CELL BIOLOGICAL MARKERS IN BREAST TUMOURS

Applications in cyto- and histopathology

Celbiologische merkers in mamma tumoren
toepassingen in cyto- en histopathologie

PROEFSCHRIFT

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De legkaart is incompleet en, hoe dan ook, altijd alleen maar een fragment van een onvoorstelbaar veel groter geheel.

Hella Haasse: Zelfportret als legkaart

Aan Gijs
Aan de kinderen

ABBREVATIONS

APAAP	alkaline phosphatase anti-alkaline phosphatase
AR	androgen receptor
BCAR gen	breast cancer anti-estrogen resistance gen
BrdUrd	bromodeoxyuridine
CI	confidence interval
DCIS	ductal carcinoma in situ
DFS	disease free survival
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EIA	enzyme immuno assay
EORTC	European Organisation for Research and Therapy of Cancer
ER	estrogen receptor
E-score	average staining intensity
FNA	fine needle aspiration
HR	relative hazard rates
IC	immunocytochemistry
IH	immunohistochemistry
LCIS	lobular carcinoma in situ
MAb	monoclonal antibody
MPI	multivariate prognostic index
MRI	magnetic resonance imaging
mRNA	messenger RNA
NOS	not otherwise specified
OS	overall survival
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PP-score	number of positive stained cells on a total of 300 cells
PR	progesterone receptor
SSCP	single-strand conformation polymorphism
u-PA	urokinase-type plasminogen activator
u-PAR	urokinase plasminogen activator receptor

CONTENTS

CHAPTER 1.

BREAST CANCER

1. INTRODUCTION

1.1. *Epidemiology and etiology*

Breast cancer is the most common malignant tumour among women in the western world, affecting 8-12 % of the female population. In the Netherlands, breast cancer occurs yearly in 1000 per 100,000 women with an absolute incidence of 9,000 new cases per year.[1] The etiology is multifactorial. Age is an important risk factor. The incidence climbs after age 30, followed by a slight dip at menopause and continues to rise during postmenopausal years.[2,3] Hormonal influences are well documented etiological factors: increasing age of the mother at birth of her first child increases the risk of breast cancer whereas late menarche and early menopause decreases the risk.[4,5,6]An extensive collaborative study reanalysing 54 epidemiological studies in 25 countries provided evidence for a small increase in the relative risk (1.24) of having breast cancer while taking oral contraceptives and in the 10 years after quitting, with no increased risk 10 or more years after stopping.[7]

Familial predisposition is considered to be an important risk factor as well. The cumulative risk of a healthy woman developing breast cancer increases from 10 to 20% if a first degree relative becomes affected. The risk is further increased (up to 40%) in families with a mean age at cancer diagnosis of less than 45 years.[8,9] In these families breast cancer is often found to be of genetic origin, and can in the majority of cases now be attributed to mutations of the *BRCA-1* gene (predisposition for breast and ovarian cancer) located on the long arm of chromosome 17, and the *BRCA-2* gene (predisposition for female and male breast cancer), located on the long arm of chromosome 13.[10] Until now over 100 mutations have been observed in these genes (252 different mutations for BRCA1, 173 for BRCA2). It is anticipated that additional mutant genes associated with familial breast cancer will be found.

Less important risk factors are ionizing radiation, including X-rays, especially when exposure has taken place during adolescence or childhood[11,12] and diets high in animal fats.[13] Obesity is shown to be a risk factor in the postmenopausal age group.[14,15] In general, environment and lifestyle contribute to the development of breast cancer. Japanese women living in US have a higher mortality rate for breast cancer than Japanese women living in Japan.[16] Recently, a comprehensive Norwegian study has confirmed the findings of others that physical exercise reduces the risk of developing breast cancer.[17,18]

1.2. *Precursor lesions.*

A history of atypical hyperplasia, whether lobular or ductal, indicates a woman's risk for the development of breast cancer of 5-10%.[19] Other histologic categories like adenosis and apocrine changes do not increase a woman's risk, meaning that the relative chance of getting breast cancer is the same as that of a reference population. Ductal carcinoma in situ (DCIS) is considered a true precursor lesion and breast cancer screening programs have led to the detection of an increasing number of DCIS: <5% in the seventies, up to 15-25% of the total number of detected cancers in the nineties.[20,21] DCIS is histologically a heterogenous group of lesions, since only one third will progress to invasion in the same breast during follow-up of 10-18 years.[20,22,23] It has therefore become increasingly important to classify these lesions in relation to their biological behaviour according to strict histological criteria. Lately a new classification,[24] based primarily on cytonuclear and architectural differentiation has been proposed. This classification distinguishes 3 categories of this lesion: well, intermediate and poorly differentiated in situ carcinomas. A highly significant correlation was found between the DCIS type and the grade of the infiltrating component arising from or located around the DCIS.[25] Moreover a strong correlation was seen between the different grades of in situ carcinomas and the presence of biological markers, the poorly differentiated being estrogen receptor (ER) and progesterone receptor (PR) negative, P53 or Her/2neu positive, the well differentiated showing the reverse phenotype and the intermediate a mixed pattern.[26,27,28] Lobular carcinoma in situ (LCIS) is rare (observed in 0.5 % of symptomatic and in 1% of screen-detected cancers[20]) and follows a different course compared to DCIS. About 15-20% of women with LCIS will develop breast cancer in the same breast, a further 10-15% will develop an invasive cancer in the controlateral breast.[20,29]

1.3. *Diagnostic procedures.*

An excisional biopsy was the only available diagnostic method for half a century. But since the sixties new diagnostic methods were developed. The introduction of fine needle aspiration cytology (FNA) has led to presurgical diagnosis of breast tumours. This simple, patient friendly, cost reducing method has an average sensitivity of 87%, specificity close to 100%, a predictive value of positive diagnosis nearly 100%, and a predictive value of negative diagnosis between 60-90%.[30,31,32,33] The introduction of mammography and ultrasound, and the acceptance of FNA as diagnostic tool, resulted in the triple diagnosis used in the pre-operative work-up of a palpable lesion. Several studies[34,35,36] have confirmed that this method detects most cancers and has reduced the number of frozen sections for the pathology department.

Breast screening programs and recent advances in mammography, ultrasound and magnetic resonance imaging (MRI), have not only led to an increasing number of DCIS but likewise to earlier diagnosis of breast cancer and therefore

to an increasing number of small <11 mm, invasive carcinomas (7% in the sixties till 25%-28% in the nineties[21,37]). These tumours are often non-palpable and therefore stereotactic or ultrasonographic localisation is needed followed by either FNA or, when this is not feasible, needle biopsy or excisional biopsy to provide a definitive diagnosis.

1.4. *Therapy*

Not only diagnostic methods have changed, also new surgical and therapeutic alternatives have been developed, based on the concept that occult metastases have already developed in about 50% of the patients at the time of diagnosis, independent of lymph node metastasis. Nowadays, breast conserving surgery, instead of mastectomy, in combination with axillary dissection, followed by postoperative irradiation of the thorax wall is generally applied to tumours less than 4 cm in diameter. Long term results after 8-10 years follow-up comparing the two treatment modalities (that is lumpectomy versus mastectomy) have demonstrated comparable disease free survival (DFS) and overall survival (OS).[38,39,40,41]In addition, systemic adjuvant therapy is given in node-positive patients: in general, chemotherapy in premenopausal patients and hormonal therapy in post menopausal patients. This approach has been shown to improve DFS and OS[42] of breast cancer patients. Neo-adjuvant chemotherapy is given to patients with large, skin or thorax wall invasive (T4) tumours.[43]

With regard to DCIS new treatment modalities are being proposed. The findings of Fisher[44] and Solin[45] suggested a beneficial effect of irradiation of the thorax in reducing the risk for development of invasive cancer after removal of the lesion by lumpectomy. Validation studies of patients with DCIS < 5cm being treated with radical excision alone or surgery plus radiation, based on the new classification, are under way (EORTC trial 10853).

Selection for node-negative patients with a poor prognosis is important, since in those cases treatment with neo-adjuvant chemotherapy is being proposed. In general, many prognostic factors have been studied in relation to the biological behaviour of breast carcinomas of which some of the most important will be mentioned in this introduction.

2. PROGNOSTIC AND PREDICTIVE FACTORS

Standard prognostic parameters

2.1. *Age.*

As mentioned before, age specific incidence displays a progressive rise with increasing age. Several studies have shown evidence that women < 35 years have higher grade primary tumours and poorer 5-year survival than older premenopausal women.[46,47,48,49] In support of these data Walker et al[50]

demonstrated that breast cancers in this age group had high proliferation rates, significantly higher incidence of P53 protein staining and more ER and PR negative tumours (see below).

2.2. *Tumour staging: tumour size, lymph node status and distant metastasis.*
Tumour size and axillary lymph node status at diagnosis are well established prognostic factors for early recurrence.[51,52,53,54,55] Lymph node status is still the major prognostic indicator with a ten-years survival of lymph node negative patients of 70% compared to less than 40% of node-positive patients[55,56]. The number of tumour positive lymph nodes also influences prognosis. Ten-year survival has been reported to be 38% if one to three nodes are positive, and 13% if four or more nodes are positive.[52] Moreover the size of the primary breast tumour is correlated to the number of nodes involved[53,56,57,58].
Presence of distant metastases indicates a poor prognosis since no cure is possible. However since breast cancer is often a slowly growing process some patients with metastasized disease may live for many years. The prognosis for patients with bone and skin metastases tends to be better than for those with visceral metastases[59].

2.3. *Histological features.*
Studies with large cohorts of patients have illustrated the importance of histological typing and grading.[60,61,62,63,64,65,66] Histological typing identifies three groups of patients with a different biological behaviour:
1. a group with a favourable prognosis (tubular, cribriform and mucinous type breast carcinomas), even if the tumour is large,
2. an intermediate group (lobular, mixed type and medullary type carcinomas),
3. a high risk group (ductal carcinomas not otherwise specified (NOS)).
In general, tumours are graded according to Bloom and Richardson[60], with modifications as described by Elston et al[63]. This system is based on the scale of tubule formation, the assessment of nuclear pleomorphism and mitotic rate. In multivariate analysis, grading, when the protocol is strictly applied as shown by the Nottingham group,[63] is an independent prognostic factor, with lymph node status second in importance. Patients with grade I tumours have a significantly better survival than those with grade II and III tumours. When grade and tumour type are used together in combination with the lymph node status,[67] the combination of these parameters even more accurately predicts prognosis.
To avoid the subjectivity of visual grading systems semi-automated and automated systems for quantitative analysis have been devised. The group of Baak[68] found the mitotic activity index to be the most accurate single prognosticator even more so when combined with lymphnode status and nuclear area (multivariate prognostic index: MPI[69]).

2.4. *Steroid receptors*

The development of normal breast glandular tissue and breast malignancies are regulated by hormonal factors, affecting cell differentiation and proliferation. Estrogens and progesterones mediate their effect on breast glandular epithelial cells and carcinoma cells by interactions with their specific (nuclear) receptor. Approximately 70% of the primary breast tumours express estrogen receptors (ER). Expression of the estrogen-regulated progesterone receptor (PR) in most of these tumours indicates that the ER is functional. In primary breast cancer high levels of steroid receptors are a favourable prognostic parameter and a predictor of response to endocrine therapy. Moreover, tumours containing both ER and PR at diagnosis have a better prognosis and show the highest frequency of reponse to endocrine therapy compared to ER-positive, PR-negative tumours.[70] Eventually however, response duration to hormonal therapy is limited and disease progression occurs in most, initially responsive, patients. Regarding the prognostic value of ER and PR in node-negative patients, controversy exists.[71] Large studies with long follow-up have demonstrated by multivariate analysis that in node-negative breast cancer ER and PR expression is a significant prognostic factor of DFS in premenopausal patients, whereas in postmenopausal patients such an association was not present.[72,73,74] In spite of these controversies, it is clear that patients with steroid receptor-positive tumours benefit from adjuvant endocrine therapy[42]. Moreover ER and PR measurements will allow us to select patients with recurrent breast cancer who are more likely to benefit from endocrine therapy[75,76], emphasizing the importance of ER and PR measurements in breast cancer.

The functional role of androgen receptor (AR) expression is less well defined. In vitro studies have suggested that androgen-induced inhibition of cell proliferation is AR mediated.[77,78] In vivo studies[79,80]on human breast cancer have shown that the combined use of androgen and antiestrogen therapy may have therapeutic advantages over anti-estrogen therapy only.

Other estrogen dependent proteins like the estrogen inducible protein pS2, are being investigated to assess their predictive and prognostic value. High levels of pS2 are related to good survival, the latter especially in the subgroup of patients with ER/PR positive tumours. Moreover pS2 has been found to be more predictive than biochemical ER measurement for the likelihood of tumour response to endocrine treatment.[81]

2.5. *Epidermal growth factor receptor*

Not only sex steroid hormones, but also polypeptide growth factors are involved in cell proliferation. Thus, epidermal growth factor (EGF) is one of the growth factors necessary for the maintenance of normal breast epithelium and can stimulate proliferation of normal mammary epithelium and human breast cancer cells in vitro. Its action is mediated by its receptor (EGFR). Approximately one-third to one-half of the breast tumours express EGFR. An inverse correlation

between EGFR and steroid receptor status in primary breast cancer and breast cancer cell-lines on the protein and mRNA level has been reported.[82] Several studies have demonstrated that tumours expressing EGFR are more likely to be resistant to endocrine therapy[83,84].

2.6. *Genes involved in development of anti-estrogen resistance.*
Development of endocrine therapy resistance in ER-positive breast cancer is a major problem in the management of patients with recurrent disease. Little is known about the mechanisms underlying this progression. It may involve alterations in the estrogen-receptor structure and function[85]and changes in the paracrine interactions with the stromal cells[86].
Recently it has been proposed (Dorssers et al[87,88]) that tamoxifen treatment resistance might well be caused by genetic alterations of the tumour cells. So far they identified three Breast-Cancer-Anti-estrogen-Resistance (BCAR 1,2 and 3) loci in a series of tamoxifen-resistant breast cancer cell lines. However, the clinical relevance of these resistance genes still needs to be established.

2.7. *Measures of proliferation*
The growth rate of tumours is determined by the ratio between tumour cell proliferation and tumour cell death as the consequence of apoptosis and necrosis. Fast growing tumours are considered to have a worse prognosis[89]. Mitotic activity, determined by counting mitotic figures, is one way of assessing proliferation and is part of the histological grading system, as described before. Baak et al[68] found the mitotic activity index to be the most accurate single prognosticator and this was even shown to be more powerful in combination with lymph node status and tumour size (Multivariate Prognostic Index (MPI)[69]). Proliferation has also been evaluated by determining the fraction of cycling cells in S-phase, either using DNA flow cytometry or ^{3}H-thymidine and bromodeoxyuridine (BrdUrd) labelling indices. Despite the lack of standardized methods of DNA content measurements, an association has been demonstrated between increased risk of recurrence and mortality of patients with high S-phase fraction with both node-negative and node-positive invasive breast cancers.[90,91,92,93] Likewise the ^{3}H-thymidine and BrdUrd labelling indices are found to be independent prognostic factors,[94,95,96] especially in node-negative patients. Antibodies directed against the proliferating cell nuclear antigen (PCNA) which appears in the nucleus primarily during S-phase have become available. However, a predictive value of PCNA labelling index for clinical outcome has not been found consistently.[97,98,99]
Other monoclonal antibodies such as Ki-67 or MIB-1, defining a proliferation associated nuclear protein have become available,[100] demonstrating cells not only in G1, but also in G2 as well as in S-phase. These markers are more easily applied to and evaluated in tumour tissues and therefore used increasingly in clinical studies evaluating growth fractions in relation to other known prognostic

indicators.[101,102,103] A better estimate of the growth potential of a tumour would be obtained if, next to a measure of proliferation, also apoptosis could be quantitated. Currently, DNA fragmentation and the presence of single strand ends of DNA can be detected by several in situ assays, employing nich-translation or terminal transferase. Recent data, however, suggest caution in the interpretation of the outcome of these assays[104].

2.8. *DNA-Ploidy*

Gross changes in nuclear DNA content can be measured by DNA flow cytometry. DNA-aneuploidy is considered as a reflection of the genetic instability of a breast cancer. The percentages of aneuploid breast cancers range between 60 and 90.

Some authors found aneuploidy to be closely related to the clinical outcome in breast cancer[105,106,107,108,109] especially in node-negative breast cancers.[107] However, due to a lack of standardized methods, results are somewhat inconsistent. In a consensus review of the clinical utility of DNA cytometry in carcinoma of the breast it was demonstrated that DNA index fails to achieve independent prognostic significance using multivariate analysis because of correlations with more powerful prognostic factors[110].

2.9. *Proto-oncogenes and tumour suppressor genes*

Molecular alterations in proto-oncogenes and tumour suppressor genes are responsible for the development of cancer. Dominant mutations can activate proto-oncogenes to become oncogenes and recessive mutations may inactivate tumour suppressor genes. The tumour suppressor gene *TP53*, located on the short arm of chromosome 17, has been implicated in the regulation of normal cell growth and division, DNA repair and apoptosis. This gene is frequently (14-52%) altered in primary breast cancer and is related to poor DFS and OS[111,112]. In general, mutations in the TP53 gene give rise to an altered protein which, due to a prolonged half-live is stably expressed at high levels. Breast cancers with high expression of TP53 protein are associated with poor response to tamoxifen and chemotherapy after relapse. This is reflected by poor progression free survival[113]. Amplification of the oncogene *MYC*, found in 10-23% of primary breast cancers, has also been associated with poor prognosis[111,113]. Since *P53* and *MYC* genes mediate drug-induced apoptosis, the effect of *P53* or *MYC* gene alterations may be related to the inhibition of the therapy induced apoptosis and therefore promote tumour cell survival. c-Myc amplification is a better prognostic factor than HER/2neu amplification in primary breast cancer.[114] Overexpression of HER/2neu protein is found predominantly in grade 3 intra ductal carcinomas[115,116].

2.10. *Tissue proteases related to invasion and metastasis.*
Cathepsin D is an estrogen inducible lysosomal enzyme, which is capable of digesting extracellular matrix and also acts as a growth factor. It may therefore play a role in determining tumour invasiveness and proliferative activity, both indicators of an aggressive biological behaviour of a tumour. Several studies have indicated that high levels of cathepsin D are related to poor survival[117,118].
Recently the Urokinase-type plasminogen activator (uPA) was found to play a central role in the multifactorial processes of invasion and metastases by activation of plasminogen which is subsequently converted to plasmin. Plasmin can activate type IV procollagenase which ultimately lead to tumour cell invasion and metastasis. The activity of uPA is enhanced after binding to its receptor (uPAR) and decreased by its inhibitors plasminogen activator inhibitor-1 and 2 (PAI-1 and PAI-2). In pre-clinical studies it was demonstrated[119,120,121,122,123]that high levels of uPA, uPAR and PAI-1 are indicative of poor prognosis also in lymph node negative patients, whereas high levels of PAI-2 are associated with a favourable prognosis in patients with tumours containing high levels of uPA. Moreover high levels of uPA, uPAR and PAI-1, were associated with poor response and survival while high levels of PAI-2 were related with prolonged survival and more favourable response following tamoxifen therapy[124]. A multicenter randomized study of node negative patients has been started to investigate the effect of adjuvant chemotherapy in patients with high levels of uPA, uPAR and PAI-I.

