Eeva Rahko

EVALUATION OF TUMOR SUPPRESSOR GENE p53, ONCOGENE c-erbB-2 AND MATRIX-METALLOPROTEINASE-9 AS PROGNOSTIC AND PREDICTIVE FACTORS IN BREAST CARCINOMA
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Oulu, Finland

Abstract

Breast carcinoma is the most common malignancy in females in western countries. Classical prognostic factors such as the size of a primary tumor and the presence or absence of axillary lymph node metastases, malignancy grade and hormone receptor status reflect the subsequent risk of disease recurrence after primary therapy and the need for adjuvant therapies. However, most breast carcinomas are detected in the early stage of the disease and the value of these classical prognostic factors is limited. There is also a great need to find new factors predicting the clinical efficacy of the anticancer drugs available. In this thesis tumor suppressor gene p53, oncogene c-erbB-2 and matrix metalloproteinase-9 were evaluated for their prognostic relevance in breast carcinoma patients treated in Oulu University Hospital, and matrix metalloproteinase-9 was also analyzed in women with premalignant lesions in the breast tissue in order to examine its role in breast carcinogenesis. Histological analyses were carried out from formalin-fixed, paraffin-embedded primary tumor specimens and p53, c-erbB-2 and matrix metalloproteinase-9 (MMP-9) statuses were systematically analyzed by immunohistochemistry.

P53 expression correlated with disease-free survival and overall survival in patients with early-stage breast carcinoma, regardless of adjuvant antiestrogen therapy. The co-expression of p53 and c-erbB-2 characterizes a tumor type with a clinically aggressive course of breast carcinoma. The clinical efficacy of anthracyline-based chemotherapy in metastatic carcinoma might be limited in patients with p53 expression in a primary tumor. When postmenopausal patients with lymph node metastases and receiving adjuvant antiestrogen therapy were examined, MMP-9 expression indicated a slightly greater risk of breast carcinoma recurrence in patients with estrogen receptor negative tumors. Hyperplastic breast tissue and invasive breast carcinoma lesions expressed some MMP-9 immunopositivity. However, the strongest positivity was seen in ductal carcinoma in situ samples, suggesting that MMP-9 participates in breast carcinogenesis in the preinvasive phase.

Keywords: antiestrogen, breast cancer, c-erbB-2, carcinogenesis, MMP-9, p53, predictive factor, prognosis
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Oulu, on Good Friday, 2007

Eeva Rahko
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADH</td>
<td>atypical ductal hyperplasia</td>
</tr>
<tr>
<td>AEC</td>
<td>3-Amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>AI</td>
<td>allelic imbalance</td>
</tr>
<tr>
<td>AI</td>
<td>aromatase inhibitor</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>ALH</td>
<td>atypical lobular hyperplasia</td>
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<tr>
<td>BM</td>
<td>basement membrane</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>C</td>
<td>cyclophosphamide</td>
</tr>
<tr>
<td>CAF</td>
<td>cyclophosphamide-doxorubicin-5-fluorouracil</td>
</tr>
<tr>
<td>CISH</td>
<td>chromogen in situ hybridization</td>
</tr>
<tr>
<td>CMF</td>
<td>cyclophosphamide-methotrexate-5-fluorouracil</td>
</tr>
<tr>
<td>CR</td>
<td>complete response</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma <em>in situ</em></td>
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<tr>
<td>DFS</td>
<td>disease-free survival</td>
</tr>
<tr>
<td>DIN</td>
<td>ductal intraepithelial neoplasia</td>
</tr>
<tr>
<td>DNA</td>
<td>deoksiribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>EIA</td>
<td>enzyme immuno assay</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor α</td>
</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor β</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>FEC</td>
<td>5-fluorouracil-epirubicin-cyclophosphamide</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HER</td>
<td>human epidermal growth factor receptor</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LCIS</td>
<td>lobular carcinoma <em>in situ</em></td>
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LDL  low-density lipoprotein
LHRH  luteinizing hormone releasing hormone
LOH  loss of heterozygosity
MTX  methotrexate
MAP  mitogen activated protein
MBC  metastatic breast cancer
MCF-7  estrogen receptor positive cell line
MDM2  mouse double minute 2
MMP  matrix metalloproteinase
mRNA  messenger ribonucleic acid
NPI  Nottingham prognostic index
NO  nitric oxid
OS  overall survival
PAI-1  urokinase-type plasminogen activator inhibitor
PBS  phosphate-buffered serum
PDGF  platelet derived growth factor
PR  partial response
PR  progesterone receptor
RR  risk ratio
SD  stable disease
SERM  selective estrogen receptor modulation
SH  zinc-interacting thiol group
SPF  S-phase fraction
TAM-TOR  tamoxifen-toremifene
TDLU  terminal duct lobular unit
TGF  transforming growth factor
TIMP  tissue inhibitor of matrix metalloproteinase
TNM  tumor nodus metastasis
T47D  mutant p53 tumor cell line
UICC  International Union against Cancer
uPA  urokinase-type plasminogen activator
VEG  Fvascular endothelial growth factor
WHO  World Health Organization
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.


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7 Conclusions

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Original papers
1 Introduction

Breast cancer represents the most common malignancy in females in Western countries (Tavassoli & Devilee 2003). During the last decades, the incidence of breast carcinoma has been increasing globally, also in Finland. In the last few years, around 4,000 patients have been diagnosed with breast cancer and around 800 women have died each year due to their malignant breast disease in Finland (Finnish Cancer Registry 2005). At the same time, thanks to screening programs primary diagnosis has become more efficient and most breast cancer cases are detected at an early stage of the disease, making the prognostic information of the classical TNM classification limited. On the other hand, screening mammography detects premalignant lesions whose clinical significance and treatment strategy is partly unclear (Ernster et al. 2002, Tavassoli & Devilee 2003).

Despite the favorable progress seen in the survival curves of breast cancer patients, one fifth of the cases with an early stage disease still experience a relapse during the follow-up (Chia et al. 2004). There is a great need for new prognostic and predictive factors to evaluate further the need and selection of anticancer therapies in adjuvant setting, not only in order to treat efficiently patients at a high risk of relapse, but also to preserve low-risk patients from potentially harmful treatments. In addition, because breast carcinoma is the most common cancer in females, the selection of patients to adjuvant treatments is significant also from an economic point of view (Oestreicher et al. 2005, Gil et al. 2006).

Despite the challenges related to the investigation of the carcinogenetic process in breast tissue and the evaluation of potential new prognostic and predictive markers, this work aimed to examine this area of interest further, particularly by focusing on p53, c-erbB-2 and MMP-9 and their immunohistochemical expression in breast carcinoma samples and MMP-9 expression in a series of benign and premalignant lesions of the breast.
2 Review of the literature

2.1 Breast cancer

2.1.1 Breast carcinogenesis

Breast cancer is one of the most common human malignancies and accounts for one quarter of all carcinomas in females. The majority of the tumors are adenocarcinomas, believed to be derived from the mammary epithelium, especially cells from the terminal duct lobular unit (TDLU) (Tavassoli & Devilee 2003). The terminal ducts are lined by a layer of luminal epithelial cells where ductal and lobular proliferations originate from, and they are surrounded by myoepithelial cells and the basement membrane. These two cell types can be distinguished by immunohistochemistry as they express different cytokeratines (Clarke et al. 2002). Both ductal and lobular cells are thought to arise from a shared, pluripotent stem cell (Pechoux et al. 1999, Clarke et al. 2002), and recent data support the idea of specific breast stem cells (Ponti et al. 2006, Shackleton et al. 2006). It has been hypothesized that cancer might result when mutations accumulate in these self-renewing stem cells (Al-Hajj et al. 2003, Dontu et al. 2003, Gudjonsson & Magnusson 2005, Woodward et al. 2005), and this hypothesis would explain the carcinoma protective effect of early full-term pregnancies. Breast tissue attains its maximum development during pregnancy and lactation, and when stem cells go through the process of permanent differentiation they become more refractory to further carcinogenesis and the remaining stem cells are also fewer in number (Russo et al. 2006). It has generally been believed that sporadic breast cancer results from the accumulation of acquired and uncorrected mutations causing activation of oncogenes (MYC, cyclin D1, c-erbB-2) and inactivation of tumor suppressor genes such as p53, which leads to abnormal cell proliferation, loss of cell cycle control and aberrant apoptosis (Barnes & Gillette 1998, Gillette et al. 1998, Andrechek et al. 2000, Kenemans et al. 2004, Way & Lin 2005, Corzo et al. 2006).

When genetic alterations develop in somatic cells it may lead to a sporadic carcinoma, but genetic damage in germ line cell causes a risk of a familial breast cancer. Further inactivation of a healthy allele of a tumor suppressor gene, for example, would be a hallmark event in an oncogenic pathway (Kenemans et al. 2004). Germline mutations in BRCA1 and BRCA2 genes account for approximately two-thirds of autosomal dominantly inherited breast cancers. The BRCA1 gene located on the long arm of chromosome 13 was isolated in 1994 (Miki et al. 1994) and the BRCA2
gene was identified soon after that, also locating in chromosome 13 (Wooster et al. 1995). In normal cells, BRCA1 and BRCA2 are nuclear proteins and their messenger-RNA (mRNA) expression is controlled during the cell cycle. Hundreds of mutations have been identified in both genes, but no single critical functional domain has been identified so far (Bove et al. 2002). However, it is very likely that additional genes predispose to breast carcinoma (Stratton 1996). Recently, the mutation rate of a BRCA2-binding protein, PALB2 (partner and localizer of BRCA2), was shown to be significantly elevated in familial breast cancer cases in Finnish population. The mutated PALB2 gene produces a truncated protein causing reduced BRCA2-binding capacity and further deficient in DNA repair capability (Erkko et al. 2007).

There are also cancer syndromes where the risk of breast carcinoma is elevated. Tumor suppressor gene p53 exists at low levels in normal cells and acts as a “guardian of genome” as it causes cell cycle arrest due to DNA damage and enables DNA repair. Li and colleagues identified germline p53 mutations in families with multiple malignancies and early onset breast cancers (Malkin et al. 1990). Healthy tumor suppressor gene PTEN regulates cell proliferation by inducing G1 phase cell cycle arrest. A rare hamartoma syndrome (Cowden’s disease) is caused by a mutation in the PTEN gene on chromosome 10 and affected patients often suffer from breast carcinoma at a young age (Nelen et al. 1996, Eng 1997). Increased risk of breast carcinoma also associates with hereditary non-polyposis colorectal carcinoma syndrome, HNPCC (Bove et al. 2002).

Carcinogenesis is a multistep process where both genetic alterations and epigenetic changes can be seen in the cells. Epidemiological studies have suggested that ductal proliferation associates with carcinogenesis in breast tissue and that the majority of breast carcinomas originate from precursor lesions of various degrees of hyperplastic changes (Allred & Mohsin 2000, Allred et al. 2001, Aubele et al. 2002), which has been supported by genetic studies. Genetic alterations cause allelic imbalance (AI) and therefore the loss of heterozygosity (LOH). A trend of LOH accumulation has been demonstrated in a process of malignant transformation (Rosenberg et al. 1997, Chauqui et al. 1997, O’Connell et al. 1998). A reduced expression of cell adhesion molecule E-cadherin has been associated with tumor dedifferentiation (Siitonen et al. 1996). Major genetic changes seem to appear during the transition from normal breast tissue to ductal carcinoma in situ (DCIS) (Ottesen et al. 1995), while the gene-expression alterations between DCIS and infiltrative cancer reveal extensive similarities (Ma et al. 2003). It is therefore presumed that most invasive breast carcinomas originate from in situ cancer (Boecker
et al. 2001, Burstein et al. 2004). Morphologically it is possible to distinguish a transition from normal epithelium to in situ ductal hyperplasia with various atypical characteristics, further to in situ carcinoma and finally to invasive carcinoma (Allred et al. 2001, Aubele et al. 2002). On the other hand, most precursor lesions will never progress to invasive carcinoma (Ottesen et al. 1995, Allred et al. 2001) and invasive cancer may develop without evidence of concurrent precursor lesions (Andersson & Miller 1994). Thus, the chronology of genetic alterations and the exact mechanism of malignant transformation in breast tissue are mainly unknown.

