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Apoptosis in Breast Cancer as Related to Histopathological Characteristics and Prognosis

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Apoptotic cells were quantitated by light microscopy in a series of 288 breast carcinomas, and their number (cells/mm² of neoplastic epithelium, i.e., the apoptotic index, AI) was related to various histopathological features and disease outcome. High AI was associated with tumour necrosis ($P = 0.003$), lack of tubule formation ($P = 0.03$), dense stromal lymphocyte infiltration ($P = 0.0009$), high grade of the tumour ($P < 0.0001$), DNA aneuploidy ($P = 0.049$), high S-phase fraction ($P = 0.010$), high mitotic rate ($P < 0.0001$), lack of sex steroid receptors ($P = 0.004$), expression of p53 tumour suppressor gene ($P = 0.004$), and high values of morphometrically measured nuclear factors ($P < 0.05$). In survival analysis, an AI greater than 3/mm² was related to short recurrence-free survival in the entire cohort ($P = 0.0079$) as well as in the axillary lymph node-negative tumours ($P = 0.0253$). Survival of the patients with node-negative tumours ($P = 0.0356$), node-positive tumours ($P = 0.0085$) and in the entire cohort ($P = 0.004$) was related to AI. Recurrence-free survival was related to the mitotic index ($P = 0.0012$), ductal type ($P = 0.011$), S.D. of the nuclear area ($P = 0.075$), and axillary lymph node status ($P = 0.096$). Cox's analysis showed that only the tumour diameter ($P < 0.001$), axillary lymph node status ($P = 0.001$), progesterone receptor content ($P = 0.004$) and ductal type ($P = 0.041$) had independent prognostic value, whereas AI did not.

Key words: apoptosis, breast cancer, prognosis
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INTRODUCTION

APOPTOSIS AND NECROSIS are the two basic types of cell death [1]. Cell death occurring during the embryonic development—during organogenesis, in several physiological processes during adult life and in pathophysiological conditions—is known as apoptosis [1–6]. The pathogenesis of apoptosis involves the cleavage of nuclear chromatin between the nucleosomes by specific endonucleases into chromatin fragments composed of approximately 200 base pairs [6–8]. The apoptotic process is controlled by inducers and repressors, the balance between these stimuli determining whether the cell cycle enters mitosis or apoptosis [3, 5–7, 9–14].

The role of apoptosis in oncogenesis is currently being studied intensely [1, 6, 13, 15–17]. According to a recent hypothesis, clonal expansion might occur as a premalignant event resulting from suppressed apoptosis, secondary to mutations in the regulatory oncogenes [2, 3–6, 10, 15–17]. Of such oncogenes, at least *bcl*-2 [5–7, 10, 15], c-*myc* [5–7, 15, 18] and *p*53 [5–7] are involved in the regulation of apoptosis.

The significance of apoptosis in breast cancer is largely unknown. Histopathological examination of human breast has already shown that the reduction of apoptosis relative to mitosis is related to fibrocystic change and carcinoma [17]. Experimental studies also suggest that the apoptotic process may be related to prognosis in breast cancer, since antioestrogens seem to stimulate apoptotis [11, 13], and the withdrawal of sex steroids might have similar functions. To date, the data related to apoptosis in breast cancer are mainly based on experimental models which clearly show the importance of the apoptotic process to growth regulation [11, 13].

Currently, there are no analyses available about the relationship between apoptosis, histopathological characteristics and prognosis in female breast cancer. The present study was conducted to correlate the number of apoptotic cells (on light microscopy) to various histopathological features and patient survival in a series of 288 breast carcinomas that were followed for over 11 years in our clinic.

MATERIALS AND METHODS

The present series consists of 288 women who were selected from the original cohort of 688 patients (from the years 1968–1990), recently subjected to detailed analysis for various prognostic factors and showing a mean follow-up of over 10 years [19–22]. The present cohort consists of consecutive patients treated and followed up between 1978 and 1990 at Kuopio University Hospital. Axillary lymph node status was determined by histological examination in 271/288 (94%) cases, and by clinical judgement in the other 17 cases. Tumour size was recorded as the maximum tumour diameter in a fresh mastectomy specimen. The follow-up was conducted at 3-month intervals during the first year, at 6-month intervals during the next 2 years, and annually thereafter. Metastases were detected by routine chest radiographs, bone radiographs, ultrasonography and laboratory tests reflecting bone and liver metabolism. Other pertinent clinical data are summarised in Table 1.