The prognostic parameters as described above, can in general also be determined using cytological material. This holds particularly true for nuclear proteins and genetic markers. Grading systems on routinely stained cytological preparations have been described[125,126]and have been found to have prognostic value in identifying high grade tumours. Likewise (semi)-quantitative studies using morphometry, image-cytometry and flowcytometry have been applied to cytology to measure nuclear size and shape[127,128,129] and/or DNA content[130,131], with results similar to those found in histology.

3. PHYLLODES TUMOURS OF THE BREAST

3.1. *Introduction*
Not only neoplasms of epithelial origin, but also benign and malignant stromal tumours may develop in the breast.
One type of stromal neoplasm, the so called phyllodes tumours, is of particular interest, since none of the histological criteria used to categorize these tumours as benign or malignant, are effective in predicting local recurren-

ce[132,133,134]. In contrast to breast carcinomas phyllodes tumours are uncommon. Only 0.3-0.5% of breast tumours in females belong to this group. The majority of cases occurs in women between ages of 35 and 55 years, the average age being around 50 years.

3.2. *Histological features*
Like fibroadenoma, phyllodes tumours are histologically characterized by a combined proliferation of stromal cells and ductal epithelium[135]. The stromal component determines the biological behaviour of the tumour. Macroscopically they form lobulated firm masses, microscopically the tumour shows cystic spaces lined by epithelium, in which the stroma classically projects in a leaflike fashion. Cytological specimens of these tumours are characterised by branching epithelial cell groups, highly cellular stromal fragments and fibroblasts in the background[136,137]. The tumour is classified according to the histological features of the stromal component: stromal cellularity, nuclear atypia and mitotic activity; stromal overgrowth, necrosis and heterologous elements. Around 20% of phyllodes tumours are considered malignant on the basis of these histological criteria. Due to the highly cellular stroma benign phyllodes tumours are often mistaken as fibroadenomas both in cytology and histology.

3.3. *Therapy*
Wide excision with a 10 mm margin of surrounding normal breast is recommended. Since some of them are clinically mistaken as fibroadenoma, the initial excision is often inadequate. Mastectomy is considered to be the treatment of choice for those women with a very large benign tumour or with malignant lesions. No axillary lymph node dissection is needed, since metastasis is almost exclusively haematogenous.

3.4. *Prognosis*
The presence of tumour at the margins of the excised specimen is found to be a major determinant of local recurrence, moreover histological characteristics have only limited prognostic significance.[134,135,136].

4. AIM OF THE STUDY

Up till now most studies evaluating cell biological markers like ER and PR have used biochemical assays. Since these methods require analysis of tissue homogenates, they do not give insight in the expression pattern of these markers at the cellular level. With the development of immunohistochemical techniques tumour markers can be directly visualized at the cellular level and consequently the distribution of prognostic markers in normal and malignant breast tissues can be estimated. The decreasing size of the tumours, and new treatment modalities together with an increasing demand of biological markers, makes the application

of immunohistological techniques even more appealing. The method is rapid, requiring a minimum amount of tissue: which permits its application even on FNA material. Good correlations between most biochemical, immunohistochemical (IH) and immunocytochemical (IC) assays have been reported[138,139,140,141,142], although discrepancies partly related to contamination with benign epithelium in the biochemical samples, and partly to heterogeneity of the tumour, loss of antigenicity and differences in techniques, may exist.
One of the purposes of this thesis was to investigate the feasibility of the use of promising immunohisto- and cytochemically determined cell biological markers in FNA material and frozen tissue samples, and to analyze their relation to clinical and histological variables in breast tumours. Furthermore, we wanted to enhance the quality of immunocytochemistry by developing a quality assured, standardized method of immunostaining. Finally we wanted to evaluate whether the detection of *TP53* gene mutations would offer new prognostic information in phyllodes tumours of the breast.

In chapter 2 we evaluated the feasibility of the use of Ki-67 assessed proliferative activity in FNA smears, obtained from benign and malignant tumours as well as from loco-regional and distant metastases and determined the relationship with clinical parameters.

In chapter 3 we compared the results of the Ki-67 determined growth fraction in FNA smears and cryostat sections of corresponding tumours. In addition we investigated the association between different histological subtypes and the level of Ki-67 reactivity.

In chapter 4 we report on a study to determine the best standardized method for material processing and IC staining of prognostic markers in FNA specimens of breast carcinomas.

In chapter 5 we investigated estrogen, progesterone and androgen receptor expression in breast cancer patients by IH on snap frozen tissue specimens. Expression of AR was compared with that of ER and PR as well as with tumour grade and age.

In chapter 6 the prognostic value of AR was analyzed in a retrospective study of 153 breast carcinomas of which the clinical, histological and IH determined cell biological data were recorded and examined in an uni- and multivariate analysis.

In chapter 7 the relation between cell biological markers and the biological behaviour of a case of a malignant phyllodes tumour, was studied. Expression

of Ki-67, TP53 and its regulated proteins, like MDM2, P21, BCL2 and BAX, in the primary, recurrent and metastatic tumour tissue was studied using IH and molecular biological techniques.

Chapter 8 gives a general discussion of the results of the studies described in this thesis and general conclusions are drawn.

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CHAPTER 2

SHORT COMMUNICATION

IMMUNOCYTOCHEMICAL STAINING OF PROLIFERATING CELLS IN FINE NEEDLE ASPIRATION SMEARS OF PRIMARY AND METASTATIC BREAST TUMOURS

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SHORT COMMUNICATION

The variability in biological behaviour of breast carcinomas is a long standing problem. Lymph node status is still the major prognostic indicator (Fisher et al 1983), but also mitotic activity appears to be of considerable prognostic relevance, either as a single parameter (Schiodt 1966; Stenquist et al 1981; Baak et al 1985), or as part of histological (Bloom & Richardson 1957; Elston et al 1982) or cytological (Mauriquand et al 1986) grading. Since only a minor fraction of proliferating cells is in mitosis, the determination of the total number of proliferating cells could be a better indicator of the growth fraction of a tumour population and therefore might give more information about the biological behaviour of a tumour.

A mouse monoclonal antibody (Ki-67) has become available defining a nuclear antigen present in proliferating cells throughout the cell cycle. The antigen is absent in G0 and early G1 (Gerdes et al 1983,1984). Thus, Ki-67 enables the immunocytochemical detection of cycling cells without the need of external administration of radioactively labelled nucleotides or mutagenic substances such as bromo-deoxyuridine or iododeoxyuridine. Until now studies on breast tumours using Ki-67 as a proliferation marker were performed on histological material. As fine needle aspiration (FNA) smears are more and more used as diagnostic tools, we investigated the feasibility of the use of this monoclonal antibody on cytological material obtained from benign and malignant tumours as well as from distant metastases of breast carcinomas. In addition, a possible relationship between the Ki-67 determined growth fraction of breast carcinomas and clinical parameters such as tumour size, lymph node status and menopausal status was investigated. The material consisted of FNA smears of 38 breast carcinomas (31 invasive ductal, 5 colloid and 2 medullary carcinomas), 20 fibroadenomas and 26 metastases of breast carcinomas (16 lymph nodes, 2 liver metastases and 8 local recurrences); the cellularity of aspirates from metastases and primary carcinomas was comparable. The air dried smears were fixed in acetone for 5 min and immunostaining with the mouse monoclonal antibody Ki-67 (Dako, Denmark) diluted 1:10 in PBS, pH 7.4, containing 0.05% gelatin and 0.1% NaN_3, was performed using the indirect conjugated immunoperoxidase method as described previously (Van der Kwast et al 1985). After addition of diaminobenzidine as substrate brown speckled staining of nuclei or nucleoli was considered as a positive reaction with Ki-67. Mitotic figures were also stained. Nuclear counterstaining was achieved by a 1 min incubation in Mayer's haematoxylin (Figure 1). The percentage of Ki-67 positive nuclei was determined by counting 500 cells at a magnification of 1000 x. The counting was done by a cytologist (VK) and care was taken to count tumour cells only or in the case of benign tumours to count breast epithelial cells only. The clinical data collected from the patients with the primary tumours are shown in Table I.

Table I. *Clinical data of 38 patients with primary breast carcinomas*

Pre	Post	T?	T1	T2	T3	T4	N?	No	N1	N2
10	28	2	11	18	3	4	3	14	11	10
Total	38					38				38

Pre/Post=pre/postmenopausal T?=Unknown; N?=Unknown.

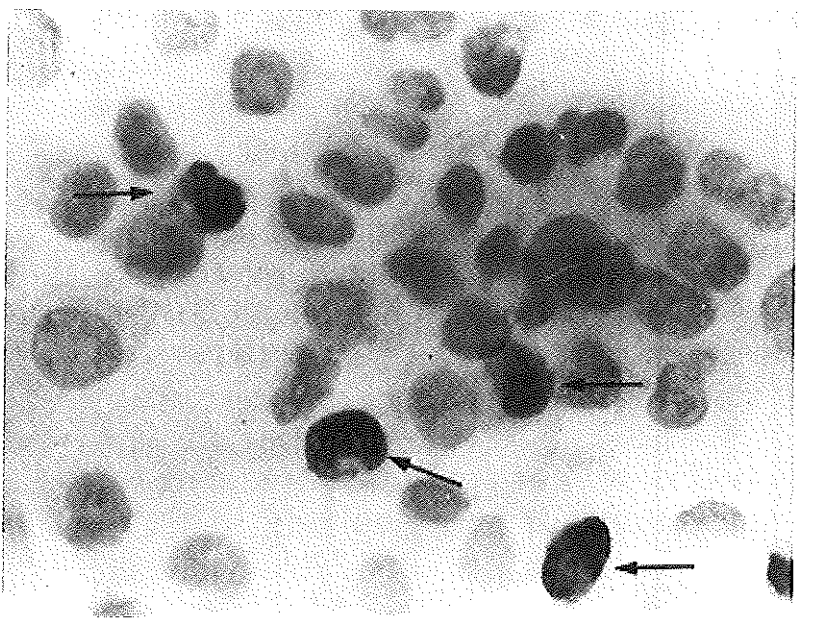

Figure 1. *Picture of FNA smear of breast carcinoma, showing Ki-67 stained nuclei (arrows).*

The FNA smears of the benign tumours contained a low percentage of immunostained nuclei, ranging from 0.0-6.7 with a mean of 1.1 and a median of 0.4. In contrast only four of the malignant tumours showed a percentage below 2, 21 between 2 and 10, and 13 even higher than this (Figure 2) with a mean of 10.5 and a median of 7.9. In comparison with the malignant primary tumours the metastases revealed even higher values with a mean of 14.3 and a median percentage of 12.3. The difference of the last two median values, assessed by the Whitney-test was statistically significant ($P < 0.05$).

Assessment of the Ki-67 determined proliferative tumour cell fraction in FNA smears of malignant breast lesions led to essentially similar results as recently

reported in several studies (Gerdes et al 1986; Lelle et al 1987; McGurrin et al 1987; Barnard et al 1987) of Ki-67 determination on cryostat sections of breast tumours (Table II).

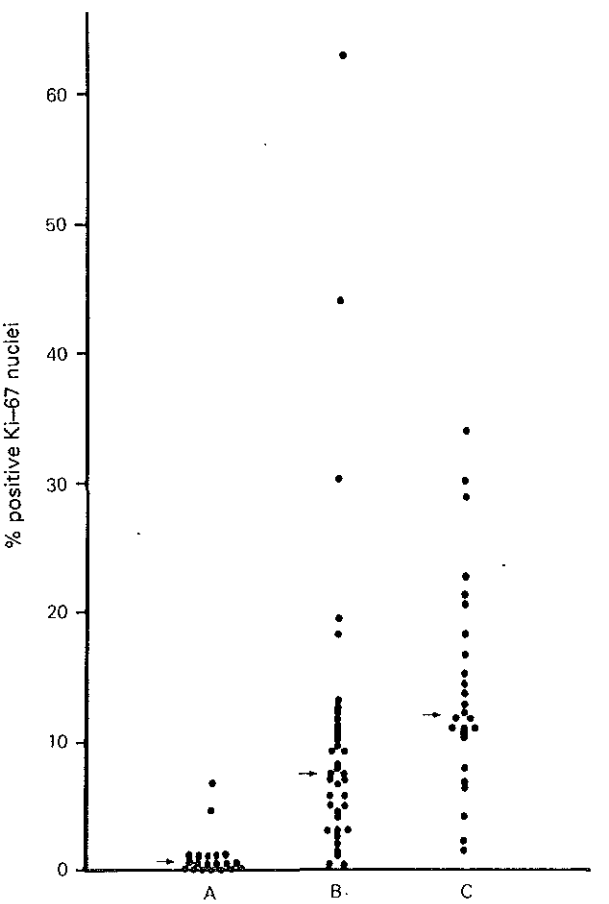

Figure 2. *The percentages of Ki-67 stained nuclei in benign,malignant and metastatic breast tumours. A=benign (n=20);B= malignant (n=38); and C= metastases (n=26). Arrow signifies median.*

Table II. *Reported data of mean percentages of Ki-67 stained nuclei in primary breast carcinomas in cryostat sections and imprints/FNA smears.*

	Histology	Cytology
Gerdes	16.6	
Lelle	16.2	14.5[a]
McGurrin	22.0	
Barnard	20.6	
This study		10.5[b]

[a]Imprints; [b]FNA smears

It appears from the data in Table II that the Ki-67 percentages of cryostat sections tend to be higher than those of the aspirates. This lower figure in aspirates may be explained by sampling differences. When the tumour is aspirated the material is derived from both peripheral and central parts of the tumour, whereas a cryostat section gives information on one part of the tumour only.

According to Baak et al (1985) and Van der Linden et al (1987) the mitotic activity index in histological material is the strongest independent prognostic factor in their group of patients both retrospectively and prospectively. Although the prognostic significance of the Ki-67 determined proliferative tumour cell fraction remains to be proven, we think that determination of the total cycling fraction of a tumour gives more information about prognosis than assessment of numbers of mitotic figures alone. This holds especially true for FNA smears, since they rarely contain mitotic tumour cells. Further support for the hypothesis that the Ki-67 determined proliferative fraction may be of prognostic relevance can be drawn from the in vitro ^{3}H-thymidine labelling studies of Meyer et al (1983) and Tubiana et al (1984). These authors incubated small specimens of freshly obtained breast tumour tissue with ^{3}H-thymidine and counted the number of labelled nuclei from autoradiographed microscopic sections. In the study of Meyer et al (1983) with a follow-up period of 4 years both the lymph node status and the ^{3}H-thymidine labelling index appeared to be the strongest independent indicators of early relapse. In the long-term prospective study of Tubiana et al (1984) the ^{3}H-thymidine labelling index appeared to be the most predictive independent indicator with respect to relapse-free survival and total survival. It should be noted that Ki-67 immunostaining is only indirect evidence of proliferation while the mitotic index and ^{3}H-thymidine incorporation directly relate to proliferative activity. Furthermore Ki-67 stains cells in G1-phase, cells arrested in this phase of the cell cycle may also be stained by Ki-67 which leads

to an overestimation of the fraction of actually proliferating cells. In contrast to the findings of Lelle et al (1987), but in agreement with the study of McGurrin et al. (1987) and Barnard et al (1987), we found no relation between lymph node status and percentage of Ki-67 labelled tumour cells. Neither did the ^{3}H-thymidine labelling studies (Meyer et al 1983; Tubiana et al 1984) show any relation to lymph node status.

No clear-cut correlation was found in this study between tumour size and menopausal status or percentage of Ki-67 immunostained nuclei. Probably the number of investigated tumours was too low to establish such a relationship.

It was interesting to see, however, that the really high values (above 40% see Figure 3) were only seen in premenopausal women. In addition, the two highest values (30.5% and 19%) in the group of postmenopausal women were medullary carcinomas. It is known that the latter tumours have a high mitotic activity and display a different biological behaviour than the infiltrating duct carcinomas (Azzopardi, 1979).

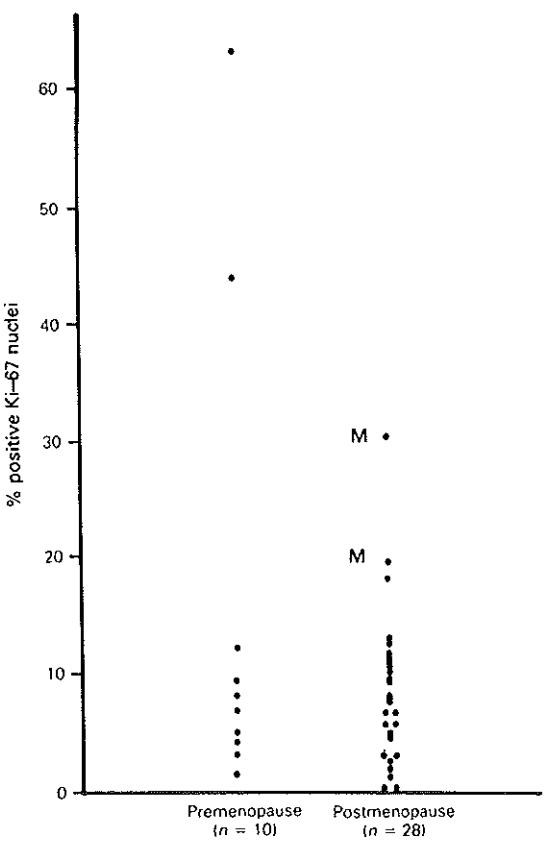

Figure 3. *Percentage of Ki-67 stained nuclei of the primary carcinomas versus menopausal status. M = medullary carcinoma.*

Our observation of significantly higher percentages of Ki- 67 labelled tumour cells in metastases compared to primary carcinomas may point to two possibilities: tumours with a higher proliferative activity may tend to a more aggressive biological behaviour, or the higher proliferative activity of metastasized tumour cells is caused by a more favourable environment. In support of the first possibility is the prospective study of Tubiana et al. (1984) which showed that the ^{3}H-thymidine labelling index is related to the probability of metastatic dissemination. In addition, Meyer et al (1983) showed that the ^{3}H-thymidine labelling index of primary breast tumours and their corresponding axillary metastases were not significantly different. Thus, it seems unlikely that the higher proliferative activity of metastasized tumours can be attributed to microenvironmental influences. Nevertheless, it would be interesting to compare the Ki-67 immunostaining results of the aspirates of the primary tumours and the metastases from the same patient. Further studies are indicated to demonstrate the prognostic relevance of the immunocytochemical assessment of Ki-67 determined proliferative fraction of tumour cells in FNA smears.