Recently, breast carcinogenesis has been regarded as a complex series of stochastic genetic events that lead to divergent and distinct pathways towards infiltrative breast carcinoma (Figure 1) (Simpson et al. 2005).

The clinical importance of tumor microenvironment for tumor progression has been discussed. One could assume that once the genetic preconditions for a malignant transformation are present, the interaction between a tumor and its microenvironment becomes more restrictive in promoting a malignant progression, such as infiltration and metastatic potential, leading eventually to a clinically evident and even metastatic carcinoma (Kurose et al. 2001, Nofech-Mozes et al. 2005, Schedin & Elias 2004). The degradation of the extracellular matrix (ECM) is crucial in this context, and the elevated matrix metalloproteinase (MMP) activity is therefore of clinical interest. A range of peptide growth factors such as transforming growth factor-\(\alpha\) (TGF-\(\alpha\)), epidermal growth factor (EGF), insulin-like growth factors (IGFs) and fibroblast growth factors (FGFs) secreted either by tumor cells or surrounding stromal cells also enhance tumor progression (Clarke et al. 2002). Estrogen can enhance the secretion of local growth factors (Clarke et al. 2002), but it also has mitotic activity itself in hormone-dependent breast cancer cell lines (Lippman et al. 1976, Osborne et al. 1985). An increased sensitivity of tumor cells to estrogen may exist due to aberrant expression of estrogen receptor \(\alpha\) (ER\(\alpha\)) (Shoker et al. 1999), due to ER\(\alpha\) mutation leading to enhanced ligand sensitivity of a receptor (Wang et al. 2001), or due to the downregulation of receptor corepressors or increased activity of coactivators; all these may promote the malignant process (Clarke et al. 2002, Anzick et al. 1997, Bautista et al. 1998).

### 2.1.2 Premalignant breast disease

There are many types of benign breast tissue lesions and only a few of them have demonstrated significant premalignant potential. The most clearly characterized premalignant lesions are referred to as atypical ductal hyperplasia (ADH), atypical...
lobular hyperplasia (ALH), ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). In these lesions one can see a loss of growth control, but no ability or sign of infiltration to adjacent tissue or metastases (Allred et al. 2001). Atypical lobular hyperplasia (ALH) is characterized by small and mildly atypical epithelial cells beginning to fill the ducts (Russo & Russo 1997), while lobular carcinoma in situ (LCIS) is diagnosed when these cells accumulate causing proximal duct distension to a large extent. Both lesions are regarded as bilateral risk factors for future infiltrative carcinoma (Page et al. 1986), presumably indicating widespread genetic damage in breast epithelial cells (Allred et al. 2001).

Atypical ductal hyperplasia is formed from a uniform epithelial population of cells that are small, round and cuboidal in shape; these hyperchromatic cells are regularly arranged. Mitoses are only infrequently seen and nuclei are evenly distributed. Cells may form bridges in cribriform type (Pinder & Ellis 2003). The ADH lesion is less than 2mm in largest diameter; greater changes, especially ones occupying several duct spaces, are more likely to be diagnosed as DCIS (Tavassoli 1992). ADH increases the risk of infiltrative breast carcinoma about four to five times compared to the general population (Dupont et al. 1993, London et al. 1992, Fitzgibbons et al. 1998) and the risk is even greater if the patient has a family history of breast cancer (Page et al. 1992). Estrogen receptor (ER) positivity is very high in ADH where almost all cells are ER positive (Barnes & Masood 1990, Mohsin et al. 2000), while in premenopausal women approximately 20% of normal epithelial cells express ER. In postmenopausal women ER positivity increases to approximately 50% of epithelial cells. Prolonged estrogen exposure and very high ER levels observed not only in ADH but also in many other premalignant lesions may contribute to increased cell proliferation (Mohsin et al. 2000, Allred et al. 2001). Oncogene c-erbB-2 positivity has only rarely been detected in ADH lesions and the p53 gene status seems to be normal as well (Gusterson et al. 1988, Lodato et al. 1990, Bartek et al. 1990, Chitemere et al. 1996). On the other hand, allelic imbalance has been detected in up to 50% of atypical hyperplasias (ADH and ALH) sharing many LOH phenotypes with invasive breast carcinoma lesions, supporting the hypothesis that atypical hyperplastic lesions might be direct precursors of infiltrative breast cancer (Lakhani et al. 1995, Rosenberg et al. 1997, O’Connell et al. 1998). The new histopathological classification for intraductal proliferative lesions has been revised by WHO using new ductal intraepithelial neoplasia terminology (DIN) (Tavassoli & Devilee 2003).
Ductal carcinoma in situ

The differential diagnosis between ADH and DCIS is often not clear because there is no general agreement on whether quantitative criteria should be used to separate ADH from low grade DCIS (Tavassoli & Devilee 2003). Ductal carcinoma in situ is generally described as a proliferation of malignant epithelial cells within the breast parenchymal structures disrupting the architecture of the breast glandular epithelium, but without basement membrane destruction, however, and preserving the surrounding myoepithelial cell layer, which remains intact (Pinder & Ellis 2003, Leonard & Swain 2004). DCIS lesions can be divided into five groups (comedo, cribriform, papillary, solid and micropapillary) according to their architectural rearrangement. There are many classification systems for DCIS (Leonard & Swain 2004). Recent classifications use only cytonuclear grade (high, intermediate or low) or in combination with necrosis (presence or absence of comedo necrosis) or cell polarization (architectural differentiation), but it is recommended that additional information on margin status, size and extension as well as abundance of calcification be documented in the histopathological report (Leonard & Swain 2004, Tavassoli & Devilee 2003). Microinvasion is defined as an extension of cancer cells beyond the basement membrane in surrounding tissue, but no single focus should exceed 1mm in greatest dimension (AJCC 2002).

Patients with DCIS are considered to have a relative risk of 8–11 for developing infiltrative cancer (Fitzgibbons et al. 1998). Available data also suggest that up to 15–30% of DCIS lesions eventually develop into invasive breast carcinoma (Betsill et al. 1978, Ottesen 2003, Sanders et al. 2005, Warren et al. 2005). However, it is postulated that the vast majority of DCIS is never detected or does not develop clinically evident malignant disease; an autopsy study suggested that around 9–20% of women will develop DCIS during their lifetime (Nielsen et al. 1987, Welch & Black 1997). Although there is a lack of biological markers to predict the clinical behavior or the malignant transformation of a single DCIS lesion, some clinical and pathological characteristics are valuable in providing prognostic information: women with a large, high-grade DCIS with necrosis and positive surgical margins are at a high risk of further local recurrence of which 50% appear to be invasive, particularly when treated primarily with lumpectomy alone (Betsill et al. 1978, Page et al. 1982, Leonard & Swain 2004, Bijker et al. 2006). Large DCIS with comedo-type histology is at risk of having microinvasive foci, which may in turn result in malignant lymphatic spread and axillary node metastasis (Wassenberg et al. 2002). Poorly differentiated histological types associate with a risk of further
distant metastases (Bijker et al. 2001). The clinical importance of DCIS has increased since the incidence of this lesion has increased dramatically due to screening mammography where calcifications that may indicate DCIS are easily detected (Ernster & Barclay 1997, Ernster et al. 2002).

Estrogen and progesterone receptors are expressed in 50–60% of DCIS lesions, and in genetic studies HER2 (human epithelial growth factor receptor 2) gene alterations are seen in approximately 40% of all DCIS, this alteration being much more frequent (60–80%) in the comedo/high-grade DCIS (Leonard & Swain 2004, Millis et al. 1996, Beckman et al. 1997). P53 mutations have also been detected in DCIS lesions, correlating highly with histological grades: p53 alterations are rare in low-grade DCIS, but exist in 5% and 40% of intermediate and high-grade lesions, respectively (Done et al. 2001, Walker et al. 1997, Allred et al. 2001). Allelic imbalance is more frequently seen in DCIS than in ADH foci. More than 100 AIs have been detected in 17 separate chromosomes studied (Radford et al. 1993, Chappell et al. 1997, Waldman et al. 2000), and especially comedo-type DCIS may reveal several LOH even in a single lesion (O’Connell et al. 1998). In most cases synchronous DCIS and infiltrative breast lesions share their LOH phenotypes, providing evidence of their evolutionary relationship (Zhuang et al. 1995, Fujii et al. 1996, Dillon et al. 1997); however, exceptions to this do exist (Allred et al. 2001).

Taken together, most of the molecular changes that can be detected in invasive breast cancer are already present in DCIS, although DCIS has not been assumed to be a clinically malignant phenotype (Bijker et al. 2001, Mommers et al. 2001, Ottesen 2003). Cytogenetic studies suggest that low-grade DCIS tends to progress into low-grade invasive carcinoma, while high-grade invasive lesions evolve from high-grade DCIS (Nofech-Mozes et al. 2005, Leonard & Swain 2004, Boecker et al. 2001, Wärnber et al. 2001, Mommers et al. 2001, Simpson et al. 2005) (Figure 1).
2.1.3 Epidemiology, etiology and prognosis of invasive breast cancer

Breast cancer is the most common malignancy affecting women worldwide (Stewart & Kleihues, 2003). According to latest estimates, more than 1,050,000 new cases are diagnosed annually, the incidence rates being the highest (>80 per 100,000 population per year) in developed countries (Ferlay et al. 2001). In 1998 more than 400,000 women died due to breast cancer globally, which represented 1.6% of all female deaths. The pattern of increasing incidence can be seen worldwide as well as in Finland, where the breast is the leading primary site of cancer in females. There were 4,027 new breast carcinoma cases in Finland in the year 2005 with an incidence rate of 86.7 per 100,000. Age-adjusted mortality rate of breast cancer per
100,000 person years has not changed markedly over the past two decades – the mortality rate was 16.1 in 1982–1986 and 16.3 in 1997–2001 – but recently breast cancer mortality rates have, however, started to decline, mainly because of early detection and advances achieved with the use of adjuvant treatment (Stewart & Kleihues 2003). The age-adjusted mortality rate of breast carcinoma was 14.8 in 2003. At present, around 85% of breast cancer patients survive five years or longer after primary diagnosis in Finland (Finnish Cancer Registry, 2005).

The growing incidence of breast carcinoma worldwide has many etiological factors, mainly hormonal and lifestyle-related. Early menarche, late pregnancy or nulliparity, late menopause and menopausal hormonal replacement therapy correlate with increased risk of breast malignancy (Stewart & Kleihues, 2003). A Western lifestyle often means a diet poor in fruit and vegetables and rich in meat and high caloric intake, as well as lack of physical activity, which all are related to an increased carcinoma risk (COMA Working Group on Diet and Cancer, 1998). Especially in postmenopausal women breast cancer risk increases with increasing body mass index (BMI), this being largely a result of the associated increase of exogenous and endogenic bioavailable estrogens, particularly estradiol (Key et al. 2003). Approximately 5–10% of breast carcinoma cases have a genetic background, and especially mutations in BRCA1 and BRCA2 genes predispose to familial type of breast cancer (Newman et al. 1988, Robson et al. 2001). A strong family history of breast carcinoma, cases in young women, multifocal carcinomas and cases with ovarian carcinoma are frequently seen in this condition.

