shown).

Correlation between steroid receptor expression, Ki-67 score and histological typing and grading

A variable steroid receptor expression and Ki-67 score were found in the group of NOS ductal carcinomas (Table 3).

Table 3. Correlation between steroid receptor expression, Ki-67 and histological typing and grading

Histology	*n*	ER (%)	PR (%)	AR (%)	Ki-67 ⩾ 20 (%)
Ductal NOS	114	75	62	84	38
Lobular	14	100	71	92	14
Mixed	14	85	64	78	29
Special types					
Colloid	5	100	100	100	0
Tubular	2	100	100	100	0
Cribriform	1	100	100	100	0
Others					
Medullar	2	0	0	0	100
Metaplastic	1	0	0	0	0
Grade					
I	20	100	75	95	0
II	79	75	63	75	15
III	54	79	39	63	66

AR, androgen receptors; ER, oestrogen receptors; NOS, not otherwise specified; PR, progesterone receptors.

Of the 17 receptor-negative tumours, 14 were NOS ductal carcinomas. Strikingly, 13 of these 14 cases expressed high (⩾20) Ki-67 values. In addition, the 14 cases negative for ER and PR and positive for AR were all NOS ductal carcinomas.

The colloid, cribriform and tubular carcinomas combined ER, PR and AR expression with low (< 20) Ki-67 values (Table 3). Figure 1 shows this variation in staining for a colloid carcinoma.

The mixed ($n = 14$) and lobular ($n = 14$) type carcinomas formed an intermediate group (Table 3); only one mixed type carcinoma lacked all three receptors and 2/14 lobular (14%) and 4/14 mixed (29%) carcinomas expressed ⩾20% Ki-67 positive cells. In contrast, the medullary ($n = 2$) carcinomas combined high Ki-67 scores with the absence of steroid receptor expression, and the metaplastic carcinoma combined receptor negativity with a low Ki-67 score (Table 3).

The correlation between immunohistochemically detected steroid receptor expression and grade is presented in Table 3. Grade I and II carcinomas showed a higher percentage of receptor-positive cases in comparison to grade III carcinomas. Moreover, all receptor-negative cases ($n = 17$) were grade III cancers, in 14 cases characterised by a high percentage of Ki-67 positive cells.

The 54 grade III tumours consisted predominantly of NOS ductal carcinomas ($n = 49$).

Correlations between steroid receptors, Ki-67 and clinical parameters

No significant association between immunohistochemically detected steroid receptor expression and lymph node status was observed. A significant correlation was seen for ER and age ($r_s = 0.28$, $P < 0.01$) and an inverse relationship for tumour size and PR (Kruskal–Wallis associated trend test, $P = 0.03$) was seen. A borderline significant inverse relationship was found for Ki-67 score and age $r_s = -0.16$, $P = 0.05$) and a significant rank correlation ($r_s = 0.21$, $P = 0.01$) for Ki-67 score and lymph node status.

DFS according to various parameters

At 60 months, the DFS of the 153 patients was 64.7% with 16 patients at risk. The results of univariate analysis (Table 4)