Acknowledgements:
We wish to express our gratitude to Miss C. Trappenburg and to ACM van Nispen for their technical assistance.

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CHAPTER 3

KI-67 STAINING IN HISTOLOGICAL SUBTYPES OF BREAST CARCINOMA AND FINE NEEDLE ASPIRATION SMEARS

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Coloured illustrations in this chapter have been placed at page 96

J Clin Pathol, **44**: 208-210, 1991

3.1 ABSTRACT

Thirty four cases of invasive breast carcinoma were analysed for heterogeneity of Ki-67 reactivity in a tumour, and proliferative activity in various histological subtypes was compared. The growth fractions determined in areas of central and peripheral tumour were the same. Mucinous and lobular carcinoma showed lower Ki-67 activity than ductal carcinomas. When ductal carcinomas were subdivided according to their dominant growth pattern, the carcinomas with a solid or comedo growth pattern showed the highest proliferative activity. These results largely confirm data from previous cell kinetic studies on the incorporation of radioactively labelled thymidine. A correlation between the growth fraction determined by Ki-67 in fine needle aspiration smears and cryostat sections of corresponding tumours was shown, implying that the immunostaining of cytological smears gives a reliable impression of the growth fraction of a tumour and may therefore be used in prospective studies.

3.2 INTRODUCTION

Cell kinetic studies have shown that there is an association between the thymidine labelling index and the biological activity of breast carcinomas (1,2). Monoclonal antibody Ki-67, which defines a nuclear antigen in proliferating cells, allows cells in the growth cycle to be detected without the need for time consuming methods such as external application of radioactively labelled nucleotides or mutagenic substances such as bromodeoxyuridine or iododeoxyuridine (3). This antibody is therefore used increasingly in studies on breast carcinoma to evaluate the growth fraction in relation to other known prognostic variables both in histological and cytological material (4-9).
As assessment of the growth fraction determined by Ki-67 immunostaining may be implicated in the prognosis of a patient it is essential to follow standardised procedures. Because it is not known to what extent discrepant data may arise due to heterogeneity of the distribution of proliferating cells in a tumour, we assessed the prevalence of Ki-67 positive cells in the peripheral and central areas of the tumour. Furthermore, we determined the association between different histological subtypes and the level of Ki-67 reactivity.
Because fine needle aspiration (FNA) smears are increasingly being used in the diagnosis of breast carcinoma and consequently for immunocytochemical analysis, we assessed the reliability of Ki-67 immunostaining of FNA smears, by comparing the results found in histological sections with those found in cytological smears of the same tumour.

3.3 METHODS

Thirty four cases of invasive breast carcinoma were evaluated. The tumours were classified according to the WHO classification (10). They included 21 ductal, six lobular, four mixed type (ductal/lobular) and three mucinous carcinomas. The 21 ductal carcinomas were consecutively classified according to the dominant growth pattern: two cribriform, three solid, four comedo, two mixed solid/comedo and 10 not otherwise specified (NOS). The comedo carcinoma was defined as a ductal in situ component in an otherwise invasive carcinoma.
A complete cross-section of resected tumour was snap frozen in liquid nitrogen. The areas of central and peripheral tumour were assessed separately.
Frozen tissue samples on glass slides coated with poly-L-lysine (11) and FNA smears on uncoated glass slides were air dried and subsequently fixed in acetone (for 10 minutes). After rinsing in phosphate buffered saline (PBS) Ki-67 (Dako, Denmark) was applied (dilution 1 in 5 in PBS with 0.02% gelatine, one hour). After rinsing with PBS a horseradish peroxidase conjugated polyclonal rabbit anti-mouse antibody (Dako, Denmark) was used as second step reagents for half an hour. After rinsing with PBS the reaction product was visualised using diaminobenzidine as substrate. After a final wash nuclear counterstaining was achieved by incubation in Mayer's haematoxylin for one minute. Positive and negative controls were included. Brown speckled staining of nuclei or nucleoli was regarded as a positive reaction. This positivity was semiquantitatively assessed in tissue sections by counting at least 300 cells in the areas with highest proliferative activity, similar to the procedure described for mitotic counting(12). In 26 cases this was done both in the central and peripheral areas of the tumour specimen.
When the invasive ductal carcinomas showed special growth patterns, these were counted separately. In FNA smears 500 cells were counted at random at a magnification of 1000 x under oil immersion and the percentage of positive nuclei was determined (8). The Ki-67 score of 14 FNA smears was compared with that of the frozen sections of corresponding tumours.

3.4 RESULTS

In 26 carcinomas the Ki-67 reactivity was assessed both in the central and peripheral areas of the same tumour. Although the central areas on average reached somewhat higher values than the more peripheral ones, a strong correlation (0.78) between the two areas was seen (fig 1).
Figure 2 shows the Ki-67 reactivity of the 34 carcinomas classified according to the WHO. The mucinous carcinomas contained a low percentage of Ki-67 stained nuclei, with a mean of 10%, whereas the ductal carcinomas showed

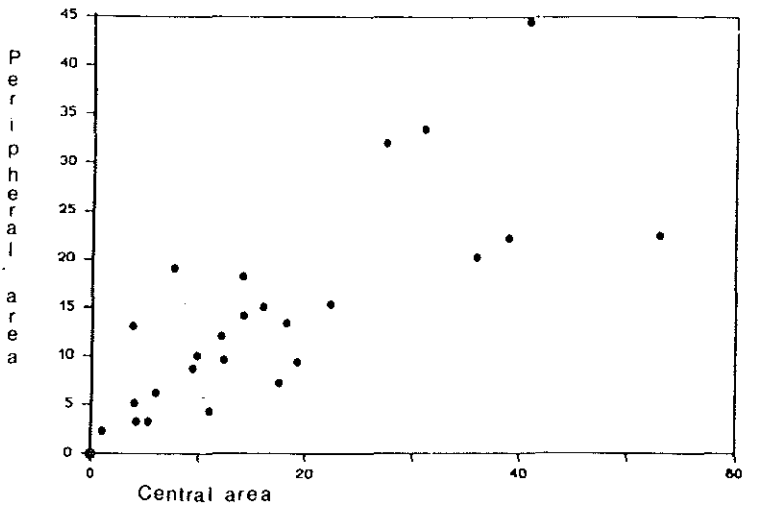

Figure 1. *The correlation between the growth fraction of central and peripheral areas in frozen cross-sections of breast carcinoma determined by Ki-67.*

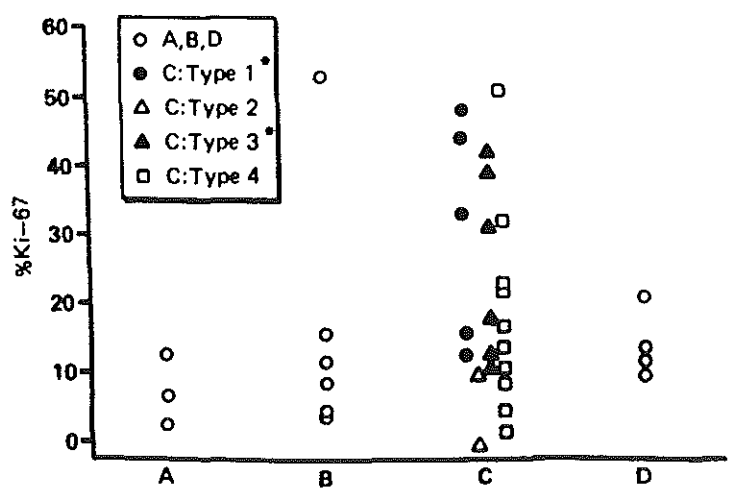

Figure 2. *The growth fraction of invasive carcinomas determined by Ki-67 and classified according to the WHO. (A) mucinous (n = 3); (B) lobular (n = 6); (C) ductal* (type I, solid n = 5; type 2, cribriform n = 2; type 3 comedo, n = 6;type 4 NOS, n = 10); and (D) mixed lobular/ductal (n = 4). * Two showed both a solid and comedo growth pattern.*

much higher values, with a mean of 23%. The lobular and mixed carcinomas showed intermediate values of 18% and 15%, respectively.
Figure 2C shows the Ki-67 determined growth fraction of the ductal carcinomas according to their dominant growth pattern. Comedo and solid growth patterns showed mean values of Ki-67 staining of 33% and 31%, respectively. The NOS carcinomas showed a mean value of 19%. Of the two ductal carcinomas with a dominant cribriform growth pattern, one showed no reactivity at all, while the other one showed Ki-67 staining of 10%. In 14 carcinomas the Ki-67 reactivity in FNA smears and frozen tissue sections of the same tumour correlated (0.71) (fig 3). In FNA smears on average lower Ki-67 values were obtained than sections of corresponding tumours. Nevertheless, a high score on FNA smears was usually associated with a high score on frozen sections (fig 4).

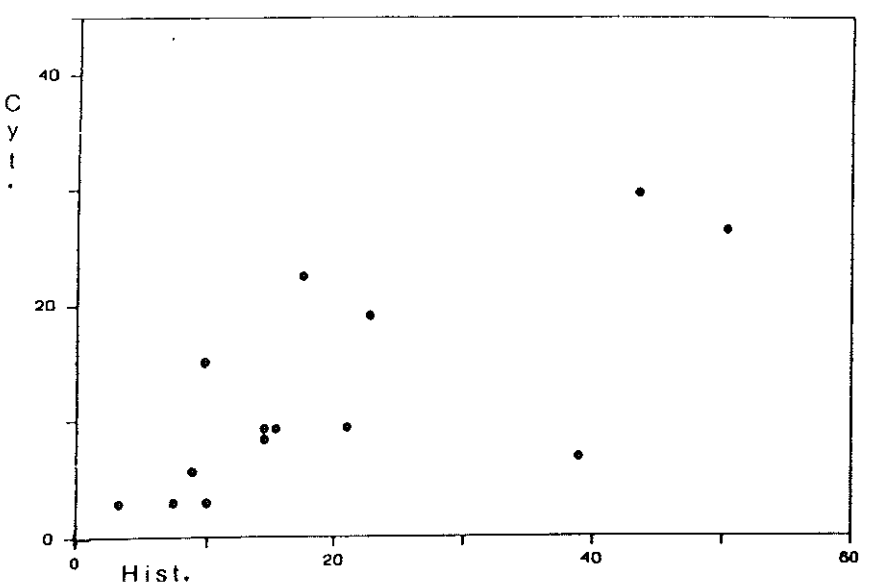

Figure 3. *Correlation between the Ki-67 determined growth fraction assessed in frozen sections and in fine needle aspiration smears of the same tumour (n =14)*

3.5 DISCUSSION

This study shows that similar levels of Ki-67 immunostaining are found both in central and peripheral areas of the tumour (fig 1). This result is at variance with the understanding that most cellular areas, and therefore the areas where active growth is most likely, are normally found at the periphery of a tumour (12,13). In our view this only holds true when the central area of the tumour is highly sclerotic due to poor vascularisation.

In this series the four histological types of breast carcinoma displayed different proliferative activity as defined by Ki-67, the ductal carcinomas showing the highest activity, the mucinous the lowest. This agrees with published findings (14-17), which state that ductal carcinomas are the most aggressive tumours compared with lobular and mucinous carcinomas, the latter two being slowly proliferating invasive carcinomas with a long survival. Similar results were obtained in the cell kinetic studies done by Meyer (1), where both lobular and mucinous breast carcinomas showed low thymidine labelling indices. Furthermore, Lelle et al compared the growth fraction, as determined by Ki-67, of ductal and lobular carcinomas and found lower values in the latter (6).

The various growth patterns of the invasive ductal carcinomas also show large differences in their proliferative activity (fig 2C). The two cribriform carcinomas showed the lowest levels of Ki-67 immuno-staining. Similarly, Meyer observed low thymidine labelling indices in the intraductal carcinomas with a cribriform growth pattern (18). The same carcinomas have an excellent prognosis (16,19). In contrast, the in situ comedo component in otherwise invasive ductal carcinomas showed high levels of Ki-67 immunostaining comparable with the high thymidine labelling indices in intraductal comedo carcinomas (18). Surprisingly, we found similar values both in comedo and solid carcinomas; Meyer observed low labelling indices in solid intraductal carcinomas comparable with the ones found in cribriform intraductal carcinomas.

Like Lelle et al we observed, on average, lower Ki-67 scores in FNA smears than in frozen sections (6). This may have been due to a different sampling and counting method. Using fine needle aspiration, cells are obtained from all parts of the tumour and counting is done randomly; frozen sections give information about only one part of the tumour and proliferative activity is determined by the areas with highest activity. Consequently, smears may give a better impression of the average proliferative activity of a tumour than histological sections.

Our results on breast cancer are in line with those found by Brown et al (20). These authors compared cryostat sections of non-Hodgkin's lymphomas with FNA smears of corresponding tumours and found an excellent correlation.

Early detection of breast cancer as a result of screening programmes will result in smaller amounts of malignant tissue available for additional techniques. The feasibility of immunocytochemical detection of proliferative activity on FNA smears shown here allows the prognostic markers on these small sized tumours to be assessed.

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CHAPTER 4

IMMUNOCYTOCHEMICAL DETECTION OF PROGNOSTIC MARKERS IN BREAST CANCER. TECHNICAL CONSIDERATIONS.

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Submitted

4.1 ABSTRACT

The purpose of this study was to establish a good technical procedure for immunocytochemical (IC) staining of prognostic markers in breast cancer specimens. The influence of various preparation, fixation and storage methods on ER, P53 and Ki-67 IC staining was assessed, using cells of two breast cancer cell lines T47D (ER/P53 +) and ZR-75-ER (ER/+, P53-). In addition we searched for a suitable transport medium. Depending on the technical procedure, great variations in expression of the tested antigens were found. Cytospins fixed and stored according to the Abbott method gave the best results. Histocon appeared to be the medium of choice. A good concordance of IC and immunohistochemical (IH) results was found when the adopted method was tested on material of 10 breast cancers. This study underlines the importance of quality controlled standardization of cell processing, fixation and storage of FNA aspirates in order to obtain reproducible and consistent IC results.

4.2 INTRODUCTION

The use of immunocytochemistry in the examination of fine needle aspirates (FNA's) of breast tumours has enhanced the diagnostic possibilities especially with regard to the assessment of prognostic markers. Up till now most prognostic markers, like estrogen receptor (ER,), Ki-67 and P53, also known as TP53, are being biochemically and/or immunohistochemically assessed on tissue specimens obtained at surgery. However, early detection of breast cancer due to breast cancer screening programmes has led to reduction of the size of breast carcinomas (1) and therefore to a reduction of the amount of tissue available for diagnostic and prognostic information. In addition, new treatment modalities, like neo-adjuvant chemotherapy for T4 tumours, necessitate the maximum yield of information from cytological specimens. Good correlations between biochemical, histological and cytological assays have been reported (2,3,4). Nevertheless discrepancies, due to heterogeneity of the tumour and to methodologically differences, may exist. Here we describe a study to establish a suitable technical procedure for immunocytochemistry (IC) of FNA material especially with regard to nuclear antigens. We compared the influence of various preparation, fixation and storage methods (study A). We made an assessment of the influence of transport media on quality of immunostaining (study B). Finally we compared IC staining of cell material and frozen tissue samples of corresponding breast cancers, using the cell material processing technique and transport medium of choice (study C).

4.3 MATERIAL AND METHODS

Study A: **The influence of various preparation, fixation and storage methods on IC staining results.**
Cell-lines T47D (ER+, P53+) and ZR-75-ER (ER+, PR-) were used for this study. Cells were harvested, pelleted by centrifugation and resuspended in RPMI 1640 medium.

I. *Cell preparation*
Two methods of cell preparation were compared:
1. A standardized method used to prepare serous effusions as described before (5). In short: Cell suspensions were diluted with 1% BSA/PBS, pH 7.4. After centrifugation (5 min at 2000 rpm) and decanting, the pellet was lysed and fixed at 4° C for 10 minutes by adding 2.5 ml lysis buffer containing isotonic ammonium chloride (4.5 g NH4CL Merck, Germany), 0.5g KHCO3 (Merck)) and 0.0186 g EDTA (Merck) dissolved in 500 ml aqua bidest, Ph 7.4; and 2.5 ml 4% paraformaldehyde (PFA) Merck). The cells were rinsed in PBS, followed by centrifugation and decanting. Then the pellet was resuspended and diluted in BSA 1% in PBS and adjusted to a cell concentration of 1x 10^6 cell/ml using a Bürker cell counting chamber (Optik Labor, Bad Homburg, Germany) to obtain reproducible specimens with optimal cell density. Finally, cytocentrifuge samples were made by centrifugation at 700 rpm for five minutes using a Shandon cytospin 2 (Astmoor, Runcorn Cheshire, England). The processed slides were air dried for a maximum period of five minutes.
2. The same as protocol 1, but without lysing and fixation of the pellet.

II. *Fixation and storage*
Different procedures were compared:
a. No fixation of processed slides. The slides were kept at room temperature (RT) overnight.
b. Fixation of cytospins with 4% paraformaldehyde followed by methanol/ acetone at -20°C (Abbott method).
c. Fixation by methanol/acetone only .
d. Cytospins directly stored at -80°and fixed afterwards according to either method b or c.
(see table I for exact procedures)

Table I. *Fixation and storage protocols (study A II)*

Method	Fixation/storage	Temp	Time
a	air-dried	RT	overnight
b	Paraformaldehyde4%	RT	10 minutes
	PBS 3X		10 minutes
	Methanol	- 20° C	3 minutes
	Acetone	- 20° C	1 minute
	Storage medium	- 20° C	
c	methanol	- 20° C	3 minutes
	acetone	- 20° C	1 minute
	storage medium	- 20° C	
d	directly stored at	- 80° C	
	direct fixation upon thawing followed by method b or c		

III. *Immunocytochemistry*

The non-fixed slides were fixed in acetone for 10 minutes prior to incubations and together with the prefixed cytospins rinsed in phosphate- buffered saline (PBS), pH 7.4, followed by pre-incubation using 1% BSA diluted in PBS for 10 minutes. The antibodies raised against ER (1D5, Dako, Glostrup, Denmark), Ki-67 (MIB-1, Immunotech/ Coulter, Westbrook, Maine, USA) and P53 (1801, Oncoscience, Cambridge, Mass, USA) were used for this study and diluted in 1% BSA in PBS. Incubation was carried out for 60 minutes at RT. The APAAP technique (Dako, Glostrup, Denmark, working dilution 1:20) was applied after incubation with the linking antibody (Ab)(working dilution 1:20 in 5% NHS in PBS) for visualising the primary antibody. All immunostained slides were counterstained with Mayer's haematoxylin (Klinipath, Duiven, Holland) for 1 min. Slides of cell-lines were included as positive controls; as negative controls the primary antibody was replaced with 1% BSA diluted in PBS in each run.