2.1.4 Histopathological types of invasive breast carcinoma

Invasive breast cancer encompasses a heterogeneous group of carcinomas that vary in terms of morphological features and clinical behaviour. Invasive ductal carcinoma represents the majority (70%) of all carcinomas and fails to exhibit sufficient characteristics to achieve classification as a specific histological type. Ductal carcinomas are thought to derive from mammary ductal epithelium, in contrast to lobular carcinoma (10–20% of infiltrative lesions) that might arise from acinar cells of terminal lobules, although this classification has been criticized, too (Tavassoli & Devilee 2003, Simpson et al. 2005, Joensuu et al. 2006ba). Ductal carcinomas often represent with a distinct tumor, and adjacent DCIS is often present. Lung and liver represent the most frequent distant metastatic sites in ductal carcinomas. Lobular type of breast cancer has a more diffuse growth pattern, with non-cohesive cells dispersing in a fibrous stroma. When developing distant relapses, lobular carcinoma
metastasizes more often into bone, the gastrointestinal tract, meninges and ovary compared to ductal type of BC (Tavassoli & Devilee 2003). Tubular, medullary, mucinous and papillary carcinomas are well differentiated subtypes of infiltrative carcinomas representing the minority (around 10%) of all invasive carcinomas and associate with a more favorable survival rate compared to ductal and lobular breast cancers (Tavassoli & Devilee 2003). In a domestic material 92% of patients with a special type of histology and node negative breast cancer were free of distant recurrences at 8 years of follow-up, the proportions being 83% and 91% for ductal and lobular carcinomas, respectively (Carstens et al. 1985, Clayton 1986, Tavassoli & Devilee 2003, Joensuu et al. 2006a).

2.1.5 Tumor parameters as classical prognostic factors

Individual risk of disease recurrence can be estimated by tumor size reflecting how long the tumor has been present and by axillary lymph node metastases reflecting the clinical stage (Bundred 2001, Carter et al. 1989, Blanco 1980). Large primary tumor burden correlates to breast cancer mortality both in node-positive and node-negative patients. The significance of axillary nodal metastases was not introduced until the 1960s (Auchincloss 1963), but it has been shown to be the most potent single prognostic factor, and even the prognostic importance of the absolute number of involved lymph nodes is supported by large clinical data (Fisher & Slack 1970). Even patients having small primary tumors but four or more positive nodes have poor survival (Carter et al. 1989, Nemoto et al. 1980). On the other hand, micrometastases (axillary lymph nodes with metastatic foci smaller than 2 millimeters in diameter) indicate only a modest survival disadvantage in the long-term follow-up (Maibenco et al. 2006). Tumor size, axillary nodal status and the presence or absence of distant metastases is the basic information for the TNM classification and for the clinical stage of the disease dividing patients into different prognostic groups. The survival rate after eight years of follow-up is 90% in Stage I patients, 70% stage II patients but only 40% in patients with stage III disease (Jardines et al. 2004). The most widely used systems to stage breast cancer are the classification from the International Union Against Cancer (UICC) (Table 1) and the American Joint Committee on Cancer (AJCC).
It was first recognized in 1890 that according to some morphological tissue features, tumors can be divided accordingly to malignancy grade (Hansemann 1890). The first formal grading of breast carcinoma into three malignancy grades and its relationship to the clinical behavior of breast cancer in patients occurred in the 1920s (Greenough 1925). Modern grading methodology is still based upon these fundamental criteria of tubule formation, nuclear pleomorphism and mitotic activity, but semiquantitative methods have also been introduced to diminish subjectivity in the interpretation (Bloom & Richardson, 1957, Black & Speer 1957, Elston & Ellis 1991). Most notably, histological grading provides independent prognostic information (Lundin et al. 2001). Clinical data confirm that high malignancy grade worsens the clinical outcome even in early-stage breast cancer patients (T1N0 or T1N1) whose 10-year survival rates are 95% in malignancy grade I, 91% in grade II and 84% in grade III tumor in a large American population (Henson et al. 1991). Malignancy grade seems to have more prognostic power than hormone receptor sta-
status in patients with a small local tumor receiving no adjuvant therapies (Samura et al. 1999).

The role of estrogen and progesterone receptors and the estradiol-induced effects in breast carcinoma cells was first reported in the 1970s (Jensen & Desombre 1972), and the prognostic importance of the hormone receptor status became evident in large later series. Estrogen and progesterone receptor negativity has been associated with a compromised clinical outcome (Chevallier et al. 1990, Castagnetta et al. 1992, Mason et al. 1983, Lundin et al. 2006), but controversial data exist as well (Aamdal et al. 1984, Butler et al. 1985). Interestingly, the prognostic relevance of hormone receptor status seems to be to some degree time-dependent, and a relation to survival might only be seen in a long-term clinical follow-up (Coradini et al. 2000). The disagreement in results may be explained by heterogeneity in analytic techniques, variety in patient populations and follow-up times (Coradini et al. 2000).

The Nottingham Prognostic Index (NPI) is a clinico-pathological classification system for breast cancer based on primary tumor size, malignancy grade and axillary lymph node status (Galea et al. 1992), where a score of less than 3.4 associates with favorable prognosis (NPI = 0.2 x tumor diameter in centimeters + lymph node stage + histological grade). High and low NPI scores have recently been shown to correlate with gene expression patterns and molecular signature of breast tumors in microarray studies (Miller et al. 2004, Yu et al. 2004).

2.1.6 Other prognostic factors

Peritumoral lymphatic vessel or vascular invasion of tumor cells correlates with compromised outcome of patients (Rosen et al. 1989, Mirza et al. 2002). The proliferation rate of a tumor can be evaluated by measuring S-phase fraction (SPF) by flow cytometry indicating cells synthesizing DNA actively or by measuring immunohistochemically the proliferation antigen Ki-67, which is a nuclear protein expressed in proliferative phases of the cell cycle. High values of both markers have indicated more aggressive behavior of breast cancer (Wenger & Clark 1998, Vero nese et al. 1993, Trere et al. 2006), as well as aberrant DNA ploidy (Kallioniemi et al. 1987, Pinto et al. 2006). However, high expression of Ki-67 antigen also seems to indicate sensitivity to chemotherapy (Urruticoechea et al. 2005). In patients who have not received any adjuvant therapies, high mitotic activity has predicted independently compromised disease-free survival (Manders et al. 2003) and overall survival (Baak et al. 2005).
The urokinase-type plasminogen activator (uPA) involving ECM degradation and its inhibitor (PAI-1) have been shown to correlate with poor outcome in early stage breast carcinoma (de Witte et al. 1999, Harbeck et al. 2002), as has the ECM component hyaluronan (Auvinen et al. 2000). CD44 is a transmembrane glycoprotein involved in cell motility, invasiveness and angiogenesis, and high expression of CD44 has indicated an adverse outcome in breast carcinoma (Ma et al. 2005, Watanabe et al. 2005). Tenascin and versican are ECM molecules with anti-cell-adhesive properties, and elevated expression of these ECM components has been associated with breast cancer recurrence and survival (Jahkola et al. 1996, Ioachim et al. 2002, Suwiwat et al. 2004). Loss of normal functioning anti-apoptotic, mitochondrial protein Bcl-2 in the tumor has been reported to predict compromised survival of patients (Callagy et al. 2006, Rolland et al. 2007). Numerous apoptosis regulators and angiogenesis-related proteins such as tumor necrosis growth factor and vascular endothelial growth factor have also been investigated in prognostic studies with varying results (Cianfocca & Goldstein 2004, Esteva & Hortobagyi 2004). Lysosomal protease cathepsin D can be overexpressed in breast cancer cells, and high levels of it have been associated with shorter breast cancer specific survival (Isola et al. 1993, Rodriguez et al. 2005). Cyclin E protein accelerates the cell cycle and has been reported to be involved in overall survival in breast carcinoma in a recent meta-analysis (Wang & Shao 2006), but no such association was reported in a large randomized adjuvant chemotherapy study (Porter et al. 2006). E-cadherin is a cell adhesion molecule that is expressed in healthy breast tissue, but a reduced expression or a total loss of expression might appear in a malignant tissue, which seems to be an indicator of poor outcome (Siitonen et al. 1996, Rakha et al. 2005).

Recently, genetic profiling of a breast tumor utilizing microarray technique identified good and poor prognostic groups in breast carcinoma patients (van de Vijver et al. 2002, Buyse et al. 2006). However, the results were not conclusive and concerns about the methodology of the studies have been put forward. The prognostic value of gene expression profiling thus remains to be validated in unselected, large patient populations (Ein-Dor et al. 2005, Ransohoff 2005). Mammography screening has also shown to be associated with prognosis in BC: breast cancer tumors detected by screening have had lower malignant potential and patients have had more favorable survival compared to cases detected outside of screening in domestic studies (Hakama et al. 1995, Joensuu et al. 2004).
2.1.7 Local therapy of invasive breast cancer

Surgical therapy

Breast cancer was regarded as an incurable disease until Halsted reported better clinical outcome in radically operated patients (Halsted 1894). Definitive local treatment by a surgical approach has remained the cornerstone of breast carcinoma treatment over the decades, but great effort has been made to find a better balance between treatment radicality and long-term side effects (Gerber et al. 1992). Resection techniques have been developed in combination with post-operative radiotherapy, and it was shown in the 1990s that survival curves of patients after mastectomy or more conservative resection procedures in combination with radiation therapy are comparable and that irradiation reduces local treatment failures (Clark et al. 1992, Veronesi et al. 1993, Fisher et al. 1995). Tumor-free margins are still essential to prevent local recurrences even if radiation therapy is delivered (Poggi et al. 2003).

Sentinel node dissection procedure in the treatment of breast cancer was first introduced in the early 1990s (Krag et al. 1993) and has potential for reducing morbidity such as lymphedema and sensory changes in the ipsilateral arm, which are common side effects related to full axillary node dissection procedure. In a recent meta-analysis the false-negative rate of sentinel node biopsy was on average 7%, but it varied from 0% to up to 29% according to the clinical experience of the surgeon; this demanding technique emphasizes the importance of a qualified team (Kim et al. 2006a). Despite limited survival data from controlled clinical trials, sentinel node biopsy has become a widely used technology in staging breast carcinoma (Kim et al. 2006a, Schrenk et al. 2001) because the quality of life of patients has been shown to be better after sentinel node biopsy than after full axillary node dissection (Leidenius et al. 2005, Mansel et al. 2006). The sensitivity of the procedure can be raised as high as 96% in experienced centers (Cox et al. 2000).

Radiation therapy

The clinical use of postoperative radiation therapy in breast cancer patients was first introduced in the 1940s by McWirtcher who reported better 5-year survival curves in patients receiving post-operative radiation therapy compared to patients who only underwent an operation (McWirtcher 1948). During the decades that followed there was great controversy in clinical studies as well as debate over the survival benefit (Fisher et al. 1970, Fletcher 1972), but later randomized trials showed inef-
vitably that irradiation after mastectomy reduced significantly the risk of local recurrence in patients with involved axillary nodes (Recht et al. 2001); the reduction of local recurrences after breast conserving surgery can be as high as 68%, and an improved local disease control also has an impact on overall survival (Arriagada et al. 1995, Early Breast Cancer Trialists 2000, 2005). The survival benefit of irradiation can be achieved despite using systemic adjuvant therapy (Overgaard et al. 1999, Recht et al. 2001). However, there is still insufficient evidence of the benefit of routine irradiation of the axilla or supraclavicular area in cases with complete axillary evacuation and only minimal nodal involvement. Patients with large primary tumors (>5cm in largest diameter) are suggested to be irradiated routinely after mastectomy, but patients with smaller tumors can also be treated if they have biologically aggressive tumors (Truong et al. 2005).