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Table 1. Patients' clinical data

Age (years) mean (S.E.)	59.9 (0.6)
Range	(27–92)
Follow-up (years) mean (S.E.)	11.6 (0.2)
Range	(5–21)
Tumour diameter (cm) mean (S.E.)	3.4 (1.2)
Range	(1–12)
Axillary lymph node negative/positive*	139/132
Metastasis at diagnosis	15
Recurrence	
Yes	112
No	176
Primary treatment	
Modified mastectomy alone	138
Mastectomy and radiation therapy	77
Mastectomy and radiation and systemic adjuvant therapy	73

*Histological examination.

Histological methods

None of the patients was treated before removal of the tumour specimen. The operative specimens from the primary tumours were fixed in buffered formalin (pH 7.0), embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Histological grading [23] and typing [24] of all tumours was completed as described previously. Mitotic figures were counted using a dual-headed microscope by two observers with an objective magnification of ×40 (field diameter 490 μm) [19]. The volume-corrected index (M/V index) method was used which expresses the number of mitotic figures/mm^2 of the neoplastic epithelium in the section [19]. Apoptotic cells were counted by using the same method in five consecutive microscope fields (field diameter 490 μm, magnification 40×, corresponds to 1 mm^2 of neoplastic epithelium in the section) and the results were corrected to correspond the number of apoptotic cells/mm^2 of the neoplastic epithelium (apoptotic index, AI). The criteria used in identifying the apoptotic cells were those previously described [25], and were (a) cells showing marked condensation of chromatin and cytoplasm; (b) cytoplasmic fragments containing condensed chromatin; and (c) intra- and extracellular chromatin fragments down to diameter of approximately 2 μm.

Flow cytometry, nuclear morphometry, steroid receptor assay and immunohistochemistry

Flow cytometry [22] and nuclear morphometry [22] were completed as described previously. The DNA index was available in 263/288 (91%) cases, and the S-phase fraction could be analysed in 184/288 (64%) tumours. Tumours with a DNA index value <1.20 were considered diploid, and those with a DNA index >1.20 were considered aneuploid. Nuclear morphometric measurements were made interactively by using the IBAS 1&2 image analysis system [22]. In this analysis, the mean nuclear area (NA), S.D. of NA, mean nuclear perimetry (PE), S.D. of PE, mean shortest nuclear axis (*D*min), mean longest nuclear axis (*D*max) and the mean area of 10 largest nuclei (NA10) were used. The sex steroid receptors were assayed biochemically using the charcoal–dextran assay as described previously [26], and the cut-off level of receptor positivity was 10 fmol/g cytosol protein. Sex steroid receptor content was available in 237/288 (82%) cases. p53 protein was detected with CM1 antibody (Novocastra Laboratories, Newcastle Upon Tyne, U.K.) in 89/288 (31%) cases using standard immunohistochemical methods as described previously [21].

Statistical analysis

In the basic statistical calculations, the SPSS-X program package was used in an IBM computer and the statistical tests used are indicated in the Results section. Frequency distributions were tested by the Chi-square test and Yate's correction was applied where appropriate. The differences between the means of continuous variables were tested by analysis of variance. The univariate survival analysis (log rank analysis, SPSS-X) was based on the life-table method with the statistics of Lee and Desu [27]. When the recurrence-free survival was analysed (univariate and multivariate analysis), the cases with a distant metastasis at diagnosis were excluded in the analysis. Multivariate survival analysis [28] was carried out with the BMDP (2L) program package in a stepwise manner and continuous variables were used as absolute numbers in this analysis. The enter limit was $P < 0.1$ and the removal limit was $P > 0.15$. Multivariate analysis also included patient age, menopausal status, the year of treatment and adjuvant therapy to control for their confounding effect. Multivariate analyses included only cases with histologically confirmed axillary lymph node status.