Study B

The influence of transport media on IC results.

The following media were compared:

1. RPMI-1640 containing phenol red, 21 mM HEPES, 10 mM NaHCO3, 4 mM glutamine, 100 U/ml streptomycin, 100 mμ/ml penicillin, 45 μg/ml gentamycin, 10 μg/ml porcine insulin, 5 μg/ml insulin, 2.5 μg/ml estradiol and 10% bovine calf serum (heat-inactivated).

2. RPMI 1640 supplemented with 10% Foetal calf serum (FCS) heat inactivated, 20.000 IE penicillin/streptomycin/ L, 20.000 IE Heparin sulphate/L, L-glutamine 300mg/l, Gibco BRL (Breda, the Netherlands).
3. PBS/BSA 1%, pH 7.4%
4. Histocon (Polysciences, Warrington USA)
Cell material of histologically proven breast carcinomas was obtained by scraping a scalpel blade firmly against the carcinoma and by directly depositing the material in the media. Cell specimens were prepared according to the best method of study A (see results). The influence of the various media on IC staining was determined.

Study C

Comparison of immunostaining of cytological and corresponding histological specimens.
Frozen tissue samples and cell scrapings of ten histologically proven breast carcinomas were obtained. Cryostat sections (4 μm thick) were fixed in formalin 4% for 10 min. Cell material was suspended in the chosen transport medium of study B and cytospins were prepared according to the adopted method of study A (see results). Immunostaining of cytospins and cryostat sections was performed using the aforementioned technique, using the antibodies anti-ER, anti-P53 and Ki-67.

Quantification
Frozen material: The percentage of IH assessed ER, and P53 stained tumour cells was calculated by scoring the number of positive cells semiquantitatively in a total of 300 cells in three different areas of the tumour section (6). The Ki-67 score was assessed by counting 300 cells in the areas with the highest activity as described previously (6,7).
Cell material: The ER, Ki-67 and p53 score was assessed by counting at random 500 cells as described before (7).
The IC and IH scores were compared.

4.4 RESULTS

Study A

Material processing and fixation:
Variations in staining intensity between the two preparation methods were seen. In general cell preparation method A.I.1 (prefixation of the cell pellet) showed little or no nuclear staining, in contrast to the strong staining of method A.I.2 (fixation of cytospins) (fig 1 A, B).

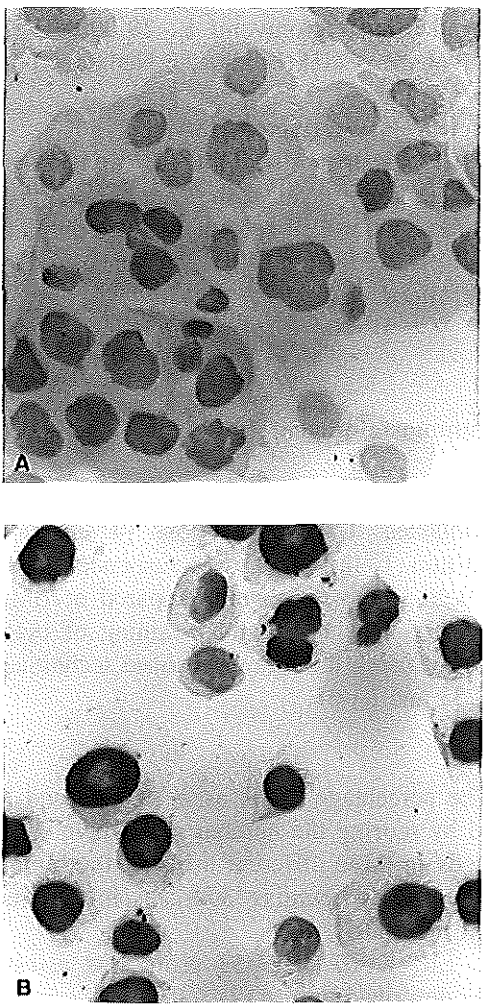

Figure 1. *Influence of cell processing on P53 immunostaining.*
A: Prefixation of suspension with 4% paraformaldehyde followed by fixation and storage of the air-dried cytospin according to the Abbott method
B: Fixation and storage of air-dried cytospin according to the Abbott method.

A rapid loss of antigenicity was seen when the cytospins were kept at RT for longer than five minutes. Direct freezing of the cytospins before fixation followed by direct fixation upon thawing according to the various tested methods gave inconsistent results. Cell processing method A.I.2, followed by fixation and storage of the cytospins according to the Abbott protocol gave the best results (demonstrated in fig 2A, B and Table II).

Table II. *Immunostaining of cell specimens of two celllines using various fixatives, prepared according to preparation method A.I.2.*

	ER		Ki-67		P53	
	A	B	A	B	A	B
fixation/storage						
a	-	+/-	-	+/-	-	+/-
b	++	++	++	++	-	+++
c	+/-	+	+/-	+	-	+
d+b*		+/-		+/-		+/-
d+c*		-		-		-

A: Cell-line ZR-75-ER (ER +, P53 -)
B: Cell-line T47D (ER +, P53 +)
method a: air-dried/RT overnight
method b: 4% paraformaldehyde+ methanol/acetone (Abbott method)
method c: methanol/acetone
method d: -80°C
** only tested with cell-line T47D*

Table III
A comparison of IC results of cell material of a histologically proven ER/P53 + breast cancer, suspended in different transport media and prepared according to the adopted method.

	medium 1	medium 2	1% BSA	Histocon
ER	+/-	+/-	++	++
P 53	-	-	+	+
Ki-67	+/-	-	+	+

Study B

Morphology and immunoreactivity of cells was best preserved when transported in either Histocon or 1% BSA (Table III).

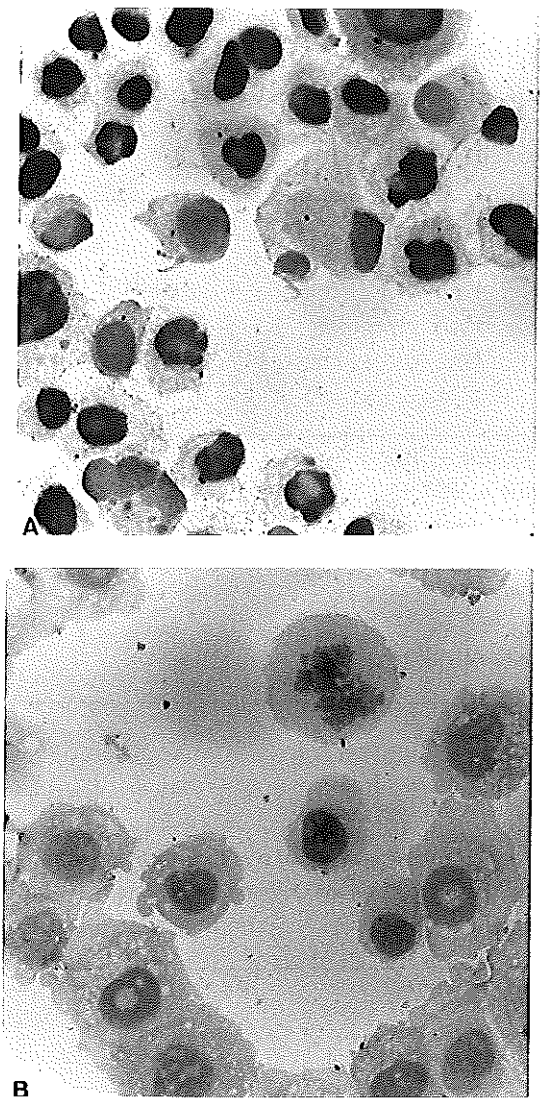

Figure 2.:*Influence of different storage and fixation methods on P53 immunostaining*
A: Fixation and storage of air-dried cytospins according to the Abbott method
B: Storage at -80°C, followed by fixation with paraformaldehyde 4%

Study C

The ten carcinomas demonstrated a good correlation between the IH staining of frozen material and IC staining of corresponding cell material, suspended in Histocon and processed according to method A.I.2 and fixed following the Abbott method (fig 3A, B and Table IV).

Table IV. *Percentages of positive IH/IC stained cells in frozen tissue samples and corresponding cytospins of ten breast cancers.*

	ER		P53		Ki-67	
	%		%		%	
sample	hist	cyt	histo	cyt	histo	cyt
1	50	<10	<10	50	50	
2	100	100	100	100	50	40
3	0	0	0	0	40	50
4	60	60	<10	<10	40	40
5	0	0	90	90	80	70
6	80	70	<10	0	30	20
7	30	40	0	<10	0	<10
8	50	30	0	0	40	20
9	80	80	30	30	10	<10
10	<10	<10	0	0	10	<10
40						

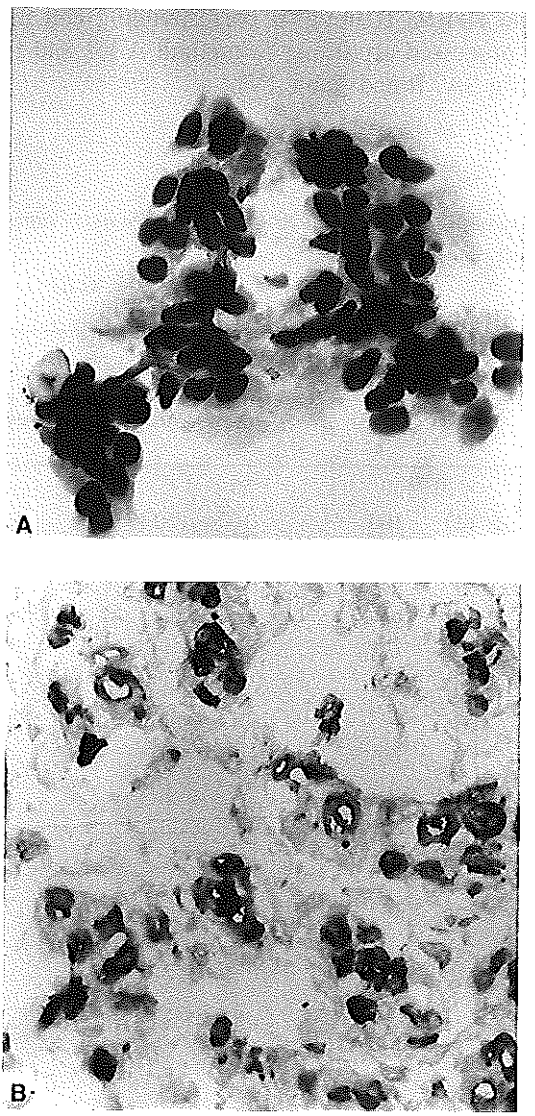

Figure 3.: *Comparison of P53 immunostaining of cytogical and corresponding histological specimens of a breast carcinoma. The cytospins are prepared, fixed and stored according to the chosen method.*

A: Cytospin

B: Frozen tissue sample

4.5 DISCUSSION

In the future IH examination of material obtained at surgery may partly be replaced by IC examination of needle aspirates, and therefore methods of investigating prognostic indicators on cytological specimens will expand. In an earlier study (8) we demonstrated that with regard to serous effusions, the most consistent immunostaining was found when lysis and prefixation of the material was applied (see method AI.1). In this study however, cell processing method AI.1 gave inferior IC results when compared to method AI.2 (see fig 1A,B). This may well be explained by the fact that nuclear antigens, like we studied, require different methods of preparation in comparison to cytoplasmic and membrane-bound antigens (9,10), in general identified in serous effusions. Therefore, prefixation of the pellet in method A.I.1, excellent for IC staining of effusions, gave poor results when used for staining of nuclear antigens.

In keeping with other reports (11,12), we observed a rapid detoriation of the immunoreactivity of the nuclear antigens after storage of air-dried slides at RT for more than five minutes. In contrast to these findings, Suthipintawong et al (13) who kept the air-dried smears at RT for up to one week did not observe any detoriation of IC staining. Here again the localisation of the studied antigens, nuclear antigens versus cytoplasmic/membrane-bound antigens respectively, may well explain this discrepancy.

In the experience of Dowell and Burton (11,12) staining results were not affected if the specimens were stored unfixed at -70°C and fixed immediately upon thawing. Applying this method, we observed negative/weak staining (Fig. 2A, B). A possible explanation for these negative results may be that due to our working situation (freezer located in other room) the air-dried cytospins were not directly frozen, nor fixed immediately upon thawing, resulting in loss of antigenicity.

Cell scrapings of breast cancers suspended in the RPMI culture media showed inferior IC results compared to IH of frozen tissue samples. For this reason we evaluated the influence of various media as well and found that preservation of cell morphology and immunoreactivity was best when suspended in either 1% BSA or Histocon. In agreement with Burton et al (12), we recommend immediate preparation (within 1 hour) of the cells to avoid declination of antigenicity. Since 1% BSA is easily contaminated by micro-organisms and fungi the use of this medium is not recommended.

By showing the influences of different preparatory, fixation and storage methods we have emphasised the need of quality controlled, standardized methods of material processing and fixation of FNA material for IC staining especially with regard to nuclear antigens. Taking these technical problems in consideration, we find IC of FNA material a reliable technique for the assessment of prognostic markers in breast carcinomas.

Acknowledgements: We thank the department of molecular biology and biochemistry for the use of their cell-lines and Professor Th. H Van der Kwast for his critical comments concerning the manuscript.

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CHAPTER 5

IMMUNOHISTOCHEMICAL DETERMINATION OF ANDROGEN RECEPTORS IN RELATION TO ESTROGEN AND PROGESTERONE RECEPTORS IN FEMALE BREAST CANCER.

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5.1 SUMMARY

The expression of estrogen (ER), progesterone (PR) and androgen (AR) receptors in female breast cancer was investigated by immunohistochemistry on snap-frozen tissue specimens of a series of 100 breast cancers. For the detection of the AR we used a recently developed mouse monoclonal antibody specific for the N-terminal domain of the human AR. Expression of AR was compared with that of ER and PR as well as with tumour grade and age. Of the breast cancers investigated, 76% were AR-positive. This high percentage corresponds well with previous data on AR expression in breast cancer determined with ligand-binding assays. In 53% of the tumours AR, ER and PR were present, while 9% of the tumours were positive for AR and negative for ER and PR. In 13% of the tumours no ER, PR or AR expression was seen; these were all grade-III tumours. A positive correlation was found between age and ER expression, but no correlation was seen between age and PR or AR. Future studies should establish the prognostic value of the combination ER, PR and AR determinations on female breast cancer with regard to biological behaviour and response rate to hormonal therapy.

5.2 INTRODUCTION.

The assessment of estrogen (ER) and progesterone (PR) receptors in breast cancers is used as a prognostic parameter and as an indicator of likely response to hormonal therapy (Thorpe, 1988). Less is known about the role and clinical significance of androgen receptors (AR) in the biological behaviour of breast cancer. A direct growth-inhibitory effect of androgens on 2 estrogen-dependent human breast cancer cell lines has been demonstrated in vitro (Poulin et al, 1988; Hackenberg et al, 1991), as well as in vivo on carcinogen-induced breast tumours in a rat model (Dauvois et al, 1989). Lea et al (1989) showed that AR is the sex steroid hormone receptor most frequently found in both primary and metastatic human breast cancers. The expression of AR appears to be best preserved during the process of metastasis. Moreover, Teulings et al (1980) and Bryan et al (1984) found that data on AR expression added significantly to the prediction of survival and response to hormonal therapy.

Up to now, AR determination has relied on ligand-binding assays. However, these methods are vulnerable due to the thermolability of the receptor, especially in combination with the action of proteolytic enzymes. Percentages of AR-positive breast cancers reported in the literature vary from 35% (Miller et al., 1990) to 84% (Lea et al., 1989). Moreover, biochemical assays do not provide any information about cellular distribution.

Recently, a monoclonal antibody (MAb) specific for the human AR has been developed. This MAb, designated F39.4, is specific for a unique epitope in the N-terminal domain of the human AR molecule (Zegers et al., 1991). Using

immunohistochemistry with this MAb, we visualized differences in percentage and intensity of AR expression in a series of 100 breast cancers. We also evaluated the clinico-pathological value of immunohistochemical AR determinations by correlating expression of AR with ER, PR, histological grading and age.

5.3 MATERIAL AND METHODS.

Patient material
The patient material consisted of tissue samples from 100 primary breast carcinomas diagnosed in the period 1989-1991. The tumours were graded according to the Bloom and Richardson system with minor modifications as described by Page and Anderson (1987). Patients' age at the time of primary surgery was recorded.

Immunohistochemistry.
Representative tissue samples were snap-frozen in liquid nitrogen and stored at -70°C until use. Cryostat sections, 4 μm thick were air-dried and subsequently fixed in formaline (4%) diluted in phosphate buffered saline (PBS) for 10 min. After rinsing in PBS, they were dehydrated in chilled methanol and acetone (-20 °C).

Immunostaining for ER and PR. After rinsing in PBS, sections were pre-incubated with normal goat serum (diluted 1:10) for 15 min. Briefly, incubation with the primary MAb was performed overnight at 4°C, using kits commercially obtained from Abbott (Abbott Park, IL). The peroxidase anti-peroxidase complex method was used employing goat anti-rat immunoglobulin (30 min) as bridging antibody (Dakopatts, Copenhagen, Denmark). After rinsing with PBS, the reaction product was visualized using diamino-benzidine- H_2O_2 as substrate.

Immunostaining for AR. After rinsing in PBS, sections were pre-incubated consecutively with avidin (30 minutes), biotin (30 minutes) and normal rabbit serum (5 minutes) all used as blocking agents (Vector, Burlingame, CA). Between each blocking step sections were rinsed in PBS (2 x 5 min). Incubation with MAb F39.4 (ascites, diluted 1:10,000) was done for 24 hr at 4°C. After rinsing with PBS, reactivity was visualized by a 30-min incubation with biotinylated rabbit anti-mouse immunoglobulin diluted 1:100 in PBS containing 4% normal human serum, followed by incubation with peroxidase-conjugated streptavidine (dilution 1:50, 30 minutes) (Dakopatts) as linking agent and diamino-benzidine-H_2O_2 as substrate. For all antibodies, after a final wash, nuclear counterstaining was achieved by incubation in Mayer's haematoxylin for 1 minute. Control sections consisted of known positive and negative specimens identified by ligand-binding assay. In addition, in one negative control section

the first-layer antibody was changed to 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 on a total of 300 cells in 3 different areas of the tumour (PP-score). The intensity of staining of each cell was estimated visually on an arbitrary scale of 0 (no staining) to 4 (very intense staining). The average staining intensity (E-score) was defined as

$$\sum_{i=0}^{i=4} iP(i) \text{ with summation over } i=0\ldots\ldots, 4$$

in which P(i)= % stained tumour cells with intensity score i (Scheres et al, 1988).