Most institutions worldwide use a total dose of 50 Gray in 2 Gray daily fractions, given five times weekly and using photon and/or electron treatment, covering the chest wall or remaining breast and lymphatic nodes in axilla, supraclavicular and parasternal area (Recht et al. 2001). The risk of long-term side effects such as lymphedema, radiation pneumonitis, rib fractures and cardiac toxicity is sufficiently low by modern computer-based radiation techniques so that radiation therapy should not be limited because of them, if otherwise indicated (Recht et al. 2001). On the other hand, recent long-term follow-up data raise concern of radiation-induced secondary malignomas such as a doubled lung cancer risk in post-menopausal patients (Rutqvist & Johansson 2006).

2.1.8 Treatment of ductal carcinoma in situ

The treatment of in situ ductal carcinomas deals with many controversies, and there are some concerns about the possible over-treatment of this good prognostic entity. In a large adjuvant study the overall survival of DCIS patients was 95% after seven years of follow-up (Ernster & Barclay 1997, Leonard & Swain 2004, Fisher et al. 1999). In conclusion, lumpectomy followed by local irradiation therapy is a recommended approach, since radiation therapy has been documented to reduce local recurrence rate by 50% after surgical excision (Fisher et al. 1998, Julien et al. 2000, Houghton et al. 2003). However, no survival advantage is gained by post-operative irradiation and the recurrence rate after radiation therapy is still 7–9% (Houghton et al. 2003, Julien et al. 2000). A large randomized trial has suggested an additional benefit of adjuvant tamoxifen therapy in terms of disease-free survival even after post-operative radiation therapy, and tamoxifen reduces particularly contralateral
breast cancer by 55% (Fisher et al. 2001, Fisher et al. 2002). However, another large trial did not show a statistically significant benefit of adjuvant tamoxifen (Houghton et al. 2003). Tamoxifen may cause severe toxicity such as thromboembolic events and endometrial cancer, which limits the wide use of adjuvant therapy in DCIS treatment, but it might benefit patients with a large DCIS of high-grade histology in terms of disease-free survival (Fisher et al. 2001).

2.1.9 Adjuvant endocrine therapy

Treatment rationale

Breast carcinoma is a classic hormone-dependent malignancy, which has been proven in both clinical and experimental conditions. The therapeutic effect of ovarian ablation in metastatic breast cancer was first reported by Beatson in 1896. On the other hand, estrogen plays a major role in normal mammary gland development. Experimental studies have shown that mammary glands are poorly developed in an estrogen null mouse (Couse & Korach, 1999). Breast tissue is regulated by a number of steroid and polypeptide hormones as well as growth factors (Dickson & Lippman 2000), particularly estrogen in menstruation cycles. Estrogen diffuses into cells and binds to the estrogen receptor (ER) protein, which leads to receptor activation as a transcription factor and subsequently expression of many target genes involved in DNA synthesis, cell cycle control, cell survival, and expression of polypeptide growth factors and angiogenic factors (Schiff & Fuqua 2002). Recently, non-genomic action of estrogen receptor activity has been described as well, where cell membrane or cytoplasm associated ER can interact directly with key growth-factor dependent kinases (Kelly & Levin 2001). It is easy to understand that withdrawal of this proliferative stimulus by therapeutic agents causes apoptosis in mammary gland cells and leads to antitumoric effect in malignant conditions. This can be achieved either by inhibiting estrogen binding at receptor level (antiestrogens) or blocking estradiol synthesis (ovarian ablation/aromatase inhibitors).

The first estrogen receptor, later called ERα, was identified in the 1960s (Jensen & Jacobson 1962) and cloned two decades ago (Green et al. 1986). ERα mediates the main effects of estradiol-induced cell proliferation and breast tissue maturation. Later, a second estrogen receptor, ERβ, has also been identified (Kuiper et al. 1996). ERβ has a limited role in breast tissue development; whether it is involved in neoplastic processes is still an open issue (Schiff & Fuqua 2002). ERβ positivity has been associated with ERα positivity and non-aggressive features of a pri-
mary tumor such as node negative disease and low malignancy grade (Järvinen et al. 2000). Progesterone receptor (PR) is also a ligand-activated transcription factor that regulates target gene expression (Tsai & O’Malley 1994) and is involved in ductal branching in breast tissue and alveolar development during pregnancy (Haslam 1988). Although progesterone is a less mitogenic compound in mammary cells than estrogen, recent studies of post-menopausal hormone replacement therapy demonstrate that exogenous progestins administered in combination with estrogen lead to an increased risk of breast cancer when compared to a single estrogen regimen (Magnusson et al. 1999, Ross et al. 2000). While there is still some debate over the prognostic relevance of hormone receptor content in a primary tumor, the predictive value of ER and PR content has been consistently recognized since the hypothesis was first introduced in the mid 1970s (De Sombre et al. 1974, Horwitz & McGuire 1975, Dowsett et al. 2006).

Most breast carcinomas (60%) express estrogen and progesterone receptors, and patients with receptor positive tumors have a 60–70% chance of benefiting from endocrine treatment, compared to less than 10% of patients with receptor negative tumors (Tavassoli & Fattaneh 2003). Estrogen receptor positivity correlates especially with post-menopausal age (Rhodes et al. 2000). However, ER and PR status are not always stable phenotypes of a tumor, since they can in fact change during the natural history of the disease and/or as a consequence of endocrine therapy (Hull et al. 1983, Kuukasjärvi et al. 1996). In clinical work, resistance to a tamoxifen therapy is a major problem and mechanisms of resistance are being studied extensively. Changes in the expression of ERα or ERβ, alterations in co-regulatory proteins and the influences of intracellular kinase signal transduction pathways may result in tamoxifen resistance (Razandi et al. 2003, Ring & Dowsett 2004, Graham et al. 2000). ER protein might mutate, altering its functionality, but this mechanism seems to represent only a minority of tamoxifen resistance (Karnik et al. 1994, Daffada et al. 1995).

**Antiestrogens**

Tamoxifen, clinically the most important antiestrogen, was discovered in the 1960s (Harper & Walpole 1967) and the drug was tested for both anticancer effect and as an ovulation inductor (Cole et al. 1971, Williamson & Ellis 1973). Tamoxifen is the most widely used drug that works through selective estrogen receptor modulation (SERM). The therapeutic effect is gained when tamoxifen antagonizes estrogen receptor function by binding competitively to it, causing the cell to be held at the
G1 phase of the cell cycle (MacGregor & Jordan 1998). On the other hand, tamoxifen is a multifunctional drug, since it has also estrogen-like, agonistic activities based on target tissue. Experimental and clinical evidence has shown that tamoxifen increases the risk of endometrial cancer and prevents osteoporosis based on estrogen-like effects on the endometrium and bone (Cuzick et al. 2003, Fisher et al. 1998). There is no simple explanation as to why estrogen-receptor mediated activation results in agonistic or antagonist effect, but the ERα/ ERβ ratio, activation of ER coactivators, ER corepressors as well as ER dimerization process may modulate the final effect in the target tissue (Levenson & Jordan 2002). Tamoxifen also reduces plasma levels of low-density lipoprotein (LDL) and total cholesterol, and may prevent myocardial infarction (Love et al. 1990, Costantino et al. 1997), but the latter could not be confirmed in a large chemo prevention study (Fisher et al. 1998). In addition to endometrial cancer risk, tamoxifen causes an excess risk of thromboembolic events (venous, pulmonary and stroke), which limits its clinical use (Cuzick et al. 2003). Tamoxifen also causes menopausal symptoms, which reduces the quality of life (Land et al. 2006).

Over the last decades the clinical use of tamoxifen in breast cancer has expanded greatly, and the drug is widely used in all age groups and in both metastatic and adjuvant settings. Indeed, its clinical efficacy even in breast cancer prevention has been established (Fisher et al. 1998, Cuzick et al. 2003, Vogel et al. 2006). Early Breast Cancer Trialists’ Collaborative Group stated that adjuvant tamoxifen reduces breast cancer mortality significantly when tamoxifen was compared to no tamoxifen, and the clinical benefit was most evident in women 50 years or older (EBCTCG 1988). Later clinical evidence showed that adjuvant treatment lasting two years or longer was more efficient than shorter tamoxifen treatment, and further, that five years of tamoxifen adjuvant treatment resulted in a 47% reduction in disease recurrence and a 26% reduction in mortality in all age groups (EBCTCG 1992, 1998). The latest meta-analysis confirmed the results, and longer follow-up times have shown that substantially reduced five-year recurrence rates also significantly reduced 15-year mortality rates (EBCTCG 2005). Until now, tamoxifen has been globally the golden standard in adjuvant endocrine treatment in estrogen receptor positive patients.

Several other antiestrogens have been synthesized, but none of them are routinely used in adjuvant treatment of breast carcinoma. Toremifene and tamoxifen have shown similar efficacy in adjuvant treatment (Holli et al. 2000). Raloxifene is being used in the treatment of osteoporosis, but it has a preventive effect on breast cancer, too (Cummings et al. 1999, Vogel et al. 2006). When treating metastatic
breast cancer, droloxifene was less effective than tamoxifen (Buzdar et al. 2002), and idoxifene treatment seems to bring no additive advantage compared to tamoxifen (Arpino et al. 2003). Fulvestrant, a pure antiestrogen, downregulates ER, reduces tumor progesterone receptor content, and has no known agonistic activity (Robertson et al. 2001). This new regimen is at least as effective as anastrozole in the second-line treatment of metastatic disease after tamoxifen failure (Robertson et al. 2003), but there is so far no clinical evidence of the use in an adjuvant setting.

Aromatase inhibitors

After menopause the ovary is no longer the source of estrogens, but they are still produced in peripheral sites such as adipose tissue and the liver (Hemsell et al. 1974) when aromatase enzyme converts androgens to estrone and estradiol. Aromatase, a cytochrome P450 enzyme complex, is also present in breast tissue, and intratumoral aromatase is a source of local estrogen production in cancerous breast cells (Brueggemeier et al. 2005). Aromatase inhibitors (AI) work by blocking estrogen synthesis (Brodie 2003), but a therapeutic effect might also be gained by preventing the synthesis of estradiol metabolites possessing genotoxic and mutagenic potential (Yue et al. 2005). A first-generation non-steroidal AI, aminoglutethimide, was introduced more than three decades ago with the same clinical efficacy as tamoxifen, but severe side effects due to suppression of corticosteroid production caused frequent treatment discontinuations (Santen et al. 1982). No superior efficacy or better tolerability was achieved by the second-generation regimen formestane (Thurliman et al. 1997). Third-generation AI regimens can be classified by their mechanism of action and by structure: anastrozole and letrozole are nonsteroidal compounds and competitive inhibitors of aromatase enzyme, while exemestane represents a steroidal structure and irreversible binding to aromatase complex (Brueggemeier et al. 2005, Strasser-Weippl & Goss et al. 2005). These third-generation regimens associate with statistically significant improvement in survival compared with tamoxifen or progestins when treating advanced breast carcinoma patients (Mauri et al. 2006). In an adjuvant setting front-line non-steroidal AI regimens anastrozole and letrozole have recently become options of adjuvant endocrine therapy in post-menopausal patients since both regimens have shown superior efficacy in terms of disease-free survival compared to tamoxifen (Baum et al. 2003, Buzdar & Cuzick 2006, Thurlimann et al. 2005). Overall survival benefit has so far been seen in extended adjuvant therapy where node-positive patients received an adjuvant letrozole treatment compared to placebo after five years of tamoxifen.
(Goss et al. 2005), and a recent meta-analysis showed a modest overall survival advantage of third-generation AI (anastrozole, letrozole and exemestane) treatment when administered after 2–3 years of tamoxifen, the absolute risk reduction being 1.2% in an adjuvant setting (Coombes et al. 2004, Jakesz et al. 2005, Bria et al. 2006b).