RESULTS

Apoptotic cells in the tumour biopsies appeared either as single cells or in small groups (Figure 1). The AI ranged between 0 and 138 with a mean (S.E.) of 11.3 (0.8).

AI was not significantly related to tumour diameter ($P = 0.8$) or axillary lymph node status ($P = 0.6$). There was a significant relationship between the histological type, tumour grade, DNA ploidy, S-phase fraction (SPF), mitotic index (M/V), presence

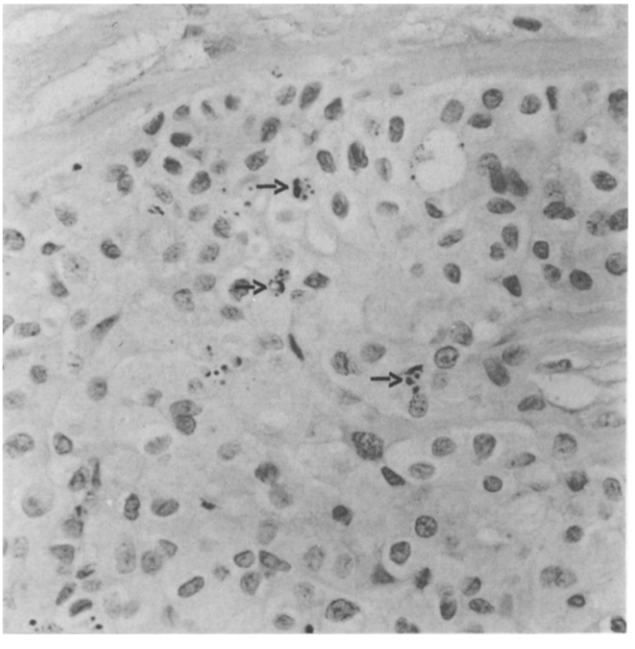

Figure 1. A grade 3 breast carcinoma containing several apoptotic cells (arrows).

Table 2. Relationship between AI and other prognostic factors

Variable	Number	Mean AI (S.E.)	Statistics
Ductal*	244	11.9 (0.9)	$t = 1.69$
Other	42	8.8 (1.5)	$P = 0.095$
Grade 1*	94	7.9 (1.3)	
Grade 2	100	9.4 (1.1)	$F = 12.7$
Grade 3	93	16.8 (1.5)	$P < 0.0001$
Diploid	131	9.6 (1.0)	$t = -1.98$
Aneuploid	132	12.8 (1.2)	$P = 0.049$
SPF <7%	131	8.0 (0.9)	$t = -2.64$
SPF >7%	53	14.4 (2.2)	$P = 0.010$
M/V <10/mm²	113	4.5 (0.5)	$t = -8.17$
M/V >10/mm²	175	15.4 (1.1)	$P < 0.0001$
PR(+)	118	9.8 (1.1)	$t = 2.15$
PR(−)	119	13.7 (1.4)	$P = 0.033$
ER(+)	127	9.4 (1.2)	$t = 2.64$
ER(−)	110	14.2 (1.3)	$P = 0.009$
p53(−)	40	7.5 (1.4)	$t = -3.01$
p53(+)	49	16.5 (2.6)	$P = 0.004$

t-test. *Some data missing.
SPF, S-phase fraction; ER, oestrogen receptor; PR, progesterone receptor; M/V mitotic index.

of sex steroid receptors, and expression of p53 (Table 2). High grade tumours which were sex steroid receptor-negative and rapidly proliferating usually had a high AI, as did tumours expressing p53 protein.

Associations between special histological features and AI are summarised in Table 3. Consistent with the quantitative measurements, tumours exhibiting signs of poor differentiation also had high AIs.