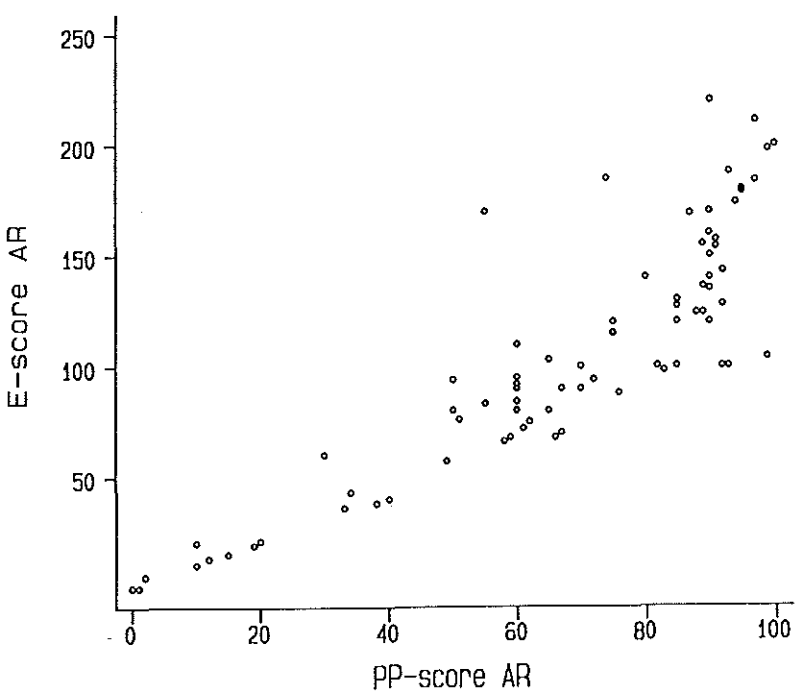

Figure 1. *Correlation of the E-score and PP-score for AR determined in 100 breast carcinomas (see text).*

For ER, PR and AR we demonstrated a close correlation (Spearman Rank correlation >0.93) between the E-score and PP-score. In Figure 1 this correlation is shown for AR. Given the strong correlation between both measures, the one that is easiest to score (the PP-score) was used for further analysis. Arbitrarily, tumours were considered to be receptor-negative if less than 10% of tumour cells were stained.

5.4 RESULTS

AR positivity was found in 76% of the tumours (Table I). Nine percent of the tumours expressed AR as the only sex steroid receptor. Although most of these AR-positive tumours displayed a selective nuclear staining reaction of the tumour cells (Fig. 2), in some tumours some cytoplasmic staining of tumour cells was present. Similarly, ER and PR were selectively present in the nuclei. Stromal cells did not react with any of the antibodies employed. Most (82%) of the AR-positive tumours showed a high percentage ($>$ 50%) of immunostained cells. Figure 3 shows the distributions of the PP-score of ER, PR and AR of all individual tumours tested. In 53% of the tumours, all 3 sex steroid receptors were present. In 13 breast cancers none of the 3 receptors was detectable. The association between AR, ER and PR status (Table I) is statistically highly significant. Among AR^- tumours the majority was also ER^- and PR^-, whereas among AR^+ tumours the majority was ER^+, PR^+. This association was also reflected in the mean PP-scores for ER and PR which were lower among AR^- than among AR^+ tumours (Fig.4). The level of AR positivity showed no further association with ER or PR. The mean PP-scores for ER and PR for tumours with a high AR level (PP-score AR $>$ 50) were no different from the mean scores for tumours with a moderately positive AR level (10 $<$ PP-score AR $<$ 50) (Fig.4). On average, the percentage of PR-positive tumour cells was lower than the percentage of AR^+ or ER^+ tumour cells.

Table I. *Expression of AR, ER and PR in breast cancer*

	ER+ PR+	ER+ PR-	ER- PR+	ER- PR-
AR +	53	12	2	9
AR -	5	5	1	13

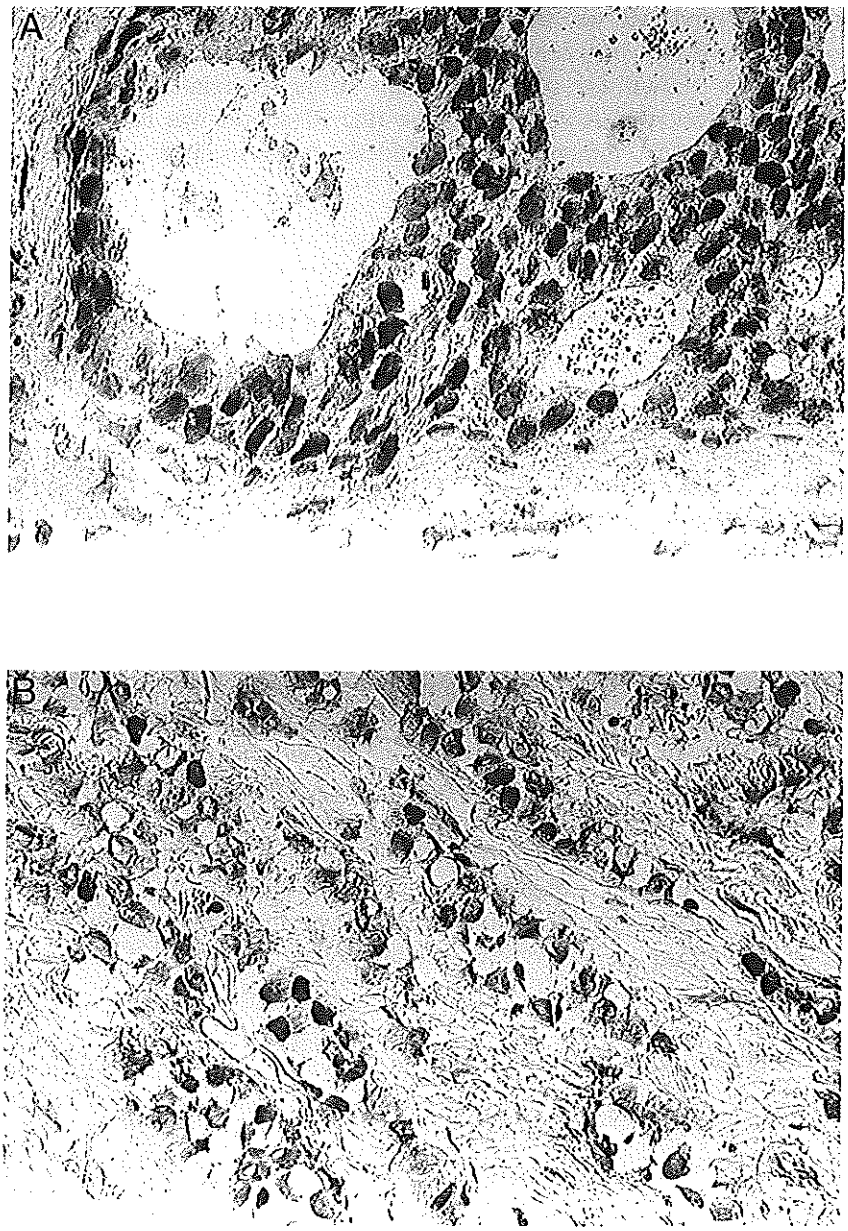

Figure 2. *Direct visualization of heterogeneity of AR expression by immunostaining with MAb F39.4 (Haematoxylin counterstaining); (a): Intraductal breast carcinoma with cribriform growth pattern. Selective nuclear staining of the majority of tumour cells. (b) Infiltrating ductal carcinoma of the breast. Dark-stained nuclei are immunolabeled for AR.*

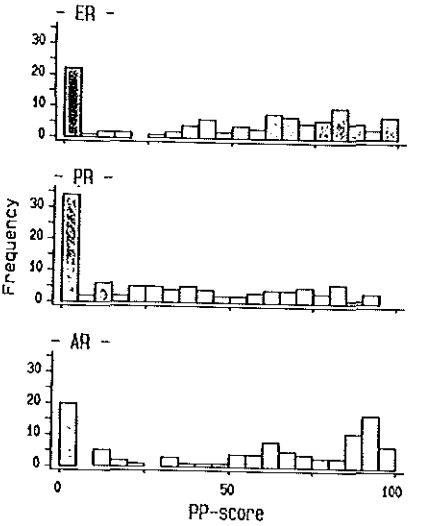

Figure 3. *Distribution of PP-score for ER, PR and AR of 100 cases of breast carcinoma.*

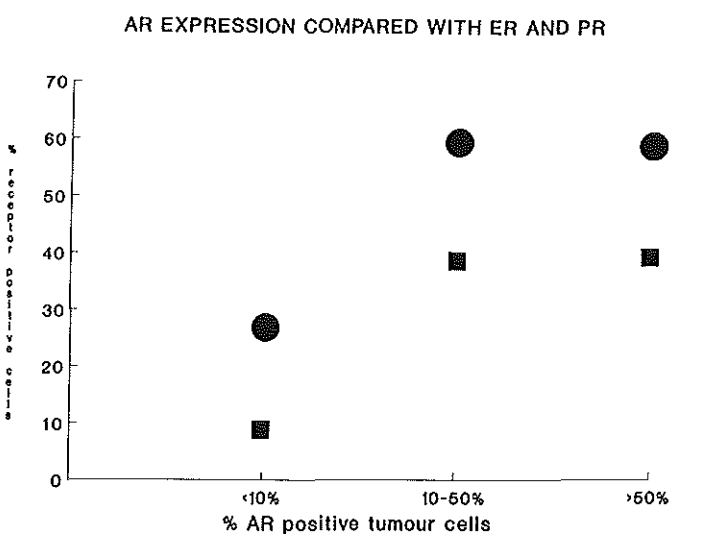

Figure 4. *Expression of AR compared with that of ER and PR. Average percentages of ER⁺ (●) and PR⁺ (■) tumour cells in breast carcinomas with less than 10%, 10-50% and over 50% of AR⁺ tumour cells.*

Correlation between grading and sex steroid receptors.
A clear correlation between grading and expression of the 3 different receptors was observed (Table II). Grades I and II showed a higher percentage of receptor-positive tumours than grade-III tumours. The 13 tumours in which none of the 3 receptors were detectable were all grade-III tumours.

Table II. *Relationship of tumour grade and sex steroid receptor expression*

Receptor status	Tumor grade			
	Grade I	Grade II	Grade III	*p*-value[1]
Total	18	46	36	
ER+ (%)	100	89	44	0.000
PR+ (%)	67	76	39	0.002
AR+ (%)	78	91	56	0.001

[1]Chi-square test

Table III. *Age and steroid receptor profile*

	Age				
	Mean	(SD)	<50yr	>50yr	*p*-value[1]
ER-	50	(10)	13	12	0.05
+	58	(12)	23	52	
PR-	55	(12)	13	26	0.66
+	57	(12)	23	38	
AR-	54	(12)	8	16	0.76
+	57	(12)	28	48	

[1]Chi-square test

Relationship of age with sex steroid receptors
Table III expresses the relation between each receptor and age. For all 3 receptors, positivity occurred more frequently among older patients, but only for ER was the association statistically significant ($p = 0.05$).

5.5 DISCUSSION.

The recent production of a MAb, designated F39.4 specific for the human AR enabled us to employ a simple immunohistochemical approach for direct light-microscopic visualization of the AR in normal (Ruizeveld de Winter et al., 1991) and neoplastic breast tissues (this study). We have previously shown that this MAb is highly specific for human AR as it shows no cross-reactivity with ER, PR or glucocorticoid receptor (Zegers et al., 1991). Immunoreactivity of various human tissues with this antibody was generally consistent with earlier biochemical and autoradiographical data (Ruizeveld de Winter et al., 1991).
By analogy with the results obtained with immunostaining for ER and PR, we found that AR is predominantly located in the nuclei of the immunopositive cells. As in earlier studies with ligand-binding techniques, we found in general a good correlation between AR, ER and PR expression, i.e. most AR-positive tumours also contained ER^+ and PR^+ tumour cells. However, the percentage of PR^+ tumour cells within a given tumour on average was lower than that of ER or AR (Fig. 4). This latter observation stands in contrast to the findings of Lea et al. (1989) who found in breast cancer cytosol preparations a lower concentration of AR than of PR or ER. In their study, a labelled ligand-binding assay was used and therefore this discrepancy with our results could be due to the difference in methodology. In agreement with the findings of Lea et al. (1989) and those of Miller et al. (1985), we observed that a high percentage of breast carcinomas contained AR^+ tumour cells (76%); 9% contained AR as their sole sex steroid receptor.
Clinical data have shown the benefits of androgen therapy in metastatic breast cancer (Manni et al., 1981; Tormey et al., 1983; Ingle et al., 1988). Teulings et al. (1980) also described a favourable response to progestin in a group of estrogen-responsive breast cancers and demonstrated that within this group the actual AR levels determined the response. Bryan et al (1984) as well as Langer et al (1990) confirmed that AR data add significantly to the prediction of survival and response to endocrine therapy. As progestin as well as progesterone can be metabolized into an androgen, these steroids may exert their effect by interaction with AR. This mechanism of AR-mediated inhibition of tumour growth may also explain the beneficial effect of high doses of progestin observed in PR^- breast tumours (Teulings et al., 1980). In our study, the AR^+ PR^- subset of breast carcinomas comprised 21% of the cases. Distinction of this AR^+PR^- subgroup of breast cancers might lead to a more specific type of endocrine therapy, including administration of androgens.
The in vitro findings of Poulin et al (1988), and Hackenberg et al (1991) on the human breast-cancer cell lines ZR-75-1 and MFM-233, respectively, support the notion of an AR-mediated growth inhibition of human breast cancer. In both cell lines the anti-proliferative effect of androgens could be completely reversed by the anti-androgen hydroxy-flutamide. Similarly, an in vivo inhibitory effect

of androgens on DMBA-induced mammary tumour growth in the rat was noted. This anti-proliferative effect of androgens could also be completely prevented by simultaneous treatment with hydroxy-flutamide (Dauvois et al., 1989). In contrast, addition of high doses (1 μM) of androgens induces a growth stimulation in the human breast carcinoma cell lines MCF-7 and EFM-19 (Hackenberg et al., 1988). To add to the complexity of the picture, it was also found that these high doses of androgen led to an increase in PR content and that in MCF-7 cells the androgen dihydrotestosterone was rapidly converted to androstane-3ß,17ß-diol, which may exert an estrogenic effect in this cell line (Zava and McGuire, 1978).

Not only do androgens act by modulating tumour growth, but also the production of peptidases by benign and malignant breast lesions is dependent on androgens. Thus, gross cystic disease fluid protein-15 and 24 production by human breast cancer cells is stimulated by androgens (Dauvois et al., 1990; Simard et al., 1990).

Earlier studies demonstrated that the presence of ER and PR is related to histological grading of breast cancers (Thorpe 1988). Indeed, in our series an opposite relationship between tumour grade and expression of ER and PR was present. The same trend also holds true for AR expression. Moreover, 13 of the 100 breast carcinomas were negative for all 3 sex steroid receptors, all of which represented grade-III carcinomas. On the other hand, about half of the grade-III breast cancers showed expression of one or more receptors. Follow-up studies should establish whether determination of the 3 sex steroid receptors is a better independent predictor of survival from breast cancer than grading.

Although with increasing age the percentage of ER^+ breast tumours was increased ($p=0.05$, Table III) this relationship was not present for PR or AR. This finding is in agreement with the data from Lea et al. (1989), Bryan et al. (1984) and Miller et al. (1985).

In conclusion, the possibility of a simple immunohistochemical detection of AR, ER and PR in breast cancers can now be used to distinguish between subsets of breast cancers which may respond to estrogens or androgens in a specific way.

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CHAPTER 6

THE CLINICAL SIGNIFICANCE OF ANDROGEN RECEPTORS IN BREAST CANCER AND THEIR RELATION TO HISTOLOGICAL AND CELL BIOLOGICAL PARAMETERS.

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Eur J Cancer, **32:** 1560-1565, 1996

6.1 ABSTRACT

To analyze 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. Estrogen (ER) and progesterone receptor (PR) levels were determined in cytosol preparations using enzyme immunoassays and in cryostat sections by immunohistochemistry. AR and Ki-67 levels were only determined immunohistochemically. Data were analyzed by uni- and multivariate models. 94/153 (61%) breast carcinomas were ER+ PR+ AR+, while 14 cases were only positive for AR.
All steroid receptor negative cases (n=17) were grade III cancers and 14 (76%) of these cases demonstrated high Ki-67 values suggestive of a 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.

6.2 INTRODUCTION

The importance of the histological subclassification of breast cancer as one of the indicators for tumour aggressiveness is well recognized. 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 estrogen (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 percent 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-estrogen therapy has therapeutic advantages over anti-estrogen 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 analyses. In addition, we correlated ER and PR detected immunohistochemically with ER and PR levels measured by a biochemical method based on the enzymeimmuno assay (EIA).

6.3 PATIENTS, MATERIALS 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 100,000 *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, 4μm thick, were air-dried and fixed in acetone for 10 min, after which the indirect immunoperoxidase technique was used for visualizing 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 the 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 summarized 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.

6.4 RESULTS

Patient and tumour characteristics.
Patient and tumour characteristics are summarized in Table 1. The histological subtypes included 114 ductal, 14 lobular, five colloid, two tubular, one cribriform, 14 mixed, two medullary carcinomas and one metaplastic carcinoma. Of the premenopausal women with positive lymph nodes (n=36), 27 received adjuvant chemotherapy, one hormonal therapy and eight 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 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).

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	No	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, estrogen 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)

Table3. *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	28
Special types					
colloid	5	100	100	100	0
tubular	2	100	100	100	0
cribriform	1	100	100	100	0
Others					
medullary	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

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

A variable steroid receptor expression and Ki-67 score was found in the group of NOS ductal carcinomas (Table 3). Of the 17 receptor-negative tumours, 14 were NOS ductal carcinomas. Strikingly, 13 of these 14 cases expressed high ($\geq$ 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.

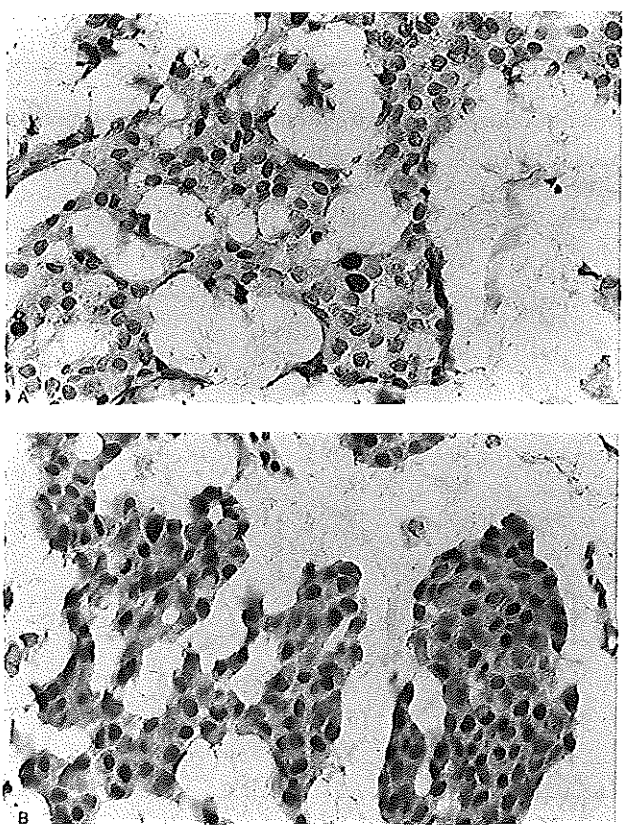

Figure 1. *Comparison of Ki-67 and androgen receptor (AR) immunostaining of frozen sections of the same colloid breast carcinoma (magnification 40x). (a) Only few tumour cells (darker nuclei) show positive staining of Ki-67. (b) Positive nuclear staining (darker nuclei) for AR is present in the majority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

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 absence of steroid receptor expression, and the metaplastic carcinoma combined receptor negativity with 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).