The survival advantage of adjuvant AI treatment has to be balanced with the observed side effects, although third generation regimens are generally well tolerated (Whelan et al. 2005). Compared to tamoxifen AIs lack the beneficial estrogen-like effect on bone, and adjuvant studies have reported an increase in fractures and osteoporosis of AI-treated patients compared to tamoxifen, which is of clinical relevance (Baum et al. 2003, Thurlimann et al. 2005). Letrozole has been has associated with a higher incidence of hypercholesterolemia and cardiac events when compared to a tamoxifen treated study arm; however, the clinical importance of this might be limited (Thurliman et al. 2005). Aromatase inhibitors cause more often musculoskeletal pain, but menopausal symptoms are usually milder compared to tamoxifen and there is no excess risk of endometrial cancer or thromboembolic events during AI therapy (Baum et al. 2003, Thurlimann et al. 2005, Bria et al. 2006b).

**Other treatment options**

In premenopausal patients with estrogen receptor positive tumors ovarian ablation is an effective and a rapid adjuvant treatment option and the efficacy of this procedure is well documented; however, allocation to ovarian ablation or suppression significantly reduces breast cancer mortality only in the absence of other systemic treatments (EBCTCG 1996, EBCTCG 2005). In premenopausal women ovariectomy or the use of LHRH agonist is as effective as chemotherapy with CMF regimen (SCTBG 1993, Kaufmann 2003). In ER positive patients a combination of ovarian ablation and tamoxifen has shown superior efficacy when compared to CMF (Jakesz et al. 2002). Quality of life and long-term side effects such as premature menopause and related bone loss have to be considered. Reversible, chemical ovarian ablation using LHRH analogy has therefore replaced ovariectomy in most cases.
2.1.10 Adjuvant chemotherapy

Treatment rationale

Despite definitive local surgical and radiotherapeutic treatments, a patient may have microscopic residual disease that subsequently develops into macroscopic metastases with lethal consequences. The ultimate goal of adjuvant chemotherapy as well as adjuvant endocrine treatment is to eliminate these undetectable deposits of residual disease (Day et al. 2005). Cancer cells are usually actively proliferating and therefore exposed to cytotoxic agents. The longest clinical experience of chemotherapeutic use is with cyclophosphamide (C), 5-fluorouracil (5-FU) and methotrexate (MTX), where cyclophosphamide acts as alkylating agent (C) causing cross-links with deoxyribonucleic acid (DNA) and therefore inhibiting DNA synthesis. Antimetabolites interfere with DNA synthesis by inhibiting thymidylate synthetase (5-FU) and dihydrofolate reductase (MTX) enzymes. Anthracyclines (epirubicin and doxorubicin) are antibiotics with cytotoxic effect by topoisomerase II inhibition, and by formation of cytotoxic oxygen-free radicals causing DNA damage. Later introduced chemotherapeutics in breast cancer adjuvant treatment are taxanes (paclitaxel, docetaxel) that bind to microtubules and inhibit mitosis in cell cycle (Chu & DeVita 2003).

Results of adjuvant chemotherapy

Bonadonna and colleagues reported in the 1970s that combination chemotherapy with cyclophosphamide, methotrexate and 5-fluorouracil (CMF) reduced significantly the recurrence rate of adjuvant treated breast cancer patients with axillary lymph node metastases, but at that time there were no long-term data on subsequent survival advantage or side effects (Bonadonna et al. 1976). Since then the clinical importance of adjuvant chemotherapy has been established in numerous clinical studies and meta-analyses. A recently published report of the efficacy of adjuvant CMF with 30 years’ follow-up time demonstrated a 29% reduction in disease recurrences and a 21% reduction in mortality, and chemotherapy seemed to benefit especially younger (premenopausal) women and node-positive patients. Dose density correlated with survival benefit, but the CMF regimen was also shown to be well tolerated in a long-term follow up (Bonadonna et al. 2005).

Anthracycline-based regimens have shown to have superior clinical efficacy over non-anthracycline therapies in a large meta-analysis. Therapies longer than
three to six months do not indicate any survival benefit over shorter courses (EBCTCG 1992, EBCTCG 1998). Adjuvant polychemotherapy is more effective in reducing breast cancer mortality than single-agent therapy. In terms of disease-free survival, polychemotherapy reduced the risk of recurrence by 35% in women aged 50 years or younger and by 20% in women aged 50–69 years. The greatest proportional mortality reduction of 11% was observed in women aged under 50 and having node positive disease. Only few women over 70 years of age have been included in study protocols, and no clear evidence of the benefit of adjuvant chemotherapy has been shown (EBCTCG 1998). In postmenopausal women with further adjuvant tamoxifen, chemotherapy reduces the risk of recurrence (EBCTCG 1992). A recent meta-analysis of 15-year follow-up further established previously described results: six months of anthracycline-based polychemotherapy reduces the annual breast cancer rate by 38% in women younger than 50 years of age, while the reduction was 20% in the age group of 50–69 years. Most notably, polychemotherapy was effective irrespective of the use of tamoxifen, estrogen receptor status, nodal status or other tumor characteristics. In conclusion, breast cancer mortality in middle-aged women with ER-positive disease could approximately be halved by six months of anthracycline-based chemotherapy and a subsequent five years of tamoxifen in 15 years of follow up (EBCTCG 2005). On the other hand, intensive therapies with high-dose chemotherapy and stem cell support bring no survival advantage in early breast cancer (Bergh et al. 2000, Hanrahan et al. 2006).

Results from taxane-based adjuvant chemotherapies have been reported in the last few years (Henderson et al. 2003, Mamounas et al. 2005, Roche et al. 2004). A recent meta-analysis of nine prospective adjuvant trials compared anthracyclines versus paclitaxel or docetaxel combinations (Bria et al. 2006a). Taxane-based combinations improved the outcome by 3.2%–4.6% (absolute benefit) in terms of disease-free survival, while overall survival improved 2%–2.8% (absolute benefit) within the median follow up time of 50–60 months (Bria 2006a). Lymph node positive patients may gain more benefit from taxane-based combination therapy, but further analyses in different subpopulations of patients are needed to evaluate the benefits and risks of taxane-based treatment in detail and with a longer follow-up time.

The benefit of adjuvant chemotherapy needs to be balanced with subsequent side effects. Besides harmful but transient side effects, such as nausea, vomiting and alopecia, also potentially lethal consequences may exist due to neutropenic infections (Chua et al. 2005, Brain et al. 2005, Martin et al. 2005). Frequent adjuvant therapy interruptions are seen particularly in elderly patients due to toxicity (Brunello et al. 2005). A subgroup of breast cancer survivors suffers from long-term
fatigue, worsening the quality of life. (Bower et al. 2000, Nieboer et al. 2005). In premenopausal women chemotherapy may induce ovarian suppression with subsequent bone loss (Vehmanen et al. 2001). Anthracycline therapy may cause cardiac toxicity, especially with higher dosages (Bonner et al. 2004). The issue of secondary malignancies is controversial, but it seems that conventional dosages are safe to use while at higher dosages, alkylating agents and anthracyclines are somewhat leukemogenic (Tallman et al. 1995, Bergh et al. 2000, Smith 2003, Praga et al. 2005).

**Treatment of metastatic breast cancer**

Metastatic breast cancer (MBC) is so far an incurable disease. The goals of systemic therapies are effective symptom palliation, better quality of life and prolongation of overall survival (Smith 2006, Mosconi et al. 2001). In the first line treatment polychemotherapy seems to produce advantages in terms of response rate and time to disease progression over single-agent chemotherapy, but contrary results exist as well (Fossati et al. 1998, Joensuu et al. 1998). Objective response to chemotherapy has been related to a longer survival when compared to non-responding cases (Bruzzi et al. 2005). Anthracycline-based regimens are more effective in terms of DFS when compared to non-anthracycline therapies, but non-anthracycline regimes are better tolerated (Lord et al. 2004). Novel taxane-containing regimens show comparative or even better efficacy and overall survival benefit over non-taxane-containing regimens (Ghersi et al. 2005). There is no standard of care in second-line or further cytotoxic treatment of metastatic breast carcinoma (Bernard-Martinez et al. 2004). A wide range of both endocrine and cytotoxic regimens is available, and patients with HER2 overexpression benefit from trastuzumab treatment (Slamon et al. 2001). In hormone receptor positive patients an endocrine approach is suggested as frontline treatment, especially in the case of non-aggressive disease progression and/or in clinically fragile patients, but a subgroup of patients expressing HER2 in a primary tumor are less responsive to endocrine treatment only (De Laurentiis et al. 2005). Aromatase inhibitors have shown better clinical efficacy than tamoxifen in the first line treatment of MBC (Mouridsen & Gershonovich 2003). Palliative radiation therapy is an effective treatment option to control local symptoms, for example due to metastatic bone disease (Wai et al. 2004). Non-oncological palliative treatment, such as controlling pain and the side effects of systemic therapies are of clinical importance (Hillner et al. 2003, Jost 2005, Donnelly et al. 2002).
2.2 Biological prognostic factors

2.2.1 P53 in breast cancer

P53 function

P53 was first described in 1979 when a protein with a molecular weight of 53,000 was found in chemically-induced sarcoma cells (DiLeo 1979). This tumor antigen was cloned in the early 1980s (Oren & Levine 1983), and soon after that p53 gene was located on chromosome 17 short arm (McBride et al. 1986). Since then p53 has been extremely actively studied and found to be the most common genetic change identified in human cancer (Gasco et al. 2002). P53 belongs to a multigene family that also includes p63 and p73. These latter genes are more often associated with embryonic development and differentiation control, but also contribute to the tumor suppressor activity of p53 (Stiewe 2007). A major role for p53 has been termed “the guardian of the genome” since a rapid increase of this protein is seen in response to DNA damage (genetic stress), hypoxia or loss of normal cell contacts (epigenetic stress) (Lacroix et al. 2006) with a subsequent cell cycle arrest, enabling DNA repair. Where the repair is not possible, p53 induces apoptosis (Shaw et al. 1992) (Figure 2). Tumor cells carrying mutations in the p53 gene are able to resist this repairing process, enabling further cell proliferation and becoming predominant cells in a tumor (MacDonald & Ford 1997). The wild-type p53 protein controls cell cycle by acting as a transcription factor for other genes such as the cyclin-dependent kinase inhibitor p21 and Bcl-2 proteins involving apoptosis. The final cell death is performed by the caspase cascade that is inducted by the release of mitochondrial cytochrome C protein (Reed et al. 2000). Loss of p53 activity induces tumors and disrupts apoptosis in p53-deficient mice (Donehower et al. 1992, Attardi & Jacks 1999).
P53 mutations have been observed in more than a half of human cancer tumor types, the incidence varying from 5% (cervical cancer) to 50% (lung cancer) and even 90% in head and neck squamous cell carcinoma (Hollstein et al. 1991, Kropveld et al. 1999, Lacroix et al. 2006). Somatic mutation of p53 is detected in approximately 26% of breast tumors according to IARC. Several hundreds of genes are either induced or repressed by p53 activity, probably in a dose-dependent manner: a lower amount of p53 may induce cell cycle arrest genes while higher levels are needed to promote apoptotic pathway in a cell (Chen et al. 1996). A microarray study estimates that p53 upregulates at least 500 genes while downregulation occurs in 260 genes by p53 function (Zhao et al. 2000). An association between breast cancer development and p53 was first recognized when a germline mutation in the p53 gene was found to be responsible for the Li-Fraumeni syndrome (Malkin et al. 1990).