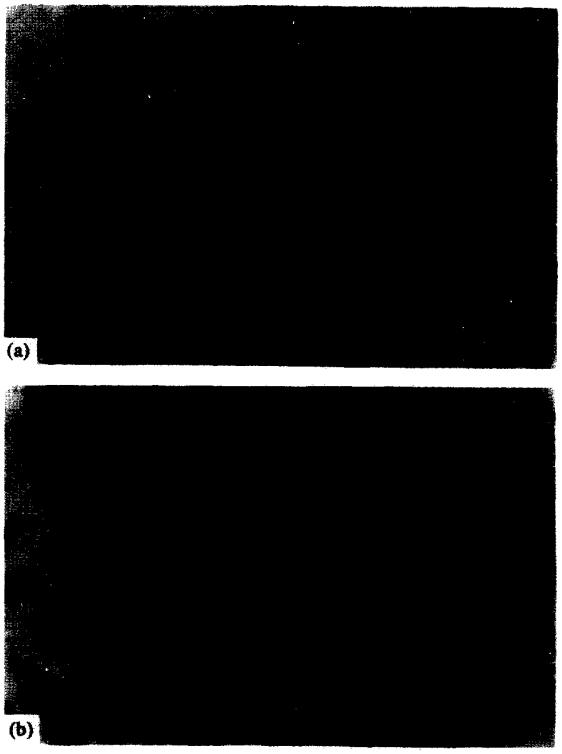

Figure 1. Comparison of Ki-67 and androgen receptor (AR) immunostaining of frozen sections of the same colloid breast carcinoma (magnification ×40). (a) Only few tumour cells (darker nuclei) show positive staining of Ki-67. (b) Positive nuclear staining (darker nuclei) for AR is present in the majority of tumour cells.

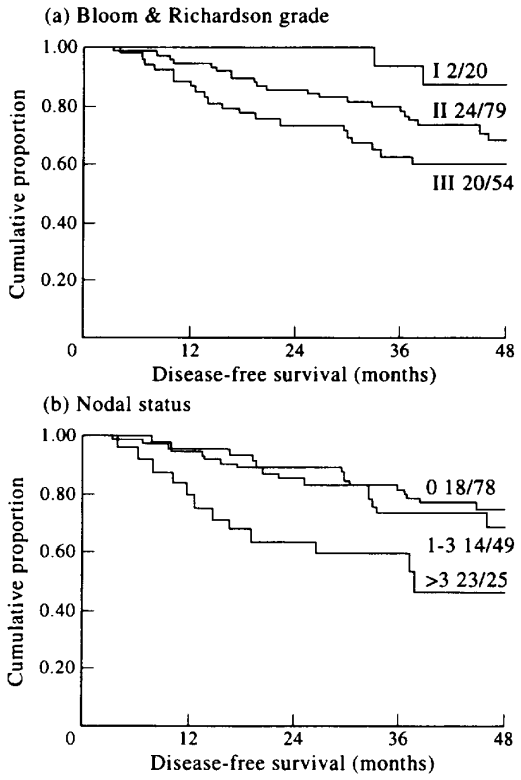

Figure 2. (a) Disease-free survival curve stratified according to grade ($P = 0.01$). (b) Disease-free survival curve stratified according to lymph node status ($P = 0.02$).

Table 4. Cox univariate and multivariate analysis in 147 patients with primary breast cancer*

Variable	Univariate HR†	Univariate 95% CI‡	Univariate P	Multivariate (initial model) HR	Multivariate (initial model) 95% CI	Multivariate (initial model) P	Multivariate (final model) HR	Multivariate (final model) 95% CI	Multivariate (final model) P
pT§	2.08	1.44–2.99	0.000	1.79	1.19–2.69	0.005	1.69	1.14–2.51	0.009
Nodal status									
1–3‖	1.37	0.68–2.76		1.46	0.69–3.11		1.53	0.75–3.09	
> 3	3.36	1.63–6.91	0.004	2.64	1.16–5.98	0.067	2.62	1.20–5.71	0.052
IH-ER¶	0.43	0.23–0.81	0.008	0.40	0.14–1.14	0.087	0.39	0.21–0.74	0.004
IH-PR¶	0.61	0.34–1.08	0.091	1.85	0.71–4.80	0.205	–	–	–
IH-AR¶	0.50	0.25–0.98	0.043	0.65	0.27–1.57	0.337	–	–	–
BR§	1.79	1.12–2.86	0.014	1.20	0.66–2.19	0.544	–	–	–
Ki-67¶	1.79	1.00–3.22	0.052	1.05	0.51–2.17	0.902	–	–	–

*5 patients tumour size unknown and 1 patient nodal status unknown; †relative hazard rate; ‡confidence interval; §test for trend; ‖tested versus node-negative; ¶dichotomised.
IH, immunohistochemically detected; BR, Bloom & Richardson grade; AR, androgen receptors; ER, oestrogen receptors; PR, progesterone receptors.

indicated an increased risk of relapse for patients with larger tumours, with high grade tumours (Figure 2a) and with more than three lymph nodes affected (Figure 2b). Similar findings were observed when patients were stratified according to ER positivity ($P = 0.008$), AR expression ($P = 0.043$) and Ki-67 score ($P = 0.052$). Not surprisingly, EIA-ER showed a similar correlation with DFS as did immunohistochemically detected ER (Figure 3).