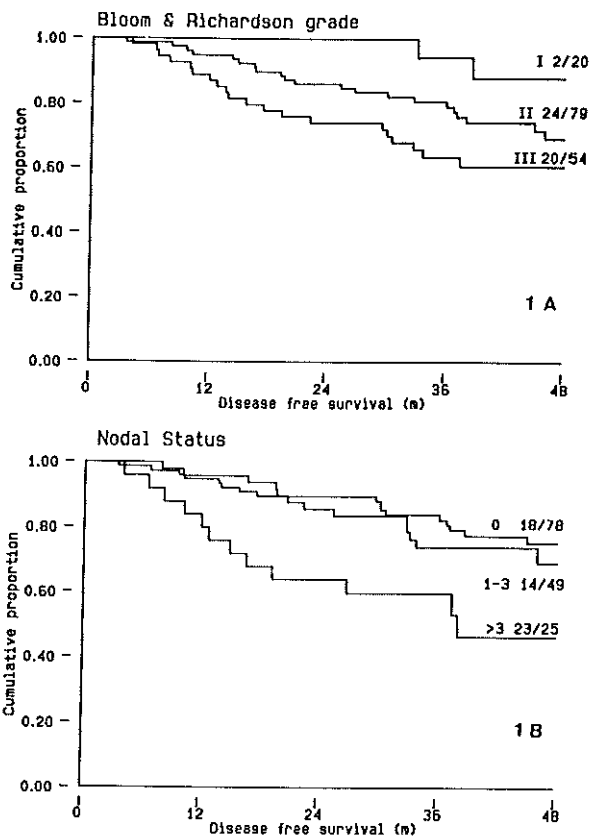

Figure 2. *(a) Disease-free survival curve stratified according to grade (P=0.01).*
(b) Disease-free survival curve stratified according to lymphnode status (P=0.02).

Table 4. Cox Univariate- and Multivariate Analysis in 147[a] Primary Breast Cancer Patients

	Univariate			Multivariate (initial model)			Multivariate (final model)		
variable	HR[b]	95% CI[c]	P	HR	95% CI	P	HR	95% CI	P
pT[d]	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[e]	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[f]	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[f]	0.61	0.34 - 1.08	0.091	1.85	0.71 - 4.80	0.205	-	-	-
IH-AR[f]	0.50	0.25 - 0.98	0.043	0.65	0.27 - 1.57	0.337	-	-	-
BR[d]	1.79	1.12 - 2.86	0.014	1.20	0.66 - 2.19	0.544	-	-	-
KI-67[f]	1.79	1.00 - 3.22	0.052	1.05	0.51 - 2.17	0.902	-	-	-

[a] 5 Patients tumor-size unknown and of 1 patient nodal status unknown.
[b] Relative Hazard Rate
[c] Confidence Interval
[d] Test for trend.
[e] Tested vs node-negative.
[f] Dichotomized.

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 < .01); 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) 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).

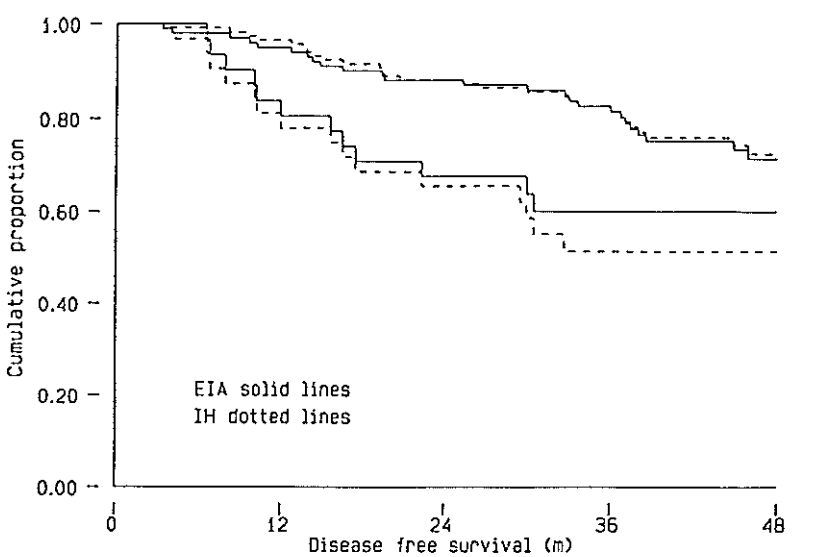

Figure 3. *Disease-free survival curve for estrogen receptors (ER) according to the method of detection.*

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 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.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.

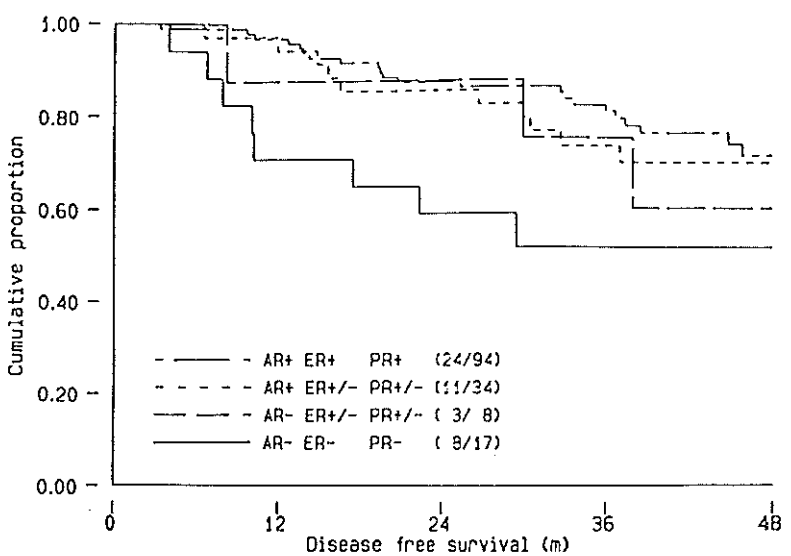

Figure 4. Disease-free survival curves for patients with tumours of different estrogen/progesteron/androgen receptor (ER/PR/AR) phenotypes. Patients with ER-, PR-, AR- tumours had the worst prognosis (p=0.026). Numbers in parentheses show failures/total amount of patients in each group.

6.5 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 anti-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 emphasized (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 emphasize the view that lack of all three steroid receptors, combined with high proliferative activity and high grading, are associated with a more aggressive tumour behaviour. This is

substantiated by the DFS data (fig 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 3 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 2 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 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 abilities.

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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CHAPTER 7

TP53 ALTERATIONS IN PHYLLODES TUMOURS

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Coloured illustrations in this chapter have been placed at page 97.

Submitted

7.1 ABSTRACT

The histological characteristics of phyllodes tumours of the breast are often not related to their clinical outcome. Additional studies are performed to investigate the possible relation of cell biological parameters to the biological behaviour of these tumours. We report a case with a histologically malignant phyllodes tumour with known clinical follow-up. Expression of TP53, Ki-67 and TP53 regulated proteins in the primary and in the recurrent and metastatic tumours as well as in adjacent benign breast tissue were studied using immunohistochemical and molecular biological techniques. A high expression of Ki-67 (30%) and TP53 (90%) was found both in the primary tumour and in the recurrent tumour sample, moreover in all tumour samples the same *TP53* gene mutation, Arg273Cys, was detected. No mutation was found in adjacent normal breast tissue indicating that it was an acquired mutation. The case presented here illustrates that detection of *TP53* gene alterations may offer new prognostic information in phyllodes tumours of the breast.

7.2 INTRODUCTION

Phyllodes tumours of the breast are characterized by leaflike projections of hypercellular stroma with cleft-like spaces lined by ductal epithelium. It is a rare fibroepithelial neoplasm of the breast that accounts for $<1\%$ of the breast tumours in females. About 25 % of phyllodes tumours are considered to be histologically malignant, thus resembling soft tissue sarcomas (1). Unfortunately the histological characteristics are usually not related to the clinical outcome *(6,12,18,22)*. Studies on modern prognostic factors in phyllodes tumours may reveal their relation to the biological behaviour of these tumours.

TP53, also known as *P53*, and *MDM2* gene alterations have been described in numerous tumours including breast cancer and sarcomas. The human *MDM2* gene, located on chromosome 12q13-14, has been found to be amplified and/or overexpressed in 15-30% of sarcomas *(7,13)* and amplified in 4-10% of human breast tumours *(9,17)*. The MDM2 protein binds directly to the TP53 protein and is able to block TP53 mediated transactivation *(4)*. *TP53*, located on the short arm of chromosome 17, has been implicated in the regulation of normal cell growth and division, DNA repair and apoptosis. This tumour suppressor gene is found to be frequently (14-52%) altered in human primary breast carcinomas *(3,8)* and in about 25% of sarcomas *(7,21)*. In general, mutations give rise to a conformationally altered protein which, due to a prolonged half-live, is stably expressed at high levels. Both MDM2 overexpression and *TP53* mutations are alternative mechanisms of TP53 dysfunction. Neither *TP53* gene alterations nor MDM2 amplification/overexpression in phyllodes tumours of the breast have, as yet, been studied.

In our ongoing studies on modern prognostic factors in phyllodes tumours four

cases out of 20 cases studied (one malignant, two borderline and one benign) showed an overexpression of TP53. In only one of these four cases, i.e. the histologically malignant phyllodes tumour, a *TP53* gene mutation in the primary tumour, was found. This patient developed a local recurrence and two distant metastases at 12, 15 and 21 months respectively after adjuvant radio- and chemotherapy of which frozen material was available for immunohistochemical and molecular biological analysis. The assessment of *TP53* gene alterations not only in the primary, but also in the recurrent and metastatic tumour tissue of this particular patient is presented in this paper. In addition we analyzed TP53 regulated proteins including $P21^{WAF/SDI/CIP1}$, MDM2 and the apoptosis related proteins BAX, and BCL2 in all tumour samples.

7.3 CLINICAL HISTORY

A 53 year old woman underwent a radical excision of a tumour in the left breast in May 1993. Histological examination showed a malignant phyllodes tumour with invasion of the deep surgical margin at the chest wall. Postoperative radiotherapy was given. After 12 months salvage mastectomy and chestwall resection was performed for local recurrence, and three months thereafter lung metastases were observed. Six courses of chemotherapy (Adriamycin/ Ifosfamide) were given, followed by surgical removal of the lung metastases and another two courses of chemotherapy. In March 1995 phase I chemotherapy was given for subcutaneous chestwall metastases without effect, followed by resection and interstitial radiotherapy with an Ir^{192} implant. In July 1995 one of the large subcutaneous metastases next to the contralateral breast was removed. The patient died in August 1995, no postmortem examination was performed.

7.4 MATERIAL AND METHODS

Histological features of the tumour were determined following standard criteria, modified according to Moffat et al 1995 (*18*) (Table 1). Frozen tissue samples of the primary tumour (I), its local recurrence (II) together with frozen tissue samples of benign breast material (III) and of two metastases (IV=lung, V=chestwall) of the same patient were used for this study.

Immunohistochemistry

Immunostaining was carried out on cryostat sections, 4 μm, which were air-dried, fixed in acetone (in case of Ki-67, P53, BCL2 and BAX staining) or in formaldehyde 4% (in case of MDM2 and P21 staining) for 10 min. Monoclonal antibodies used: Ki-67 (Ki-67 DAKO, Glostrup, Denmark; working dilution 1/10); P53 (1801, Oncoscience, working dilution 1/500 and DO-1, Santa Cruz, Santa Cruz, Cal, USA, working dilution 1/100)), MDM2 (Ab-1, Calbioch,

Cambridge MA, USA, working dilution 1/40), P21 (2G 12, Pharmingen, San Diego, Ca, USA, working dilution 1/20), BCL2 (124, Dako, Glostrup, Denmark, dilution 1/100) and BAX (4F11, Immunotech/Coulter, Westbrook, Maine, USA; ready for use). The APAAP technique was applied for visualising the antibody, as described previously (*23*) A colon cancer (Ki-67 and P53), lymphnode (BAX and BCL2), rhabdomyosarcoma (MDM2) and P21 positive mammary cell line were used as positive controls. In addition, as a negative control, the primary antibody was replaced with PBS or non-immune ascites fluid.

Quantification
The percentages of P53, MDM2, P21, BAX and BCL2 positive (tumour) cells were estimated, by two independent observers (VKB and SHL), by counting the number of positive cells in a total of 300 cells in three different areas of the tumour. The Ki-67 score was assessed by counting 300 cells in the areas with the highest proliferative activity, as described previously (*16*).

DNA extraction, PCR-SSCP and sequencing analysis.
The frozen (tumour) samples were pulverized and homogenized in phosphate buffer according to the EORTC procedure (EORTC Breast Cancer Corporative Group, 1980). High molecular weight chromosomal DNA was isolated and exons 4 through 9 of the *TP53* gene were analyzed using polymerase chain reaction and single-strand conformation polymorphism analysis (PCR-SSCP), as described previously (*3*). The breast cancer cell-lines SKBR-3 (mutated at codon 175 in exon 5), T47-D (mutated at codon 194 in exon 6), EVSA-T (mutated at codon 241 in exon 7) and the colon cell-line HT-29 (mutated at codon 273 in exon 8) were used as positive controls, whereas ZR75-1 was used as a negative control. Samples showing an altered migration pattern were analyzed again, using an independent PCR product, and subsequently sequenced. Sequencing was performed using the "Ampli Cycle^{tm} sequencing" kit from Perklin Elmer (Branchburg, New Jersey, USA) and 5-prime ^{33}P end-labelled primers. The DNA sequence was determined by electrophoresis of the terminated products on a 6% denaturing polyacrylamide gel, containing 8M urea, followed by autoradiography.

7.5 RESULTS

The primary tumour was characterized as malignant (Table 1), due to high cellularity of the stroma with nuclear polymorphism, stromal overgrowth and high mitotic rate (> 10 mitoses per 10 high power field (HPF)), shown in figure 1A. The samples of the local recurrence and the metastases showed similar features.

Table 1. *Histological features*

stromal characteristics	Primary tumour(I)	recurrence (II)
stromal cellularity	+++	+++
nuclear polymorphism	+++	+++
stromal overgrowth	+++	na[1]
tumour margin invasive	> 10%	na[1]
mitoses/ 10 HPF[2]	> 10	> 10
necrosis	-	+/-
heterologous stromal elements[3]	-	-

[1] : *"not applicable"*

[2] : *number of mitoses per 10 HPF = high power field*

[3] : *malignant bone and cartilage*

Immunohistochemistry

The staining patterns of the primary tumour (sample I), its local recurrence (sample II), benign breast tissue (sample III) and lung and chestwall metastases (samples IV and V respectively) with regard to different antibodies are summarized in Table 2. Ki-67 expression was absent in normal tissue, similar for both the primary (I) and the recurrent tumour (II) (30% versus 30%), but higher in the metastases (60%-50%). A heterogenous staining pattern was observed for P53 in the primary tumour, with 60-90% moderate/strong (++/+++) nuclear staining, in contrast to the more homogenous strong nuclear staining of the recurrence and the metastases (100%)(Figure 1, C-F). Cytoplasmic localisation of the P53 protein was only observed in sample I for P53, when using antibody 1801, in samples I and II, when using antibody DO1. A sporadic nuclear overexpression of MDM2 was seen in the metastatic samples (regarded as negative, table 2) but no nuclear staining was observed in sample I and II. However a granular cytoplasmic staining was observed in sample I. BAX was highly (90%) expressed in the cytoplasm of the primary, local recurrence and first metastatic tumour but the intensity decreased from strong in sample I to moderate in samples II and IV; chest wall tumour sample V however demonstrated a low BAX expression (< 10%). BCL2 was found to be highly expressed in sample I (70%), in contrast to a low expression of the same antibody in the other tumour samples. No expression of P21 was observed in all tumour samples, although granular cytoplasmic staining was noticed in sample I. In the benign breast material only expression of BAX and BCL2 in the ductal epithelium was observed (Table 2).

Table 2. *Immunohistochemical expression of the different markers in the tissue of the various tumour samples and benign breast tissue.*

		primary tumour	recur-rence	benign breast	metastasis lung	metastasis chestwall
markers		I	II	III	IV	V
		%	%	%	%	%
Ki-67		30	30	neg	60	50
P53	1801	60-90*	100	neg	100	100
	DO1	90	90	neg	100	100
MDM2		neg	neg	neg	neg	neg
BAX		90	90	30	90	<10
BCL2		70	<10	100	20	<10
P21		neg	neg	neg	neg	neg

**in this sample a heterogenous staining pattern was found*

PCR-SSCP and sequencing analysis

TP53 gene mutation analysis was performed using PCR-SSCP and sequencing techniques. *TP53* base alterations in exons 4 through 9 were tested. The primary tumour (I), local recurrence (II), adjacent normal (III) and the two metastases (IV, V) were examined. An altered migration pattern on SSCP, indicative of a mutation, was observed in exon 8 of samples I, II, IV and V. Sample III, which contained DNA from adjacent normal breast tissue showed no alteration in the migration pattern. PCR products with this altered migration pattern were subsequently analyzed by cycle sequencing. A missense point mutation CGT>TGT at codon 273, shown in Figure 1B, resulting in an amino acid change from Arg->Cys (R>C) was observed in those tumour samples (I, II, IV and V).