Fig. 2. Activation of p53 and cellular responses. (Modified from Blackburn & Jerry 2002.)
P53 gene and protein regulation

The TP53 gene contains 11 exons encoding a protein that has three functionally distinct regions: an acidic N-terminal region which plays a major role in p53 degradation and interaction with other regulatory proteins, a central DNA-binding region and a C-terminal region that involves in tetramerization and regulation of p53 activity; p53 is active only in its homotetrameric form (Lacroix et al. 2006) (Figure 2). Post-translational modification is the key mechanism regulating p53 function. Examples of p53 activation are phosphorylation that associates with p53 protein stabilization, cis/trans isomerization that causes conformal change needed for p53 activation, and acetylation that augments DNA binding. Multiple pathways are also employed to abolish p53 function when its transcriptional activity is no longer needed: for example deacetylation provides a quick mechanism to stop p53 activity, while ubiquination targets p53 for degradation. In cancer the biological effect and activity of p53 depends not only on post-translational modifications but also on its mutations status, the amount of gene product as well as interaction with many co-regulatory proteins. Alterations of p53 regulators and target genes can also lead to compromised p53 function (Lacroix et al. 2006). P53 mutants might show dominance over coexpressed wild-type p53; there are data indicating that the mutation type of p53 gene may determine whether loss of the remaining wild-type p53 allele is necessary for the malignant process (van Oijen & Slootweg 2000).

P53 mutations and diagnostics

In breast cancer about 1,400 distinctive mutations have been identified in the p53 gene (Olivier et al. 2004). The great majority of them (>90%) affect the central part of the gene in exons 5–8 encoding the protein section interacting with DNA, and 90% of them are missense mutations. DNA sequencing provides accurate information of mutation status (Romano et al. 1989, Runnebaum et al. 1991, Bergh et al. 1995). Despite the vast majority of p53 mutations resulting in a negative effect of p53 function, not all mutations are inactivating. Mutant p53 may lose only part of its DNA binding activity (Rowan et al. 1996) and indeed, there exists mutant p53 expressing higher apoptotic activity than normal p53 (Saller et al. 1999). DNA sequencing provides more accurate information on p53 mutation status, but the complete gene should be examined to get reliable information (Bergh et al. 1995). False positive results may also occur with this technique, for example as a consequence of contamination of samples during processing. On the other hand, false
negative result might occur if the mutation is located in a position disadvantageous for a proper primer (Sjögren et al. 1996).

In normal cells the level and activity of p53 is low and virtually undetectable, because the half-life of p53 protein is only 20 minutes (MacDonald & Ford 1997). Mutated p53 proteins usually have increased stability, probably because mutant p53 protein no longer triggers normal degradation, and this leads to p53 accumulation in cells, which can be detected immunohistochemically (Lacroix et al. 2006). The result of immunohistochemical staining correlates with gene mutation status in less than 75% of breast tumors since not every mutation produces a stable protein (IHC false negative finding) and because normally functioning p53 protein might bind to cellular proteins causing protein accumulation (IHC false positive finding) (Norberg et al. 1998, Lacroix et al. 2006).

Finally, there are a number of proteins able to interact with p53 and the mutation status and expression pattern of these proteins also modifies p53 function; for example the MDM2 protein is involved in p53 degradation in cells and is amplified in 5.7% of breast cancers (Al-Kuraya et al. 2004). The expression of these interacting molecules may vary in different tumor types, making the regulatory system of p53 even more complex (Lacroix et al. 2006).

**P53 as a marker of prognosis in breast cancer**


The prognostic value of p53 alterations seems to be different in node-negative and node-positive subpopulations: no prognostic power has been seen in several studies in node-negative patients (Ferrero et al. 2000, Reed et al. 2000, Korkolis et
while some clinical studies report worse clinical outcome in patients with p53 alteration in the tumor in node-positive patients (Pietiläinen et al. 1995, Chappuis et al. 1999, Eissa et al. 1997). In a large meta-analysis of more than 9,000 patients, p53 immunopositivity correlated only weakly with clinical outcome (Barbareschi et al. 1996), but a more recent meta-analysis studying p53 status by mutation analysis strongly supports the adverse effect of p53 mutations on breast cancer specific survival (Pharoah et al. 1999, Borresen-Dale 2003). Again, there was a difference between patients with local or locally advanced disease: in more than 3,500 patients the risk ratio (RR) of dying of breast carcinoma in mutant p53 group was 1.7 in node-negative patients and 2.6 in node-positive patients (Pharoah et al. 1999).

The prognostic significance of all types of mutations varies; mutations in genes involving DNA binding have been associated with the poorest prognosis (Berns et al. 1998, Alsner et al. 2000). Co-expression of alterations in gene status of p53 and c-erbB-2 seems to characterize a subgroup of breast cancer patients with poor prognosis (Nakopoulou et al. 1996, Bebenek et al. 1998, Beenken et al. 2001).

P53 as a predictive marker in breast cancer

Preclinical studies. O’Connor and colleagues studied the sensitivity of 58 different cell lines to anticancer drugs in routine clinical use, and found that endogenous mutant p53 cell lines tended to be more resistant to anticancer agents than cell lines with wild-type p53 in vitro. On the other hand, microtubulin inhibitors (paclitaxel and vincristine) that cause mitotic arrest in cells instead of DNA damage were found to act independently from p53 status (O’Connor et al. 1997). Numerous in vitro studies have reported very conflicting results, and a recent meta-analysis comprising 356 in vitro studies tried to evaluate further sensitivity or resistance to chemotherapeutic agents in cancer cell lines (Cimoli et al. 2004). P53 knockout cells were found to be more drug-resistant than their normal p53 counterpart; however, the role of p53 alone in determining sensitivity/resistance to cytotoxic agents was limited, because the individual molecular pathology and differentiation of a cancer line is also crucial in terms of sensitivity to anticancer agents (Cimoli et al. 2004). The predictive value of p53 has also been studied in an in vivo setting. Lowe and colleagues showed that immunocompromised mice bearing p53 mutated tumors were associated with both resistance to adriamycin therapy and radiation; relapses were also seen more often compared to mice with normal p53 status (Lowe et al. 1994). Tumor growth inhibition rate has been shown to be lower in nude mice.
bearing p53 mutant tumors, with the exception of cyclophosphamide exposition (Koike et al. 2004).

Preclinical data investigating p53 function and sensitivity or resistance to antiestrogenic compounds are more limited when compared to studies investigating cytotoxic agents. There is evidence that estrogen-depriving compounds in estrogen-responsive breast cancer cells induce growth suppression and cell cycle arrest at the G0-G1 phase with simultaneous upregulation of p53 and p21 protein and downregulation of cyclin D1 and c-myc (Thiantanawat et al. 2003). However, tamoxifen-induced apoptosis in an estrogen receptor positive breast cancer cell line (MCF-7) did not affect p53 expression at the mRNA or protein level (Zhang et al. 1999), and apoptosis may happen without p53 activity: tamoxifen is able to induce apoptosis in normal human mammary epithelial cells that have suddenly lost their p53 function, but resistance to tamoxifen-triggered apoptosis is developed within 10 passages in vitro (Dietze et al. 2001, Seewald et al. 2001). Tamoxifen exposure can promote tumor progression in a mutant p53 breast tumor cell line (T47D) (Schafer et al. 2000).

Sensitivity to radiation therapy is partly mediated by p53 function, but data concerning in vitro breast cancer lines are limited. Gamma radiation in mice with normal p53 status induced rapid apoptosis while the same did not happen in p53 deficient animals (Komarova et al. 2000). Increased sensitivity to radiation was seen in human esophageal carcinoma cells that originally bore a p53 mutation, but were retrovirally transduced with wild-type p53 gene (Matsubara et al. 1999). However, clinical studies that have examined the possible relationship between clinical sensitivity to radiation and primary tumor p53 status have not shown a significant effect (Lacroix et al. 2006).

Clinical studies

Despite some promising data of previously described preclinical studies, the results of predictive studies in breast cancer patients have been highly controversial. Geisler et al. reported doxorubicin resistance in patients with p53 mutations and locally advanced breast cancer, but also suggested that other genetic defects act together with loss of p53 mutation (Geisler et al. 2001). The clinical outcome was compromised in patients with p53 mutated primary tumor receiving CMF adjuvant therapy (Andersson et al. 2005), but in other studies especially patients with p53 mutation/and or immunopositivity benefited from a CMF adjuvant regimen (Stal et al. 1995, Askmalm et al. 2004). In immunohistochemical studies Clahsen and colleagues
showed that patients with p53-negative tumors benefited significantly from perioperative CAF chemotherapy, whereas patients who had p53 immunopositive tumors did not (Clahsen et al. 1998). In addition, p53 immunoreactivity has been related to poorer clinical outcome in antracycline-based therapies in many other adjuvant studies as well (Bottini et al. 2000, Kandioler-Eckersberger et al. 2000, Mieog et al. 2006). However, in a neoadjuvant setting no connection has been shown between p53 expression and sensitivity to primary chemotherapy in breast cancer (MacGrogan et al. 1996, Rozan et al. 1998, Prisack et al. 2005). Despite the encouraging preclinical findings, p53 status has recently failed to predict the benefit from dose-dense adjuvant chemotherapy containing paclitaxel (Malamou-Mitsi et al. 2006). When treating metastatic breast cancer, Sjöström and colleagues did not find any connection between p53 immunopositivity and response to taxane-based chemotherapy either (docetaxel or methotrexate in combination with 5-fluorouracil) (Sjöström et al. 2000). In a study comparing the efficacy of high-dose adjuvant chemotherapy to standard-dose therapy, patients with p53 immunopositive tumors had better outcome after a high-dose regimen, whereas higher doses gave no survival advantage in p53 negative cases (Kroger et al. 2006).

The sensitivity to tamoxifen therapy in terms of p53 status has been evaluated in some studies and the results have been inconclusive. In two large adjuvant studies p53 accumulation did not predict the clinical outcome in tamoxifen-treated patients (Berry et al. 2000, Knoop et al. 2001) Linke and colleagues assessed p53 status by immunohistochemistry and/or mutation analysis and reported p53 alterations to significantly predict poorer survival after adjuvant tamoxifen (Linke et al. 2006). When treating recurrent or metastatic breast carcinoma, mutation in the p53 gene seems to weaken the sensitivity to tamoxifen therapy (Berns et al. 2000, Berns et al. 2003). On the other hand, Elledge et al. reported that response to tamoxifen therapy was not associated with p53 IHC accumulation, but p53 immunopositivity was still a significant indicator for shorter overall survival (Elledge et al. 1997).

Clinical studies that have examined the possible relationship between clinical sensitivity to radiation and primary tumor p53 status have not shown a significant association (Lacroix et al. 2006).
2.2.2  c-erbB-2 in breast cancer

c-erbB-2 gene and protein product

Schechter and colleagues first described in rat glioblastoma tissue the neu oncogene product with a molecular mass of 185 kDa (Schechter et al. 1984), and soon after that a monoclonal antibody (anti-p185) treatment was found to reverse neu-transformed cells into a non-transformed phenotype (Drebin et al. 1985). This oncogene named c-erbB-2 was found to encode a glycoprotein with tyrosine kinase activity (Akiyama et al. 1986). Immunohistochemical staining was reported to detect the c-erbB-2 gene protein expression (Venter et al. 1987). HER2 (human epidermal growth factor receptor-2) and its relatives HER1 (epidermal growth factor receptor; EGFR), HER 3 and HER4 all belong to the HER family receptors. In normal cells, activation of these receptors’ tyrosine kinases triggers a complex network of signaling pathways, such as mitogen-activated protein (MAP) kinase cascade, that control cell growth, differentiation, motility and adhesion (Akiyama et al. 1986, Mansour et al. 1994, Menard et al. 2004, Zaczek et al. 2005) (Figure 3). When cells are transfected with HER2/neu, they acquire a more malignant phenotype, with stimulation of cell proliferation, invasion and metastasis (Benz et al. 1993). In addition to the immunohistochemical method, the alterations of c-erbB-2 can be analyzed with fluorescence in situ hybridization (FISH) or chromogenic in situ hybridization (CISH) from formalin-fixed paraffin-embedded tissues (Kallioniemi et al. 1992, Laakso et al. 2006). Strong HER2 immunohistochemical positivity correlates with c-erbB-2 gene amplification and indicates better responsiveness to trastuzumab therapy (Baselga 2001, Vogel et al. 2002, Carlson et al. 2006).
c-erbB-2 as a prognostic and predictive factor

Varley et al. reported preliminary clinical results of prognostic value of HER2 in breast carcinoma patients: amplification of neu was seen in 19% of invasive breast carcinomas with a subsequent poor short-term prognosis (Varley et al. 1987), but Slamon and colleagues established the prognostic power of this gene alteration in a larger material, where HER2 amplification resulted in shortened disease-free and overall survival in breast carcinoma patients (Slamon et al. 1987, Slamon et al. 1989). Since then both c-erbB-2 gene and its protein product have been intensively investigated with similar results (Kallioniemi et al. 1991, Pinto et al. 2001, Joensuu et al. 2003, Yamashita et al. 2004, Kroger et al. 2006), but a large number of studies mainly examining HER2 status by IHC have revealed conflicting results as well (Ravdin et al. 1995, Revillion et al. 1998, Mirza et al. 2002). There is a clear relationship between HER2 positivity and negative hormone receptor status, as well as an association with certain histological subtypes of mammary tumors (ductal inva-
sive and in situ), poor histological and nuclear grades, aneuploidy and a high rate of cell proliferation, but whether HER2 overexpression has any independent prognostic value is less clear (Revillion et al. 1998, Mirza et al. 2002).