Figure 4 gives an indication of the DFS according to the various immunohistochemically determined receptor combinations. For DFS, the patients with ER+, PR+, AR+ tumours had the best prognosis, and the group with a combination of negative AR, ER and PR had the worst prognosis ($P = 0.026$), but the number of patients was low ($n = 17$).

The results of the Cox regression analysis are shown in Table 4. Relative hazard rates (HR), the 95% confidence

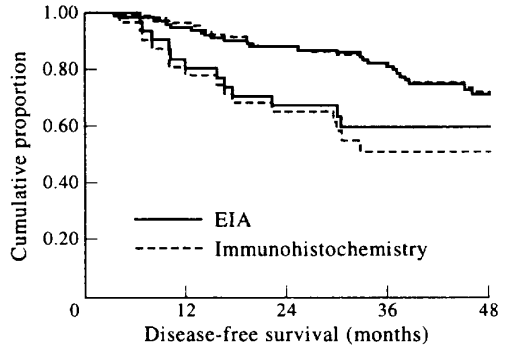

Figure 3. Disease-free survival curve for oestrogen receptors (ER) according to the method of detection.

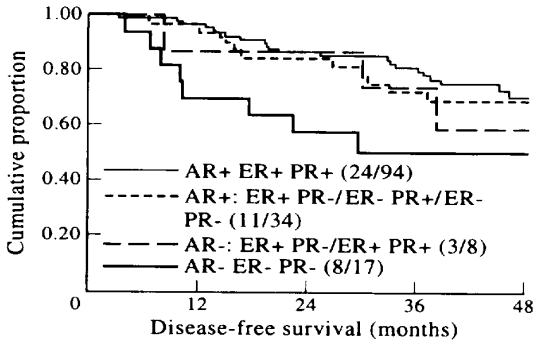

Figure 4. Disease-free survival curves for patients with tumours of different oestrogen/progesterone/androgen receptor (ER/PR/AR) phenotypes ($P = 0.026$). Numbers in parentheses show failures/total amount of patients in each group.

intervals (95% CI) and P values are given for both the univariate and multivariate analyses. The results of both the initial and final multivariate models are shown. The addition of adjuvant therapy, which was not statistically significant in the univariate analysis, had no influence on the estimations of the initial model. It appeared that tumour size and nodal status were the major prognostic factors, with ER status showing an additional prognostic value. Given these factors, the others showed no statistically significant associations in the final model. Estimations of the relative HR of tumour size (HR 1.70, 95% CI 1.15–2.51) and immunohistochemically detected ER (HR 0.36, 95% CI 0.19–0.69) were not influenced by adding adjuvant therapy as an indicator variable to the final model as compared to Table 4.

DISCUSSION

In recent years, the EIA has become an alternative method to the conventional dextran-coated charcoal (DCC) assay. Like others [22, 23] we found good correlation ($P < 0.0001$) between values obtained by EIA and those using immunohistochemistry. In addition, the prognostic impact of ER/PR detected by either method was similar (Figure 3). The observed discrepancies between EIA and immunohistochemical determination may be partly related to variations in the proportion of tumour tissue in the specimens examined, and partly to the presence of receptor-positive benign epithelium in EIA samples, resulting in false positive ER and PR values. Loss of antigenicity due to material processing, especially in the case of the known labile PR, may give an additional explanation for the discordances. No biochemical data for AR were available for this material, but Ruizeveld and associates [24] observed that immunoreactivity with this antibody was generally consistent with earlier biochemical and autoradiographical data. As primary tumours are of increasingly smaller size at primary surgery, due to breast cancer screening programmes, immunohistochemical measurements of steroid expression may, in the future, become an acceptable alternative to the binding assay, particularly as monoclonal steroid receptor antibodies for use on paraffin sections have become available.