7.6 DISCUSSION

Recent studies (*6,12,18,22*) on phyllodes tumours have shown that the presence of tumour at the surgical margin is the major determinant of local recurrence or development of metastasis, whereas the histological features of these tumours are of secondary importance with regard to prognosis. This prompted our study on modern prognostic features in phyllodes tumours to investigate their possible use in prediction of more aggressive tumour subsets. The case presented here, was one of a group of 20 patients with phyllodes tumours of which frozen material was available for immunohistochemical

and molecular biological analysis. In four cases (one histologically malignant, two borderline and one benign) an overexpression of P53 (>10%) was found. Frozen material was available of only three tumour samples for gene analysis (data not shown). Only the case presented here (histologically malignant) showed a *TP53* gene mutation. Over-expression of P53 protein was not only seen in the primary but also in the recurrent and in the two metastatic tumour samples. Furthermore in all tumour samples of this patient, the same *TP53* gene mutation, e.g. Arg273Cys, was detected. A sample of benign breast tissue of this patient showed no gene alteration, indicating that this was an acquired *TP53* gene mutation and not a germline mutation. This is the first report on TP53 mutations in phyllodes tumours. Analysis of the TP53 crystal structure indicated that some of the commonly mutated basic residues either are important for stabilization of the p53-DNA complex (e.g. Arg175 and Arg249) or directly interact with the DNA (e.g. codons Arg248 and Arg273)(*5*). Codon 273 of *TP53* is one of the most frequent missense mutation sites found in human cancers. Wild-type TP53 has been shown to be a sequence-specific transactivator for promotors containing the TP53 binding site, including GADD45, cyclin 9, MDM2, P21 and BAX (*11*). Therefore we have studied P53-dependent induction of MDM2 and of $P21^{waf1/cip1;}$ a potent inhibitor of cyclin dependent kinases. P21 has been shown to be induced by radiation *(10)* or transforming growth factor ß (TgFß). In contrast to the high P53 expression sporadic nuclear overexpression of MDM2 was only seen in the two metastatic tumour samples. This is consistent with the analysis of the *TP53* and *MDM2* genes in sarcomas which shows that no MDM2 alterations are found in sarcomas with *TP53* gene mutations (*20*). The cytoplasmic staining of MDM2 which we observed in sample I has not been described elsewhere in sarcomas, although a faint cytoplasmic staining is mentioned in epithelial breast cancer studies (*17*). In the presence of mutated *TP53* gene no induction of P21 protein is expected, which is consistent with our findings of negative nuclear staining of P21 in both primary and metastatic tumour tissue, see Table 2. Cytoplasmic staining of P21 was observed in sample I, and not present in the other tumour samples.

The second possible mechanism of growth suppression by wild-type P53 is explained by its promotion of apoptosis. The sensitivity of cells to apoptotic stimuli is considered to be regulated by the ratio of BAX:BCL (*2,14*). Interestingly, cells with high BAX expression are found to be associated with high response to chemo- and radiotherapy (*15*).

We have studied BAX expression patterns and the expression of its dimerization partner BCL2. The observed overexpression of BCL2 in sample I may have caused blocking of apoptosis and therefore clonal selection of radiotherapy resistant cells, however this is not in concordance with the negative BCL2 expression found in the recurrences. In this case also strong cytoplasmic BAX staining was found in the primary tumour different from the mode-

rate staining in the other tumour samples. As yet, we have no unequivocal explanation for the strong BAX staining in the primary tumour and the lack of response to radiotherapy.
In conclusion, mutated P53 in this tumour, with its associated incompetence for DNA repair, cycle arrest and apoptosis may have lead to a clonal selection of more aggressive tumour cells after DNA damage (*19*) and therefore different expression levels of the studied proteins in addition to radioresistance. The case presented here illustrates that detection of *TP53* gene alterations may offer new prognostic information in phyllodes tumours of the breast. Further and larger studies on TP53 and its regulated proteins will provide more insight of the prognostic value of these proteins in phyllodes tumours.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUDING REMARKS

In this chapter the clinical significance of histological, IH and IC determined parameters in breast tumours analyzed in the foregoing studies, will be discussed.

8.1. *Introduction*

Early detection of breast cancer has reduced the size of the average breast tumour at diagnosis. in the future new diagnostic and surgical procedures will again influence the amount of tissue available for diagnosis and IH and biochemical assays. For instance, ultrasound localised biopsies used for the diagnosis of small non-palpable tumours detected by mammographic screening may in the future be replaced by diagnosis with stereotactic core needle biopsy, leading to a further reduction of tumour tissue.
Lately, the need of complete axillary dissection has been questioned, because of high morbidity of the procedure caused by lymphedema and neuropathy of the involved arm. For this reason examination of the so-called sentinel node(s), defined as the first node draining the primary tumour in the regional lymphatic basin[1], is being advocated and may well replace total axillary dissection in the future. As lymph node status has long been the golden standard in determining the prognosis of a breast cancer patient, not knowing the nodal status of a patient will have important repercussions for breast cancer management, and will enhance the need for other valid prognostic factors.
In addition to these changes in diagnostic and surgical procedures, peri-operative chemotherapy is being advocated even in the group of node-negative patients and peri-operative hormonal therapy in postmenopausal women. These developments again will reduce the amount of tumour tissue available for additional techniques, especially for biochemical assays. These considerations will make the use of immunological techniques more appealing for assessment of clinically significant cell biological markers, also on cytological material, particularly when no primary surgery is performed. Moreover, these markers will gain impact when strong prognosticators like lymph node status are no longer available.

8.2. *Histo/cytological grading and typing.*

The prognostic value of histological grading has been studied widely and been accepted as an important predictive parameter of the biological behaviour of breast carcinomas, provided that strict criteria are followed [2,3,4]. Likewise, it has been demonstrated that histologically typing of breast carcinomas has prognostic significance[5,6]. In addition Pereira et al has shown that these parameters are used together even more accurately predicted prognosis[7]. In view of these findings, we graded the different types of breast cancer according to Bloom and Richardson and modified according to Elston[4] and found grade to

be an important parameter in univariate analysis (chapter 6). Moreover, we found the 17 receptor negative tumours to be grade III tumours. They were characterised by a high percentage of Ki-67 positive cells. In addition, histological subtypes, such as cribriform, colloid and tubular were all found to be receptor positive (chapter 5 and 6) while demonstrating low Ki-67 activity. These observations emphasise the actual importance of grading and typing. In the light of future developments, when lymph node status is not longer known, it may be important to implement these parameters in clinical breast cancer management.

8.3. *Proliferative activity*

The proliferative activity of tumour cell populations, as defined by different approaches has been found to provide information about prognosis. Since some of the methods like the labelling techniques are laborious and not suited for routine laboratory practice, the use of immunohisto/cytochemistry has become more appealing. The monoclonal antibody Ki-67 defining a nuclear antigen associated with proliferation, was at first only applied to frozen tissue. We found that this antibody (chapter 2) could easily be used on cytological material and that cytological assessment of Ki-67 growth fractions led to similar results as found in cryostat sections of corresponding tumours (chapter 3). Moreover, in concordance with thymidine labelling studies[8], a relationship between subtypes of breast cancer and proliferative activity was demonstrated (chapter 3 and 6). Infiltrating ductal carcinomas were showing the highest activity, and subtypes like tubular and colloid carcinomas were showing the lowest. Numerous papers have been published about the prognostic value of the Ki-67 determined growth fraction [9,10,11,12]. Correlations have been demonstrated between Ki-67 scoring, the S-phase fraction as determined by flow cytometry and mitotic count [9,13,14]. Considering these facts, Ki-67 immunostaining can be seen as a rapid and inexpensive method of assessment of the proliferative index, and may be helpful in selecting patients for endocrine therapy (slowly proliferating tumours) or chemotherapy (rapidly proliferating tumours).

8.4. *Steroid receptors.*

The biochemical assessment of ER and PR is well recognized as aid for predicting prognosis and choice of therapy. We found, like in other studies[15,16] a significant correlation between biochemically obtained data and immuno-histochemically obtained data for ER and PR (chapter 6). Likewise, the prognostic value, as for disease free survival, of ER/PR detected by either method was similar. We confirmed the prognostic value of ER in multivariate analysis. A reliable detection of these receptors not only in histological but also in FNA material is feasible, as shown in chapter 4. The clinical significance of AR is poorly understood. The widespread expression of AR in breast cancer

(incidence of 76%), demonstrated in chapter 5 and 6, and later confirmed by Isola[17] and Hall[18] suggests that this receptor may be of biological importance in breast cancer. Unfortunately, we were not able to determine the additional therapeutical relevance of AR positivity as the number of patients receiving hormonal treatment for advanced disease was too small to study this relationship. Since Birrell et al.[19] found a correlation between AR level in the primary tumour and response to medroxyprogesterone acetate therapy in lymphnode positive breast cancers, this finding may indicate that activation of AR function may act as an important inhibitor of breast cancer growth in vivo.

8.5. *TP53 alterations*

TP53 alterations have been described in numerous tumours including breast cancer and sarcomas. Mutations give rise to a conformationally altered protein, stably expressed at high levels, which can currently be routinely determined by immunohistochemistry. *TP53* alterations appear to independently predict poor prognosis in breast cancer patients[20,21], but have, as yet not been studied in phyllodes tumours of the breast. In our ongoing studies on phyllodes tumours, not only TP53, and Ki-67 but also TP53 regulated proteins were examined using IH and molecular biological techniques. In one case of a patient with a malignant phyllodes tumour of the breast (chapter 7), we found not only high expression of both Ki-67 and P53 protein in the primary tumour, recurrence and metastases, but also the same *TP53* gene mutation in all samples. Since this patient died within two years after diagnosis, *TP53* gene alterations, like in breast cancer, may represent an important prognosticator, indicating a poor prognosis in phyllodes tumours.

8.6. *Quality assurance in immunohisto/cytochemistry*

When we wish to implement immunohisto-/cytochemical detected parameters in breast cancer management, several problems have to be considered. Among others, we mention the considerable variation in scoring methods and cut-off points for various parameters used in the literature. An example of this problem is the fact that we, like Bouzubar and Veronese[10,11] have taken a Ki-67 score equal to or over 20% as a cut-off level of high and low proliferative activity while Railo and Isola have used a cut-off level of ten percent in their assays[9,13]. These differences may have considerable impact in survival analyses, and inter-laboratory studies are needed to validate the different methods.

Not only differences in cut-off points, but also in detection techniques and in methods of material processing, fixation and storage do exist, all influencing the detectability of an antigen. This latter is especially true for immunostaining in cytology[22], and makes comparison of studies cumbersome, if not impossible. To standardize immunostaining in cytology, we embarked on a study to analyze the influence of different working procedures. We found that, depending on the localisation of the antigen (nuclear, cytoplasmic or membranous) different

methods of material processing are required. These observations illustrate the need for quality controlled standardized procedures to ascertain reproducible immunostaining results. In view of these facts, the pathology committee of the EORTC Breast Cancer Cooperative Group has set up a EORTC pathology collaborative group to coordinate activities concerning standardization of methodologies, cut-off points etc.

8.7. *Implementation of cell biological markers in clinical decision-making*
Many of the cell biological markers described have prognostic value in univariate analysis but when multivariate analysis is applied these parameters in general, add very little to prognostic models. This was again demonstrated in chapter 6: in univariate analysis AR as well as ER, tumour size, lymph node status, grade and Ki-67 proved to be significant prognostic factors for 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.

Table 1

authors	time (yrs)	long-term prognostic factors				
		N status	grade	type	size	growth fraction
Rosen	18			+	+	
Dixon	20			+		
Galea	15	+	+		+	
Meyer	10	+			+	
Silvestrini	6-10					+
Lipponen	4	+			+	

Table 2

authors	time(yrs)	short-term prognostic factors	
		ER	PR
Raaymakers	3	+	
Schmitt	2	+	+

It should be noted however, that *time* after first treatment is another important factor in determining the prognostic value of a parameter. Several authors have demonstrated the changing importance of prognostic factors during longterm follow up. Tumour size, lymph node status, grade and growth fraction are found to be long term prognostic factors[3,23,24,25,26](Table 1). Estrogen receptor and PR lose their prognostic significance after the first few years after surgery[27,28](Table 2).

These observations emphasize the fact that for implementation of new parameters follow-up studies over longer periods of time in combination with multivariate studies are necessary to validate the use of these parameters. Moreover quality assurance programs will be needed to standardize the various procedures.

8.8. CONCLUDING REMARKS.

In the last decade our knowledge of the biological behaviour of breast cancer has increased remarkably. Moreover, refinements of treatment, have resulted in improvement of five year survival. Nonetheless overall breast cancer mortality rates have not improved significantly in the last 20 years. Conventional parameters, like lymph node status, tumour size, grading and typing are still of great importance, but additional prognostic parameters are urgently needed, especially for node negative patients because of a trend toward a general use of adjuvant therapy[29]. As stated earlier, new treatment modalities and reduction of tumour tissue available for diagnostic and prognostic information, necessitate the maximum yield of information from histological but also from cytological specimens. This will especially be true when systemic therapy is going to be applied prior to surgery and aspirates will be the only tissue available for determination of predictive and prognostic factors. We have demonstrated that proliferative activity, can easily be determined on cytological/histological material, provided that standardized methods, also in scoring are being applied. The same is true for the detection of hormone receptors and TP53. The determination of a combination of these parameters may help to identify patients with a low/high probability of risk of tumour recurrence. In addition the benefit of hormonal or chemotherapy can be anticipated. The Nottingham group has indeed shown in a large group of patients over a long period of time (15 years) that a combination of different independently significant prognostic parameters (lymph node status, grade and size) can predict survival of an individual patient more accurately than using any of these factors individually[23]. Likewise they demonstrated that another index (a combination of grade, ER, site of initial metastasis and disease free interval) could be used for a group of patients with metastatic disease to predict the effect of hormone therapy[30]. Clark[29] on the other hand combined age, lymph node status, S-phase, tumour size and ER/PR values in a model to define the poor risk group. In addition new parameters like TP53 expression may be used to predict resistance to specific therapies. We conclude therefore that time has come for the clinical implementation of those parameters, which have already proven their prognostic/predictive value, in order to avoid unnecessary treatment, especially in the case of node negative patients.

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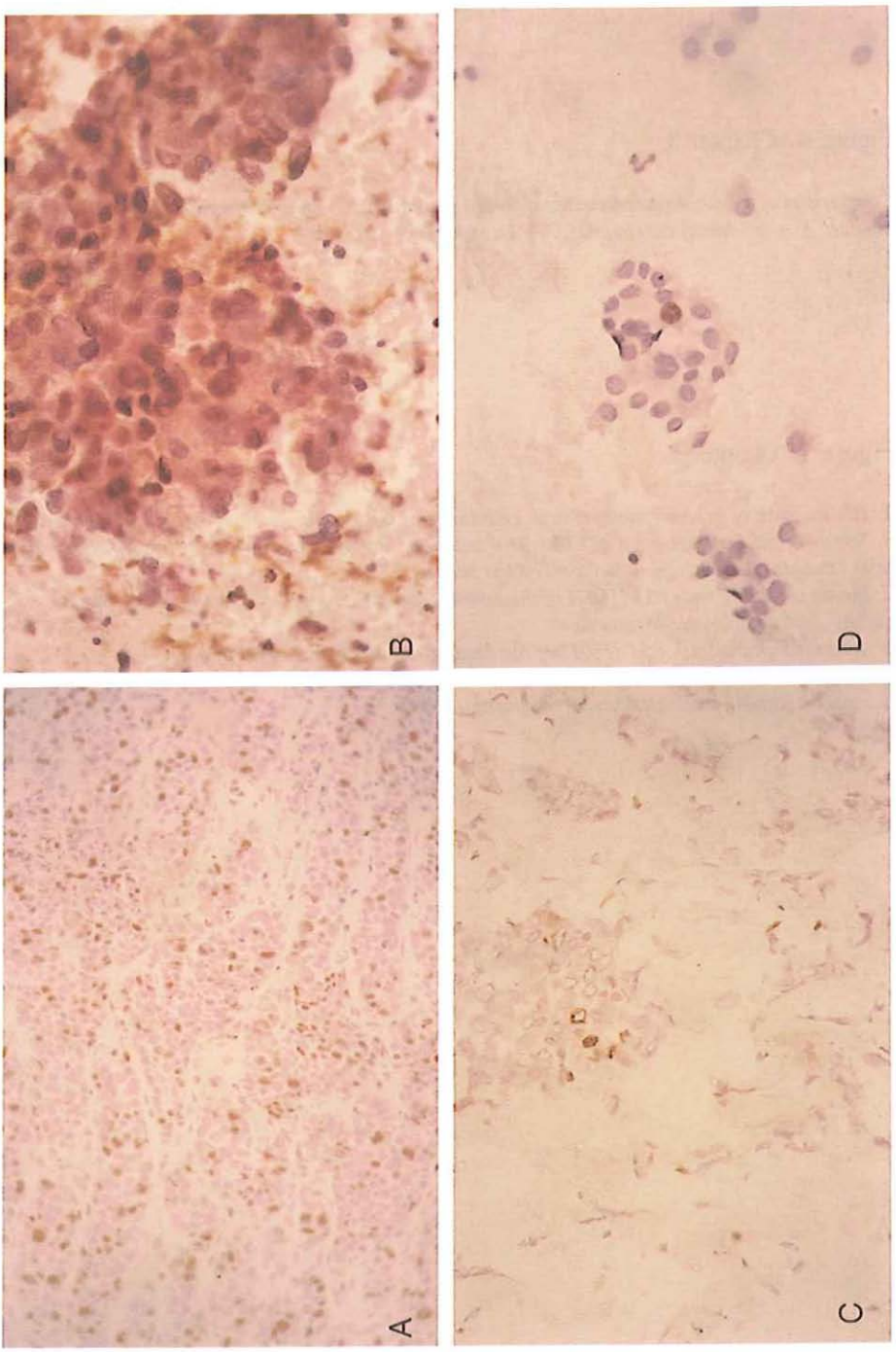
Figure 4. Chapter 3

Comparison of Ki-67 immunostaining of frozen sections (A,C) and FNA smears (B,D) of the same tumour. A + B: ductal carcinoma, C + D: mucinous carcinoma

Figure 1. Chapter 7

A: HE-staining of primary tumour: high cellularity of the stroma with nuclear polymorphism
B: Sequence analysis of exon 8 of TP53. The mutation is indicated by the arrow (Arg273Cys) in the right lane. In the left lane wild-type TP53 is shown.
C: Immunostaining with P53 (1801) of the primary tumour: moderate, heterogenous staining with nuclear and cytoplasmic localisation.
D: Immunostaining with P53 (1801) of the recurrence: strong nuclear staining
E and F: Immunostaining with P53 (1801) of the two metastases, E lung (sample IV), F chestwall (sample V): strong nuclear staining comparable to D.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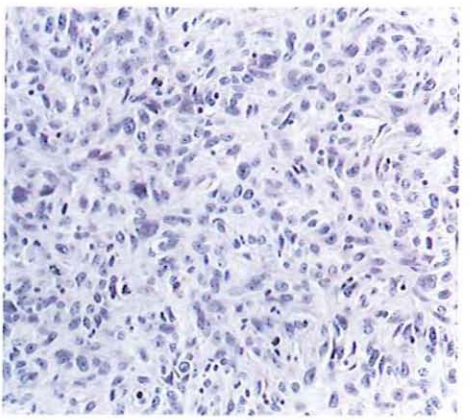