There are clinical studies suggesting that HER2 alterations lead to poor response to tamoxifen (Berry et al. 2000, Ferrero-Pous et al. 2000, Pinto et al. 2001, Jukkola et al. 2001, Dowsett et al. 2006), but this has not been proven in all studies (Elledge et al. 1998, Berry et al. 2000). However, preclinical findings have shown evidence for interactions between estrogen receptor and HER2 signaling pathways (Jones 2003, Ocana et al. 2006). Tamoxifen therapy may have an estrogen-like, agonistic effect in HER2 positive endometrial and ovarian cells, and tamoxifen exposure induces excessive cell proliferation in vitro in tamoxifen-resistant breast carcinoma cells expressing HER2 (Lee et al. 2000, Shou et al. 2004). ER molecules located near the cell membrane are able to activate growth factor receptor tyrosine kinases (EGFR and HER2) leading to (resulting in?) the agonistic effects of estrogen (Razandi et al. 2003). Overexpression of HER2 and subsequent excessive growth factor signaling may cause progesterone receptor loss and lower sensitivity to tamoxifen therapy. However, HER2 signaling does not correlate with resistance to antiaromatase therapies (Osborne et al. 2005). Combination therapy with the monoclonal antibody trastuzumab has recently been shown to have superior efficacy over single endocrine treatment: time to treatment failure was longer in patients receiving both trastuzumab and anastrozole compared to anastrozole alone when treating metastatic breast cancer in HER2 positive and estrogen receptor positive patients; however, the patients suffered in general from a very rapidly progressing disease (Kaufman et al. 2006).

There are very few randomized controlled trials evaluating the predictive value of HER2 amplification/overexpression in breast cancer patients receiving chemotherapy. HER2 positive tumors have responded more often to anthracycline regimen than non-anthracycline combinations in some studies (Paik et al. 2000, Di Leo et al. 2002, Cooke et al. 2001), but studies have not been able to confirm this in a trial with high-risk patients (Kroger et al. 2006) or with a high-dose anthracycline regimen (Faneyte et al. 2004) or weekly doxorubicin therapy (Geisler et al. 2001). Indeed, some studies report loss of efficacy of anthracycline-based therapy in metastatic breast carcinoma in c-erbB-2 positive tumors (Järvinen et al. 1998, Jukkola et al. 2001). So far, primary tumor HER2 status has not predicted the clinical efficacy of taxane-based regimens (Sjöström et al. 2002, Konecny et al. 2004). However, patients with elevated pre-treatment serum HER-2 levels have been reported to
benefit from taxane-based chemotherapy in a metastatic setting (Muller et al. 2004).

Strong HER2 expression and/or gene amplification are powerful predictive factors when selecting patients for targeted, monoclonal antibody therapy with trastuzumab. An antibody binding to HER2 receptor leads to internalization of receptor-antibody complexes and inhibition of HER2 mediated intracellular signaling pathways, and it also triggers immunologic responses in the host, which explains the antiproliferative effect (Harari 2004). As a single agent trastuzumab shows moderate clinical efficacy in MBC (Vogel et al. 2002), but higher response rates and survival benefit are seen in combination with anthracycline or taxane-based chemotherapeutics (Slamon et al. 2001, Marty et al. 2005). According to recent data, trastuzumab therapy in combination with chemotherapeutics reduces the risk of disease recurrence by approximately 50% in HER2 positive cases in an adjuvant setting in three years of follow-up (Piccart-Gebhart et al. 2005, Romond et al. 2005, Slamon et al. 2005, Joensuu et al. 2006b). Cardiac toxicity (decrease in left ventricular ejection fraction and congestive heart failure) is increased especially when trastuzumab is administered with a concurrent anthracycline regimen and may cause discontinuation of treatment; it is therefore recommended that cardiac function be monitored during therapy (Slamon et al. 2001, Than-Chiu et al. 2005). In a neoadjuvant setting a large primary tumor, expression of basal markers and insulin-like growth factor receptor membrane expression may lead to trastuzumab resistance in HER2 positive tumors (Harris et al. 2007).

2.2.3 MMP-9 in breast cancer

Extracellular matrix and the MMP family

Extracellular matrix (ECM) plays a major role in tissue architecture and homeostasis. It is under a constant remodeling process where ECM components are synthesized and deposited as well as proteolytically degraded. The principal ECM components are collagens, proteoglycans and hyaluronan, constituting a structure that regulates cell migration and provides a reservoir of cytokines and growth factors (Stamenkovic 2003). Changes in ECM can be seen as a response to host cellular stimulus. Matrix metalloproteinases (MMPs or matrixins) are endopeptidases that in part maintain tissue homeostasis by turnover of ECM and are capable of cleaving virtually any components of the extracellular matrix. However, MMP function is not limited to regulation of ECM composition only, since MMPs also functionally
regulate non-ECM molecules such as growth factors, chemokines and cytokines and therefore mediate cellular interactions with their environment (Stamenkovic 2003). Normal cell-matrix composition takes place in physiological conditions such as embryogenesis, wound healing, bone resorption and mammary involution (John & Tuszynski 2001, Chakraborti et al. 2003), but uncontrolled ECM remodeling by MMPs takes part in pathological processes in either benign or malignant situations such as rheumatoid arthritis, coronary artery disease and tumor progression (Sternlicht & Werb 2001, Nagase et al. 2006). Most of the evidence supporting the MMP activity in cancer development comes from experimental studies, where MMP expression has promoted tumor growth and invasion, which can be reduced by synthetic MMP inhibitors (Coussens & Werb 1996, Ala-aho & Kähäri 2005). The multistage process of cancer metastasis requires MMP activity at least in part, but the most critical point in this sense is MMP activity for primary tumor growth and invasion; reliance on MMP activity may decrease in the evident metastatic stage of malignant disease (Stamenkovic 2003).

The first mammalian MMP was discovered in early the 1960s in amphibian tissue (Gross & Lapiere 1962), and so far 24 matrix metalloproteinase genes that code 23 different MMPs have been found in humans. Matrixins can be divided by enzyme substrate specificity into five groups named collagenases, stromelysins, gelatinases, matrilysins and membrane-type MMPs, but there is some overlapping in substrate specificity (John & Tuszynski 2001) (Table 2). All MMPs share several structural characteristics: an inactive form of a matrixin contains a hydrophobic pre-peptide domain required for signal secretion, an aminoterminal propeptide domain that is removed upon MMP activation and a zinc-binding catalytic domain (John & Tuszynski 2001). MMPs are very strictly regulated; to accomplish their functions, MMPs must be secreted and regulated both locally and temporarily at the right time (Strenlicht & Werb 2001).
Table 2. Vertebrate MMPs and some of their substrates (Modified from Sternlicht & Werb 2001, Visse & Nagase 2003, Nagase et al. 2006).

<table>
<thead>
<tr>
<th>MMP</th>
<th>Common names(s)</th>
<th>Some substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase-1</td>
<td>Aggregan, collagen I, II, III, VII, entactin, laminin, tenascin, vitronectin, casein, fibrin, fibrinogen</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase-A</td>
<td>Collagen I, III, IV, V, VII, X, XI, decorin, fibronectin, gelatin, laminin, osteonectin, tenascin, fibrin, fibrinogen, proTNF, proTGF</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin-1, Transin-1</td>
<td>Aggregan, collagen I, IV, V, X, XI, laminin, tenascin, casein, e-cadherin, substance P</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin</td>
<td>Laminin, gelatin, collagen I, IV, V, IX, XI, XVIII, tenascin, vitronectin</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase-2, Neutrophil collagenase</td>
<td>Collagen I, II, III, XI, XIV, aggregan, fibrillin, laminin, 2-macroglubulin</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase-B</td>
<td>Aggregan, collagen IV, V, XI, XIV, elastin, gelatin, vitronectin, fibrin, plasminogen, proTNF, proTGF, fibrin, proMMP-2, -9</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin-2, Transin-2</td>
<td>Aggregan, collagen III, IV, V, elastin, gelatin</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>Aggregan, gelatin, 2-macroglubulin</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Metalloelastase, Macrophage elastase</td>
<td>Elastin, collagen I, IV, laminin, vitronectin, plasminogen</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase-3</td>
<td>Collagen I, II, III, fibronectin, gelatin, fibrinogen</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>Collagen I, II, III, gelatin, laminin, proMMP-2</td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>Proteoglycans, proMMP-2</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>Collagen III, fibronectin, proMMP-2</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td>Gelatin, fibrinogen, proMMP-2</td>
</tr>
<tr>
<td>MMP-18</td>
<td>Collagenase-4</td>
<td>Collagen I, II, III, gelatin</td>
</tr>
<tr>
<td>MMP-19</td>
<td>Stromelysin-4</td>
<td>Collagen I, IV, gelatin, laminin, tenascin</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
<td>Amelogenin, aggregan, laminin</td>
</tr>
<tr>
<td>MMP-21</td>
<td>XMMP (Xenopus)</td>
<td>Gelatin</td>
</tr>
<tr>
<td>MMP-22</td>
<td>CMMMP (Chicken)</td>
<td>Amelogenin, aggregan, laminin</td>
</tr>
<tr>
<td>MMP-23</td>
<td>Cysteine array (CA)MMP</td>
<td>Gelatin</td>
</tr>
<tr>
<td>MMP-24</td>
<td>MT5-MMP</td>
<td>Fibronectin, gelatin, proMMP-2</td>
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<td>MMP-25</td>
<td>MT6-MMP</td>
<td>Collagen IV, gelatin, proMMP-2, -9</td>
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<tr>
<td>MMP-26</td>
<td>Matrilysin-2</td>
<td>Collagen IV, gelatin, proMMP-9</td>
</tr>
<tr>
<td>MMP-27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-28</td>
<td>Epilysin</td>
<td>Casein</td>
</tr>
</tbody>
</table>

**Tissue inhibitors of matrix metalloproteinases**

Endogenous tissue inhibitors of MMPs (TIMPs) play an important role in MMP regulation. The TIMPs are a family of 21–34 kDa proteins capable of inhibiting matrixins reversibly by forming noncovalent 1:1 stoichiometric complexes (Lambert et al. 2004). Of the four TIMPs identified in vertebrates (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) the most efficient MMP-9 inhibitor is TIMP-3, while TIMP-2 and TIMP-4 bind less potently to MMP-9 (Baker et al. 2002). In addition to binding
MMPs, tissue inhibitors are also able to form complexes with the proMMPs and therefore regulate the activation process of MMPs. TIMPs differ in terms of gene regulation and tissue-specific expression, and their biological function is also very complex, because they have both growth-promoting and growth-suppressive activities that cannot be explained by MMP inhibition only (Lambert et al. 2004). While TIMPs are supposed to act locally, a plasma protein α2-macroglobulin forms irreversible complexes with MMPs, representing the most important MMP inhibition system in tissue fluids (Sternlicht & Werb 2001). Finally, relatively little is known about MMP catabolism, but a complex of MMP/α2-macroglobulin can be endocytosed and cleared permanently. Thrombospondin 2 has also been implicated in the clearance of matrixins (Sternlicht & Werb 2001).