The inverse relationship of Ki-67 positivity with ER, PR and AR expression was similar to the results obtained in other studies for ER and PR [10, 25, 26]. However, Wintzer and associates [27] found no correlation between Ki-67 and ER status, and an inverse relationship between Ki-67 and PR; Gasparini and associates [28] made the opposite observation. These controversies may partly be explained by differences in the assays used and partly by differences in cut-off levels. Isola [29] correlated AR with proliferation as determined by the S-phase fraction, but found only a weak inverse association. The relationship between pure histological parameters, such as typing, grading and the biological behaviour of breast carcinomas, has already been emphasised [1–5]. As the aggressiveness of a tumour may also be reflected by the presence or absence of a number of other tumour characteristics, it seemed logical to relate the histological parameters to these markers. In agreement with an earlier study [15], we found that the Ki-67 defined proliferation index was related to particular types of breast cancer (see Table 3), and that most cases with high Ki-67 positivity were found in the group of NOS ductal carcinomas. No significant correlation was demonstrated between either Ki-67 expression or steroid receptor expression and grade. Yet all steroid receptor negative tumours ($n = 17$), that is AR–, ER–, PR–, appeared to be grade III tumours. Moreover, 14 cases of this group appeared to be NOS ductal carcinomas and 13 demonstrated high (≥ 20) Ki-67 activity. These findings emphasise the view that lack of all three steroid receptors, combined with high proliferative activity and grading, are associated with a more aggressive tumour behaviour. This is substantiated by the DFS data (Figure 4). In contrast, and in line with published results [1–5, 25], we found that the colloid, tubular and cribriform tumours combined steroid receptor positivity, including AR, with low Ki-67 values and low grading (Table 3). In addition, consistent with the view that the group of mixed and lobular carcinomas represent a group of tumours with intermediate prognosis, we observed that only one of 28 mixed and lobular type carcinomas lacked all three steroid receptors (Table 3) and only six of 28 contained $\geq 20\%$ Ki-67 positive tumour cells. The same relationship was found with respect to grading: only two of 28 mixed and lobular type carcinomas were grade III tumours.

Teulings and associates [30] stated that the positive response to treatment with high-dose progestin (megestrol acetate) in a group of patients with metastatic breast cancers was determined by the AR level. Moreover, Hackenberg and colleagues [31] demonstrated *in vitro* that the progestin medroxyprogesterone acetate inhibits proliferation of a ER- and PR-negative cancer cell line via AR. Therefore, AR positivity may have additional therapeutic consequences, particularly in

the group of carcinomas with high proliferative activity, and with the presence of only the AR sex steroid receptor, as was observed for 14 NOS ductal carcinomas. Unfortunately, in our series, the number of patients receiving hormonal treatment for advanced disease was too small to study this relationship.

The prognostic impact of tumour size, lymph node status, ER, AR, Ki-67 and grade in univariate analysis emphasises the importance of these parameters. In the multivariate analysis, we confirmed [6, 26] that lymph node status, tumour size and ER expression were the only independent prognostic factors and that neither AR nor Ki-67 offered additional discriminating ability.

In contrast to the large studies of Dixon and associates [2] and Pereira and associates [5], our study included a relatively small group of 34 patients with non-ductal carcinomas. Consequently, a reliable statistical analysis of these subsets of breast cancers was not possible. Nevertheless, we feel that our results emphasise the importance of histological typing and grading of individual tumours. Moreover, they indicate that these histological parameters in combination with a few common cell biological parameters, including AR, may help in the initial selection of high- and low-risk patients. Further refinement in choice of treatment for each individual patient may then be realised by using these parameters.

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