Table 3. Apoptosis related to special histological features

Feature	Number	Mean AI (S.E.)	Statistics*
Proportion of intraductal growth			
None	174	10.7 (1.0)	$F = 1.7$,
Some	88	11.3 (1.4)	$P = 0.173$
Principal	21	16.5 (3.9)	
Tubule formation			
Extensive	4	13.5 (5.1)	$F = 2.4$,
Some	23	5.5 (1.6)	$P = 0.093$
None	256	11.8 (0.9)	
Tumour necrosis			
No	224	10.2 (0.9)	
Scanty	41	12.9 (1.6)	$F = 4.7$,
Moderate	13	24.1 (3.5)	$P = 0.003$
Severe	5	13.6 (2.8)	
Nuclear pleomorphism			
Slight	12	5.8 (1.7)	$F = 8.1$,
Moderate	158	8.4 (0.9)	$P < 0.0001$
Severe	111	16.1 (1.6)	
Density of tumour infiltrating lymphocytes			
None and weak	43	6.7 (1.0)	$F = 7.1$,
Moderate	88	8.8 (0.9)	$P = 0.0009$
Dense	152	13.7 (1.3)	

*Analysis of variance.

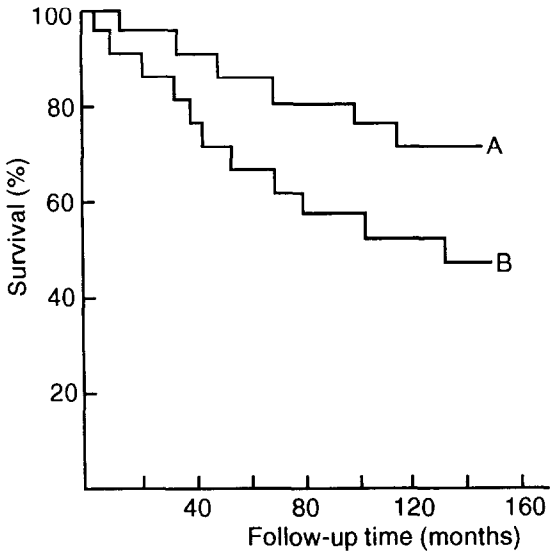

Figure 2. Survival of the breast cancer patients categorised according to AI. The curves are significantly different ($\chi^2 = 12.5$, $P = 0.004$). Curve A: AI < 3, $n = 73$; Curve B: AI >3, $n = 215$.

AI was positively correlated with M/V ($R = 0.410$, $P < 0.01$), Dmin ($R = 0.283$, $P < 0.01$), Dmax ($R = 0.280$, $P < 0.01$), NA10 ($R = 0.271$, $P < 0.01$), PE ($R = 0.262$, $P < 0.01$), SDNA ($R = 0.257$, $P < 0.01$), SPF ($R = 0.231$, $P < 0.01$), SDPE ($R = 0.193$, $P < 0.01$) and NA ($R = 0.119$, $P < 0.05$). Multivariate linear regression analysis (all the histopathological data included) showed that mitotic index was the only explanatory factor for AI (dependent variable in the analysis) (βB(S.E.) 0.283 (0.057), $P < 0.0001$).

In survival analyses, AI predicted prognosis in the entire cohort (Figure 2), in axillary lymph node-negative (Figure 3)

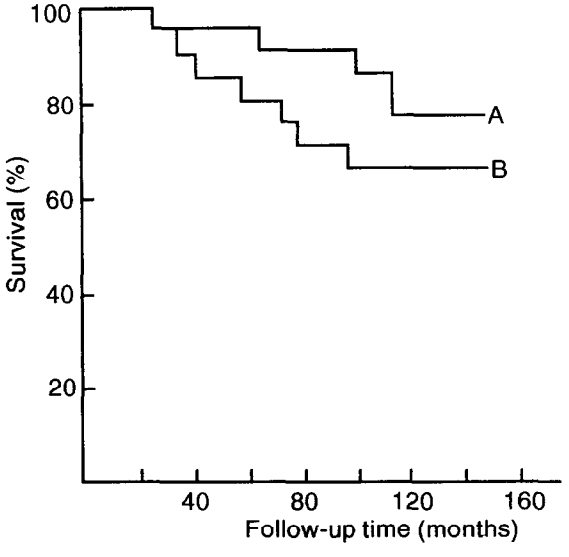

Figure 3. Survival of the axillary lymph node-negative breast cancer patients categorised according to AI. The curves are significantly different ($\chi^2 = 4.4$, $P = 0.0356$). Curve A: AI < 3, $n = 36$; Curve B: AI > 3, $n = 103$.