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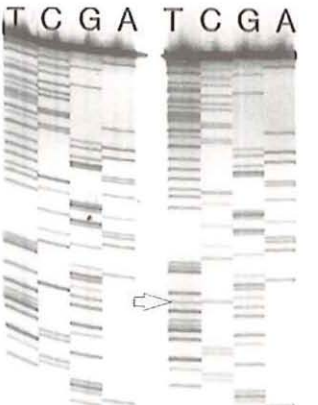

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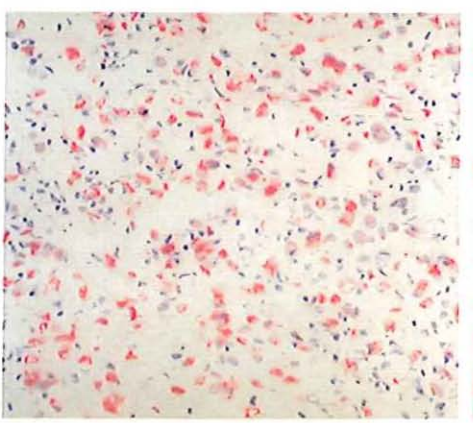

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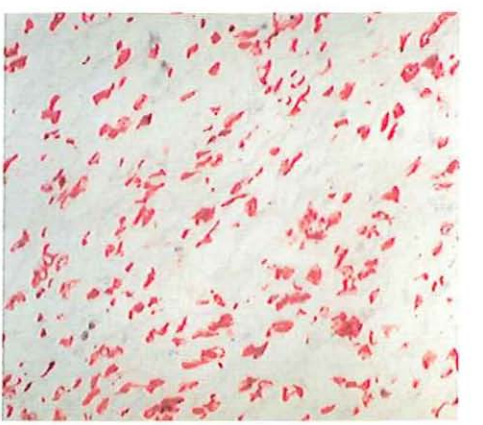

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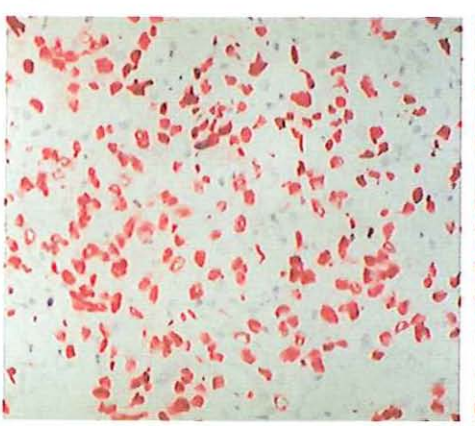

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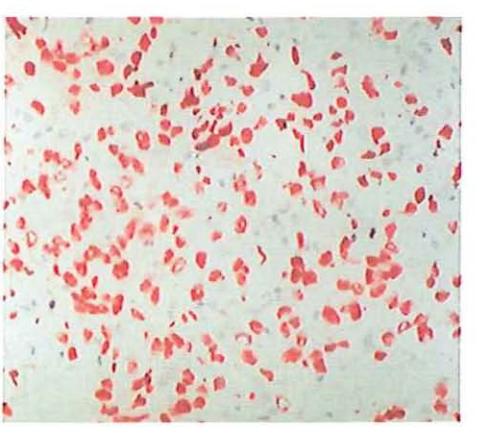

F

CHAPTER 9

SUMMARY

The aim of this thesis was to analyse the applicability of immunocytochemistry (IC) on fine needle aspirations (FNA) of breast tumours and to assess the clinical value of not only IC, but also IH determined cell biological parameters. The growth fraction of a breast tumour as reflected by its mitotic activity is considered to be an important prognostic indicator. The application of the monoclonal antibody Ki-67, defining a nuclear antigen present throughout the cell cycle enables a simple evaluation of the growth fraction of a tumour. Initial studies with this antibody were confined to tissue sections of breast tumours.
Since the initial diagnosis of breast cancer in general, is made on FNA smears and patients are increasingly given chemotherapy prior to surgery, the feasibility of the determination of proliferative activity in FNA smears was examined. In *chapter 2* we describe a preliminary study in which FNA smears were IC stained with monoclonal antibody Ki-67. A well-defined nuclear immunoreactivity was obtained in a variable proportion of the cells allowing a reproducible quantitation of Ki-67 positive cells in these smear preparations. A low median percentage of 0.4 % Ki-67 positive cells was found in benign aspirates as compared to the much higher median Ki-67 score of 7.9 in aspirates derived of breast cancers. A significantly higher median Ki-67 score of 12.3 was demonstrated in the FNA smears of breast cancer cells that had metastasized. This latter finding is in line with the view that rapidly proliferative breast cancers have a prognostically less favourable behaviour.
The reliability of Ki-67 immunostaining in FNA material was subsequently assessed in *chapter 3*. We compared the results of the Ki-67 determined growth fraction in FNA material and cryostat sections of corresponding tumours. Ki-67 scores of FNA smears correlated well ($r = 0.79$) with the values obtained in the frozen sections. In addition, we observed both on frozen sections and FNA smears that the Ki-67 scores differed for the various histological types of breast cancer. These latter results confirmed previous published data employing in vitro incorporation of radioactively labelled thymidine in breast cancer tissue fragments.
The potential implementation of IC determined prognostic factors in clinical decision-making requires a highly reproducible and reliable technology, which can only be achieved by standardization of handling and processing of the diagnostic material and a robust technique of immunostaining based on well-characterized and generally available reagents. In addition, the scoring system should not be subject to great inter-observer variation. Actually, substantial variations in the values of a given parameter can be found in the literature. In *chapter 4* we analysed the influence of material processing, fixation and storage of FNA aspirates on IC staining results. We developed a standardized method

for achieving reproducible immunostaining results of optimal quality for nuclear antigens. Employing this method, a high concordance of IC results obtained in histological material and corresponding cytological material of 10 breast carcinomas was observed.

The presence and frequency of estrogen receptors (ER) and progesterone receptors (PR) in breast cancer has been examined extensively both by biochemical and immunohistochemical (IH) methods. Much less was known about the frequency of androgen receptors (AR) in breast cancers. In *chapter 5* AR expression in 100 breast carcinomas was determined using a recently developed monoclonal antibody specific for AR. Seventy-six percent of these breast cancers were AR positive, while nine percent of the tumours expressed AR without concomitant ER or PR expression. Tumour grade was not only inversely related to ER and PR expression, but also to AR expression. Moreover, all receptor negative tumours were poorly differentiated (grade III) tumours.

Although the prognostic role of oestrogen receptors (ER) and progesterone receptors (PR) in breast cancer has long been established, both with regard to endocrine therapy response and survival, little information was available as to the prognostic impact of AR expression in breast cancers. To establish the clinical significance of the presence of AR in breast carcinomas we studied 153 cases, with a median follow-up time of 46 months, as described in *chapter 6.* In addition, the prognostic impact of the Ki-67 score was assessed and several clinico-pathological parameters were recorded. The latter included tumour stage, patients' age, and disease free survival (DFS). ER and PR expression was quantified both by IH in frozen tissue sections and by biochemical enzyme immunoassay in cytosol preparations, while AR expression was assessed semiquantitatively only by IH. Biochemically assessed ER and PR levels correlated well with IH determined ER and PR scores, respectively. In univariate analysis AR as well as ER, and Ki-67 score in addition to the pathological parameters tumour size, lymph node status, and tumour grade all proved to be significant prognostic factors for DFS. Multivariate analysis, however, showed lymph node status, tumour size and ER status to be the only independent prognostic factors. Unfortunately, we could not study the relationship between AR expression and response to hormonal therapy, since in this series the number of patients receiving hormonal treatment for advanced disease was too small.

A nowadays frequently investigated prognostic parameter is the TP53 protein, a product of the *TP3* tumour suppressor gene. Although its overexpression at the protein level and the occurrence of point mutations in the *TP53* gene has been studied extensively in breast cancers, the occurrence of similar aberrations in the less common phyllodes tumours of the breast has not been reported. In *chapter 7* a case of a metastasized phyllodes tumour of the breast was studied with regard to IH determined Ki-67 score, TP53 protein expression and its

regulated proteins, and *TP53* gene alterations. Next to TP53 overexpression, a high Ki-67 score was observed not only in the primary tumour but also in the recurrent tumour and in the tumour metastases. In all tumour samples an identical *TP53* gene mutation, i.e. Arg273Cys, was detected. Apparently, primary tumour, recurrent tumour and metastases represent the progeny of a single precursor cell. In a larger series of phyllodes tumours the prognostic impact of *TP53* gene mutations remains to be established.

In *chapter 8* we discuss the consequences of new diagnostic and therepeutical procedures with regard to breast cancer management. We conclude that these changes will make it even more important to use IH and IC for the determination of cell biological parameters in breast cancer.

SAMENVATTING

De bedoeling van het in dit proefschrift beschreven onderzoek was om de bruikbaarheid van immunologische technieken in de cytologie, met name in punctie materiaal van mamma tumoren, te onderzoeken en de klinische betekenis van immunohisto-/cytochemisch bepaalde celbiologische parameters te testen. De groeisnelheid van een tumor gekarakteriseerd door het aantal cellen in deling, wordt als een belangrijke prognostische parameter beschouwd. Het gebruik van het monoclonale antilichaam Ki-67, dat een kern antigeen aantoont dat uitsluitend in de kern van delende cellen wordt gevonden, stelt ons in staat om op een simpele manier de groeifractie van een tumor te bepalen. Aanvankelijk werd dit antilichaam door andere onderzoekers alleen gebruikt bij onderzoek van histologisch materiaal van mammatumoren. Aangezien de eerste diagnose van borstkanker in het algemeen gesteld wordt op punctie materiaal, werd door ons onderzocht of ook in cytologisch materiaal met behulp van Ki-67 de delingsactiviteit van een mammacarcinoom kon worden bepaald. In *hoofdstuk 2* beschrijven we een eerste studie waarin cellen afkomstig van puncties immunocytochemisch werden onderzocht met behulp van het monoclonale antilichaam Ki-67. Er werd in een wisselend aantal van de cellen een duidelijke immunoreactiviteit van de kern verkregen, wat een reproduceerbare bepaling van de hoeveelheid Ki-67 positieve cellen mogelijk maakte. Een laag mediaan percentage van 0.4 Ki-67 positieve cellen werd gevonden in uitstrijken afkomstig van punctaten van goedaardige mamma lesies in vergelijking met de veel hogere mediane Ki-67 score van 7.9 in punctie materiaal afkomstig van mamma carcinomen. Een significant hogere mediane Ki-67 score van 12.3 werd gevonden in punctie materiaal van uitgezaaide mammacarcinomen. Deze laatste bevinding past bij de gedachte dat snel delende mammacarcinomen een prognostisch minder gunstig gedrag vertonen. De betrouwbaarheid van de immunocytochemische kleuring met Ki-67 op punctaten werd vervolgens getest in *hoofdstuk 3*. Wij vergeleken de resultaten van de met Ki-67 bepaalde groeifractie in punctaten met die bepaald in vriescoupes van weefsels van dezelfde tumoren. De Ki-67 scores van het punctie materiaal correleerden goed ($r=0.79$) met de waarden verkregen in vriesmateriaal. Bovendien vonden we zowel in de vriescoupes als in cytologische uitstrijken dat de gevonden Ki-67 scores verschillend waren voor de verschillende typen mamma carcinoom. Deze laatste resultaten bevestigden eerder gepubliceerde data waarbij radioactief gelabelde thymidine in vitro in mamma carcinoom weefsel werd geincorporeerd, en waarbij eveneens bleek dat bepaalde typen mammacarcinoom een lagere groeisnelheid vertoonden.
De mogelijke implementatie van immunocytochemisch bepaalde prognostische factoren in de klinische besluitvorming vereist een goed reproduceerbare en betrouwbare techniek. Dit kan alleen worden bereikt door standaardisatie van het verwerken van het diagnostisch materiaal en een betrouwbare techniek van

immunokleuring gebaseerd op goed gekarakteriseerde en algemeen verkrijgbare reagentia. Aangezien belangrijke variaties in waarden van de verschillende parameters worden gevonden in de literatuur, moet bovendien het scoring systeem niet onderhevig zijn aan een te grote variatie tussen waarnemingen gedaan door verschillende onderzoekers. In *hoofdstuk 4* analyseerden wij de invloed die de materiaal verwerking, fixatie en de manier van bewaren hebben op de expressie van in punctie materiaal, immunologisch bepaalde prognostische parameters. Wij ontwikkelden vervolgens een gestandaardiseerde methode om kernantigenen immunologisch te kleuren, die goed reproduceerbaar was en van optimale kwaliteit. Bij toepassing van deze methode, werd een goede overeenkomst gevonden tussen gevonden immunocytochemische resultaten bepaald in histologisch materiaal en die bepaald in het corresponderende cytologische materiaal bij 10 mamma carcinomen.
Er is zowel biochemisch, als immunohistochemisch uitvoerig onderzoek gedaan naar de aanwezigheid, ook kwantitatief, van oestrogeen -(ER) en progesteron receptoren (PR) in mamma carcinomen. Veel minder is bekend ten aanzien van de aanwezigheid van androgeen receptoren (AR) in deze carcinomen. In *hoofdstuk 5* werd de AR expressie bepaald in 100 mamma carcinomen met behulp van een recent ontwikkeld monoclonaal antilichaam specifiek voor AR. Zes en zeventig procent van deze mamma carcinomen toonde AR expressie, bovendien werd in negen procent van de tumoren AR expressie gevonden, zonder dat er sprake was van ER of PR expressie. Daarnaast werden alle tumoren histologisch gegradeerd. Het bleek dat de tumor graad omgekeerd was gerelateerd aan ER en PR expressie maar ook aan AR expressie en dat alle ER, PR en AR negatieve tumoren, graad III tumoren bleken te zijn.
Hoewel de prognostische betekenis van ER en PR in borstkanker reeds lang bekend is, zowel wat betreft de respons op hormoon therapie (betere respons bij positieve ER en PR), als wat betreft overleving, is nog weinig bekend ten aanzien van de prognostische betekenis van AR expressie in mamma carcinomen. Om de klinische betekenis van de aanwezigheid van AR in borstkanker te analyseren, onderzochten we 153 mammacarcinomen met een gemiddelde follow-up van 46 maanden, zoals beschreven in *hoofdstuk 6*. Daarnaast onderzochten we de prognostische betekenis van de Ki-67 score en werden verschillende klinische en pathologische parameters bepaald, zoals leeftijd, de tumor stagering en de ziektevrije periode. ER en PR expressie werd kwantitatief bepaald zowel immunohistochemisch op vriesmateriaal als biochemisch met de enzyme immunotechniek, terwijl de AR expressie alleen immunohistochemisch werd bepaald. Biochemisch bepaalde ER en PR expressie correleerde goed met immunohistochemisch bepaalde ER en PR scores. In univariaat analyse bleken AR, evenals ER en Ki-67 score alsmede de pathologische parameters tumor grootte, lymfklier status en tumor graad allemaal significant prognostische factoren wat betreft de ziekte vrije periode te zijn. Multivariaat analyse toonde echter dat alleen de lymfklier status, tumor

grootte en ER status onafhankelijke prognostische factoren waren. Helaas konden we niet de relatie tussen AR expressie en respons op hormonale therapie onderzoeken, aangezien in deze patienten serie het aantal patienten dat hormonale therapie had gekregen voor gemetastaseerde ziekte, te klein was.
Bepaalde genen, de zogeheten tumor suppressor genen, onderdrukken het ontstaan en/of de progressie van kanker cellen. Een voorbeeld van een dergelijk gen is het *TP53* gen. Ofschoon zowel de overexpressie van het TP53 eiwit (een produkt van dit gen) als de mutaties die in dit gen kunnen voorkomen, uitgebreid zijn onderzocht in mammacarcinomen, zijn er nog geen studies gedaan naar dergelijke mutaties in de minder voorkomende phyllodes tumoren van de borst. In *hoofdstuk 7* wordt een geval van een gemetastaseerde phyllodes tumor beschreven waarbij niet alleen de immunohistochemisch bepaalde Ki-67 score werd bepaald maar ook de TP53 eiwit expressie en de door dit gen gereguleerde andere eiwitten. Bovendien werd gekeken naar *TP53* gen mutaties. Behalve een TP53 overexpressie werd er ook een hoge Ki-67 score gevonden niet alleen in de primaire tumor, maar ook in het tumor recidief en de metastasen. In al het tumor weefsel werd dezelfde *TP53* gen mutatie gevonden, nl Arg273Cys. Blijkbaar waren zowel de primaire tumor als het recidief en de metastasen afkomstig van dezelfde voorloper cel. De prognostische betekenis van *TP53* gen mutaties zal in een grotere serie van phyllodes tumoren moeten worden aangetoond.
In *hoofdstuk 8* wordt besproken wat de gevolgen kunnen zijn van de veranderende diagnostische en therapeutische procedures ten aanzien van het algemeen beleid van borstkanker. Wij concluderen daarom dat het in de toekomst steeds belangrijker zal worden om op histologisch maar ook op cytologisch materiaal met behulp van immunologische technieken celbiologische parameters te bepalen.

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"Last but not least" alle mensen van de dr Daniel den Hoed kliniek, die het werken ter plekke zo plezierig maken. Laten we hopen dat dit ook in de toekomst zo mag blijven.

Curriculum vitae.

De schrijver van dit proefschrift werd 15 juni 1940 in den Haag geboren. In 1959 behaalde zij het diploma gymnasium ß aan het 1e Vrijzinnig Christelijk Lyceum aldaar en begon haar studie geneeskunde aan de universiteit in Leiden. Na het behalen van het doctoraal examen in 1965, vervolgde zij haar studie aan de universiteit in Groningen, waar het artsexamen werd afgelegd in 1968. Na werkzaam te zijn geweest als huisarts, leidde zij enige jaren buro's voor zuigelingen en kleuters. In 1975 aanvaardde zijn een parttime (40%) aanstelling aan het toenmalige Rooms Katholiek Ziekenhuis in Groningen op de afdeling Pathologie (hoofd J.C. van de Griendt), tegelijkertijd volgde zij een cursus cytologie in Leiden (dr M.E. Boon). In 1980 volgde een parttime (40%, vanaf juli 1988 70%, vanaf november 1992 80%) aanstelling op de afdeling cytologie (hoofd dr D.I. Blonk) van de dr Daniel den Hoed kliniek, vanaf mei 1989 afdeling pathologie DDHK (hoofd dr Th. M. Vroom, vanaf 1992 dr S.C. Henzen-Logmans). Tevens is zij vanaf 1983-heden, als docent cytologie verbonden aan de Leidse Hogeschool, afdeling laboratorium onderwijs, in Leiderdorp. Daarnaast is zij vanaf september 1996 consulent cytologie van de Stichting Cytodiagnostisch Onderzoek (CDO) in Rotterdam (hoofd dr H. Beerman).
Zij is getrouwd met Gijs Kuenen, en heeft 3 kinderen en 2 kleinkinderen.

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