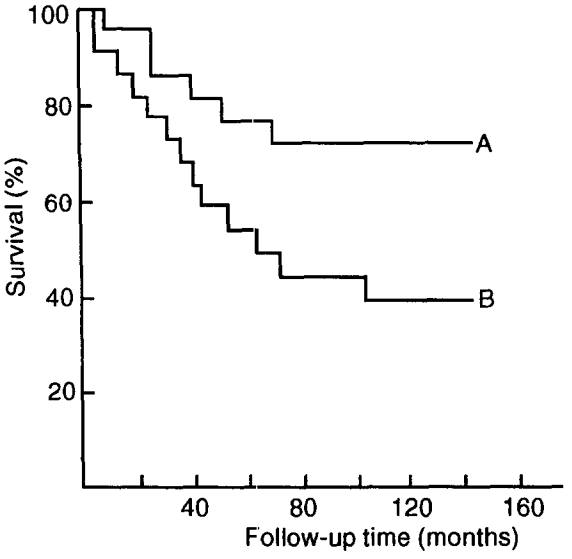

Figure 4. Survival of the axillary lymph node-positive breast cancer patients categorised according to AI. The curves are significantly different ($\chi^2 = 6.9$, $P = 0.0085$). Curve A: AI < 3, $n = 31$; Curve B: AI > 3, $n = 98$.

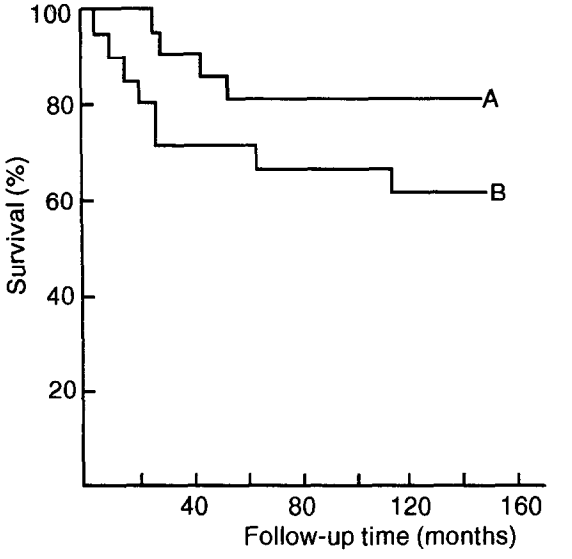

Figure 6. Recurrence-free survival of the patients with axillary lymph node-negative breast cancers categorised according to AI. The curves are significantly different ($\chi^2 = 5.0$, $P = 0.0253$). Curve A: AI < 3, $n = 36$; Curve B: AI >3, $n = 103$.

and node-positive tumours (Figure 4). Recurrence-free survival in the entire cohort (Figure 5) and in axillary lymph node-negative tumours (Figure 6) was related to AI, whereas in the axillary lymph node-positive tumours, AI was not related significantly to the recurrence-free survival ($P = 0.18$).

Multivariate analysis showed that AI had no independent prognostic value in the entire cohort or in axillary lymph node-negative or node-positive tumours. Recurrence-free survival was also not related to AI. Independent predictors were tumour diameter ($P < 0.001$), axillary lymph node status ($P = 0.001$), progesterone receptor positivity ($P = 0.004$) and ductal type ($P = 0.041$). Recurrence-free survival was independently predicted by mitotic frequency/mm^2 ($P = 0.012$), ductal type ($P = 0.011$), S.D. of NA ($P = 0.075$) and axillary lymph node status ($P = 0.096$).

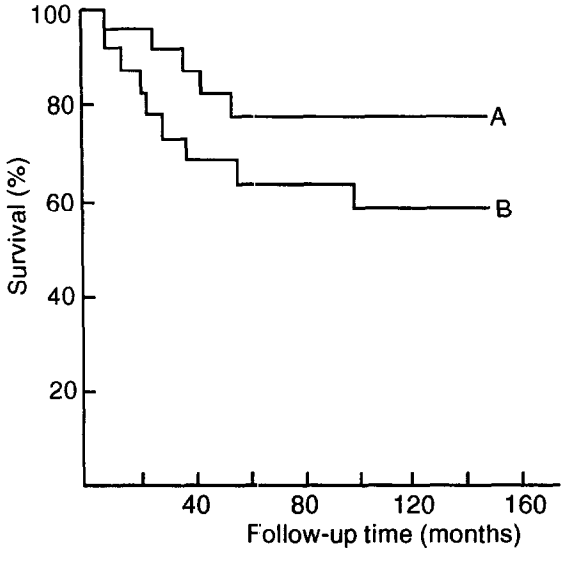

Figure 5. Recurrence-free survival of the patients categorised according to AI. The curves are significantly different ($\chi^2 = 7.0$, $P = 0.0079$). Curve A: AI <3, $n = 69$; Curve B: AI > 3; $n = 202$.

DISCUSSION

The present results confirm previous observations on the relationship between apoptosis and mitosis [11, 17, 29]. The close relationship between apoptosis and mitosis suggests common regulatory mechanisms for these events. The final direction of the cell cycle is probably regulated through growth factors, and also affected by oncogene activation in cancer, the role of c-*myc* being of particular interest. Expression of c-*myc* can stimulate either cell proliferation or apoptosis [3, 5–7, 18, 30], depending on whether the cell cycle is supported by growth factors [12, 14] or is limited by growth factor deprivation or treatment with other cell cycle blocking agents [3, 9, 12, 14]. *In vivo* c-myc expression may be related to high cellular turnover in which cell proliferation and apoptosis co-exist [3, 5, 6, 15]. Alternatively, the oncogenes *ras* and *bcl-2* may protect cells from susceptibility to apoptosis which may result in rapid cell proliferation and tumour formation [5, 6, 10, 15, 16]. These complex mechanisms are currently incompletely understood in breast cancer.

The *p53* gene may initiate apoptosis by causing G1/S arrest in cells expressing c-myc [5–7]. The present results showed that apoptosis was clearly more common in tumours with a high fraction of p53-positive nuclei. However, the results are complicated, since the CM1 antibody recognises both the wild and mutant types of the p53 protein. In p53-positive tumours, the concentration of p53 protein in the nucleus is greatly increased, probably due to accumulation of the p53 protein (mutated or normal) which may even be inhibitory for apoptosis.

Thus, the results might suggest that stabilised p53 is the result of DNA damage which might be the reason for its positive correlation with apoptosis and poor prognosis.

High AI was closely related to several histological features of malignancy, including high tumour grade and other signs of poor differentiation. However, in multivariate analysis, the mitotic index was the only explanatory factor for AI, which

further confirms the close relationship between mitosis and apoptosis [11, 17, 29]. Thus, tumor growth/regression is dependent upon balance between cell gain (proliferation) and cell loss (by apoptosis and necrosis). Accordingly, the present results concur with previous observations in breast cancer [17] and other neoplasia [29].

The growth of cancer cells is dependent on various viability factors including hormones [11–14]. The withdrawal of these factors stimulates apoptosis [11–14] and accordingly, sex steroid receptor-negative tumours had higher AIs. However, in practice, this accelerated apoptosis is not reflected in the clinical behaviour of sex steroid receptor-negative tumours since these tumours are rapidly growing and clinically malignant [22]. This means that other growth regulating factors are actively involved. Under experimental conditions, the linear growth rate of the cells is related to high apoptotic activity, while the exponential growth phase is associated with low apoptosis rate [31]. These experimental results are not in accordance with observations in the present clinical series, where apoptosis was clearly more frequent in poorly differentiated tumours with oncoprotein expression, rapid growth and poor outcome. The regulation of apoptosis in hepatoma cell lines [31] may be totally different to that in human breast cancer which may account for these variable results. Thus, it seems that a direct comparison of human cancer *in vivo* and *in vitro* is not relevant, and other mechanisms, like cell necrosis, are likely to be involved. Cell necrosis may contribute to tumour regression in cases in which AI is small and proliferation rate is also low. Moreover, it is difficult to distinguish between conventional necrosis and necrosis subsequent to the breakdown of apoptotic cells, which have failed to have been cleared by phagocytic cells.

The results of survival analysis showed that AI as a prognostic factor gives similar survival estimates as the mitotic index in this same cohort [19, 20]. However, the multivariate analysis clearly showed that mitotic index is a more accurate prognostic parameter than AI, which is in agreement with results in transitional cell bladder tumours [29]. This means that, in untreated human breast cancer, cell proliferation rate is the main determinant of the prognosis independence of apoptosis. Accelerated apoptosis induced by endocrine or cytotoxic therapy may overcome the net effect of cancer cell proliferation and accordingly, beneficial therapeutic results can be obtained [3 ,9, 11]. Recently, the apoptotic process has been particularly studied in the context of therapeutic trials [3, 9].

The present analysis is based on light microscopic quantitation of the apoptotic cells [25]. Scoring of apoptotic cells shows some inter-observer variation since the identification of apoptotic cells is more difficult than that of the mitotic figures in conventional histopathological sections. The large number of cases included in this study reduces the impact of random variation, and accordingly, renders the correlations between histopathological features, prognosis and AI relevant. It also became clear that the assessment of AI on light microscopy does not offer any prognostic information in breast cancer additional to the standard prognostic factors. The prognostic potential of the established predictors in this same cohort has been discussed in a recent series of reports [19–22].

The results suggest that the biological regulation of apoptosis in breast cancer is clearly a subject for further research, particularly from a therapeutic point of view [7, 11, 13]. Accordingly, further analyses are needed to clarify the relationship between apoptosis and response to therapy in neoplasia at the molecular level.

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Patient Population Analysis in EORTC Trial 22881/10882 on the Role of a Booster Dose in Breast-conserving Therapy

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The changing composition of the patient population in breast cancer, which has been reported over the last decade, has important consequences for prognosis. In the present trial, an analysis of the population in an EORTC trial (22881/10882) on breast-conserving therapy was conducted. A shift towards earlier stages has been seen stage per stage, therefore better survival and local control rates are likely to be expected in comparison to previously published series. The majority of tumours in this trial were small, with a median clinical size of 2 cm and a median pathological size of 1.5 cm. A substantial number of lesions were detected in a pre-clinical stage (17.8%). Nodal involvement was present in only 19% of all patients and usually in only a low number of nodes (only 4% of all patients had four or more nodes invaded). The median number of nodes examined was 12, the difference between institutions was large. There was a significant correlation between the number of nodes examined, the percentage of patients with positive nodes ($P = 0.03$) and the percentage of patients with massive axillary invasion ($P = 0.003$). The correlation between clinical evidence and pathological invasion of the axillary nodes showed that 15% of the clinical examinations were false-negative and 51% were false-positive. Pathological nodal invasion could be clinically predicted in only 31% of patients, and consequently clinical examination of the axilla was a poor predictor of prognosis in this study. Pathological invasion of axillary lymph nodes was better correlated to pathological tumour size than clinical or radiological size.

Key words: breast cancer, radiation therapy, tumorectomy
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INTRODUCTION

BREAST-CONSERVING THERAPY (local tumour excision with consecutive radiotherapy) is at present considered to be the standard of care for early breast cancer, making it possible to avoid ablative surgery. During the last decades, evidence has emerged proving the validity of this approach. The first trial which demonstrated the equivalence of mastectomy and breast-conservative treatment was an Italian series, only for tumours less than 2 cm, randomising between Halsted radical mastectomy and quadrantectomy plus radiotherapy to the whole breast (50 Gy) and a booster dose of 10 Gy to the scar [1, 2]. Although the conservative approach proved to be equivalent to radical surgery, it was only confirmed for patients with very small (< 2 cm) tumours. A trial on a similar group of patients in