**Gelatinases**

Gelatinases are zinc-dependent endopeptidases belonging to the MMP family and they have been linked to tumor progression due to their tissue remodeling capacity, induction of angiogenesis as well as basement membrane degradation ability (Egeblad & Werb 2002). There are two members of gelatinases. MMP-9 (gelatinase A) is a 92 kDa collagenase that was first identified as a gelatin-binding protein in the 1980s (Vartio et al. 1982). Along with MMP-2 (gelatinase B), which is 72 kDa collagenase, MMP-9 digests type IV collagen, and structurally these gelatinases differ from other matrixins by their three repeats of type II fibronectin domain into the catalytic domain (Allan et al. 1995) (Figure 4). MMP-2 and MMP-9 are closely related proteins, but there are several differences in their expression and activation patterns as well as substrate selectivity (Allan et al. 1995, Sternlich & Werb 2001) (Table 2).

![Fig. 4. Structure of gelatin-binding MMPs (MMP-9 and MMP-2), (modified from Visse & Nagase 2001).](image-url)
MMP-9 regulation

MMP-9 function is regulated by several mechanisms: on gene expression level, by post-transcriptional activation, cell-ECM or cell-cell contacts and by tissue inhibitors (John & Tuszynski 2001, Lambert et al. 2004). The precise signaling pathways leading to induction of MMPs is generally not fully understood, but numerous cytokines and growth factors including interleukins, interferons, VEGF, PDGF (platelet derived growth factor) and steroid hormones such as estrogen (Razandi et al. 2003) as well as cell stress are known to regulate the transcriptional activity of the MMP-9 gene (Parsons et al. 1997). However, MMP gene response depends not only on transcriptional factors, but also on cellular context and the presence or absence of other signals (Sternlicht & Werb 2001). Neutrophils store gelatinase B, but other cells synthesize and secrete MMP-9 into the ECM upon stimulation (Chakraborti et al. 2003).

Most MMPs including MMP-9 are secreted as inactive zymogens (pro-MMP-9) containing a secretory signal sequence and a propeptide whose proteolytic cleavage is essential for MMP-activation. The latency is maintained until proteolytic removal of the propeptide domain or perturbation of the cysteine-zinc interaction of the propeptide (Sternlicht & Werb 2001) (Figure 4). On the other hand, MMPs, once activated, are also capable of activating other MMPs. Gelatinases can activate each other (Stamenkovic 2003) and MMP-3 activates proMMP-9 both in vitro and in vivo, thus promoting invasiveness of breast cancer cells (Ramos-DeSimone et al. 1999). Plasmin has been regarded as a potent MMP activator in vivo, including the activation of MMP-9 (Lijnen et al. 2001). Chemical activation of MMPs has also been demonstrated to occur in vivo, for example nitric oxid (NO) can activate proMMP-9 during ischemia (Gu et al. 2002) and reactive oxygen species in inflammatory processes (Weiss 1989). Certain estradiol metabolites also generate free radicals that are able to activate proMMP-9 (Paquette et al. 2003).

MMP-9 function and carcinogenesis

Normal MMP-9 expression is mainly limited to osteoclasts, macrophages, keratinocytes of healing wounds and trophoblasts of placenta (Mohan et al. 1998, Munaut et al. 1999), but it is also regarded as one of the most potent MMPs involved in tumor progression and metastasis (Hua & Muschel 1996, Tryggvason et al. 1987, John & Tuszynski 2001, Sternlich & Werb 2001, Turpeenniemi-Hujanen 2005).
Excessive MMP-9 can be expressed by carcinoma cells or by adjacent stromal cells induced by malignant cells, leading to breakdown of basement membrane, which is an early phenomenon in the neoplastic process (Chambers & Matrisian 1997, Nielsen \textit{et al.} 1997, Sternlich & Werb 2001). Tumor cells are also capable of stimulating tumor-associated macrophages to increase MMP-2 and -9 expression and thereby enhance invasion in vitro (Hagemann \textit{et al.} 2004). Normal myoepithelial cells derived from benign breast tissue reduce breast cancer cell invasion in vitro via MMP-2 and -9 down-regulation in tumor cells and fibroblasts (Jones 2003). MMP-9 expression can also be seen in benign breast tissue cultures, depending on the growth factors present in serum-containing media (Kousidou \textit{et al.} 2004). MMP-9 has been shown to promote tumor growth by several mechanisms: it mediates insulin-growth factor signaling in prostate carcinoma cells (Manes \textit{et al.} 1999) and modulates host immune suppression by activation of TGF-β (transforming growth factor β) that is a potent inhibitor of T cell function (Gorelik & Flavell 2001). MMP-9 is also involved in malignant breast cell migration in vitro: carcinoma cells form membrane spicules or “invadopodia” structures, where active MMP-9-mediated degradation of the plasma membrane and CD44-linked cytoskeletal changes together promote tumor cell migration during cancer progression (Bourguignon \textit{et al.} 1998).

MMP-9 also contributes to neovascularization. MMP-9 knockout mice display a delay in endochondral bone formation that is related to delayed neovascularization (Coussens \textit{et al.} 2000) and MMP-9 activates TGF-β that promotes capillary tube formation in vitro (Yu & Stamenkovic 2000). MMP-9 also participates in angiogenic switch in transgenic mice by releasing VEGF (vascular endothelial growth factor) during carcinogenesis (Bergers \textit{et al.} 2000). Breast cancer cells exposed to hypoxia induce a potent angiogenic factor, connective tissue growth factor (CTGF), which in turn modulates degradation of ECM via MMP-9 expression (Kondo \textit{et al.} 2002). However, MMP-9 also produces antiangiogenic compounds such as angiotatin and endostatin, thus inhibiting neovascularization. MMP-9 may thus either promote or inhibit angiogenesis, the net effect on tissue depending on other regulators and ECM compounds (Cornelius \textit{et al.} 1998, Ferreras \textit{et al.} 2000, Stamenkovic 2003).

Some studies have examined the MMP-9 status in both benign and malignant breast tissue. Jinga \textit{et al.} evaluated MMP and TIMP activity and protein expression by gelatin zymography, immunoblotting and the ELISA method in benign and malignant breast tissue, finding increased MMP-9 activity in malignant samples. The results also suggested that abnormal MMP-9/TIMP-1 balance plays a role in
breast carcinogenesis (Jinga et al. 2006). In another study the enzymatic activity of MMP-9 was studied by zymography in benign and malignant breast tissues, and pro-MMP-9 was clearly more often expressed in carcinoma samples compared to non-cancerous samples. Only pro-MMP-9 production was elevated in DCIS and small (T1) carcinomas, but in larger (T2-T4) tumors or in cases including skin invasion, both production and activation of MMP-9 was increased (Rha et al. 1997). MMP-2 and -9 activity is reported to be elevated in breast carcinoma compared to benign fibroadenoma tissue (Hanemaaijer et al. 2000). There are also some data showing that expression levels of MMP-9 along with MMP-26, TIMP-2 and TIMP-4 were highest in ductal carcinoma in situ specimens when compared to normal breast, hyperplastic tissue or invasive carcinomas (Zhao et al. 2004).

**MMP-9 as a prognostic factor in breast cancer**

While there is evidence that MMP-9 function involves tumor invasion in general, suggesting indirectly MMP-9 function to be an adverse prognostic factor, the issue has been little studied in clinical breast cancer populations, and the results obtained have been contradictory. The variation in the results may be partly explained by different study methods: MMP-9 function can be evaluated in a primary tumor by immunohistochemistry where antibodies may bind selectively to an activated or latent protein, or the antibody may bind both forms of MMP-9. Messenger RNA of MMP-9 mirrors the transcriptional activity of the MMP-9 gene in a primary tumor and can be detected by the in situ hybridization method. MMP-9 protein activity can, on the other hand, be analyzed by gelatin zymography of tumor extract. The enzyme-linked immunosorbent assay (ELISA) can be used to measure MMP-9 immunoreactive protein in circulation or tissue extracts (Allan et al. 1995).

High MMP-9 expression in cancerous cells has been associated with favorable disease-free survival (Scorilas et al. 2001, Pellikainen et al. 2004), but opposite data exist as well (Li et al. 2004). Positive MMP-9 status in stromal cells has mainly predicted poor overall survival (Pellikainen et al. 2004, Mylona et al. 2007), whereas no specific effect on overall survival was reported elsewhere (Li et al. 2004). On the other hand, some data do not indicate any connection between MMP-9 status and breast cancer-related survival in either tumor or stromal cells (Remacle et al. 1998, Tetu et al. 1998). The prognostic significance might also vary depending on the patient population; in one study better DFS and OS associated with MMP-9 positivity in only node-negative patients, while no association was detected in node-positive patients (Scorilas et al. 2001). Furthermore, frequent co-expression
of MMP-9 and HER2 in stromal cells has been reported, which might explain the aggressive phenotype of these tumors (Pellikainen et al. 2004, Mylona et al. 2007). van’t Veer’s data included up-regulated MMP-9 to in other genes involved in cell invasion, cell cycle control, signal transduction and angiogenesis, which are associated with a poor clinical outcome (van’t Veer et al. 2002). Despite the conflicting results in breast cancer, MMP-9 overexpression has appeared to indicate poor prognosis in lung cancer, gastric cancer, osteosarcoma as well as head and neck carcinoma (Cox et al. 2000, Sier et al. 1996, Foukas et al. 2002 and Ruokolainen et al. 2004). Clinical findings in other malignancies are still inconclusive; for example, stromal MMP-9 expression has been shown to correlate with a more favorable outcome in colorectal cancer (Takeha et al. 1997).
3 Purpose of the present study

This work has aimed to examine further the prognostive and predictive value of p53, c-erbB-2 and MMP-9 in breast cancer patients, and MMP-9 expression in benign, premalignant and malignant breast tissue, particularly by focusing on the following issues:


2. The prognostic and predictive significance of p53 immunopositivity in postmenopausal, node-positive breast cancer patients treated with an adjuvant anti-estrogen therapy.

3. The prognostic value of MMP-9 immunoreactive protein in postmenopausal, node-positive breast cancer patients treated with an adjuvant anti-estrogen therapy.

4. The immunohistochemical expression of MMP-9 in benign, pre-malignant and malignant breast tissue.
4 Materials and methods

4.1 Patients and tissue samples

The studies presented are based on breast cancer patients treated at Oulu University Hospital. Tissue samples were collected from the files of the Department of Pathology at Oulu University; only a few samples were collected from District hospitals of Kajaani and Kokkola (I–IV). In the fourth study patients with hyperplastic breast tissue or in situ cancer were also included (65 patients) while 64 samples represented infiltrative breast carcinoma (Table 3). Histological analyses were carried out from formalin-fixed, paraffin-embedded primary tumor specimens obtained from diagnostic and therapeutic procedures. p53, c-erbB-2 and MMP-9 status in primary tumours were systematically analysed by immunohistochemistry. The stage of disease was determined according to the TNM classification of tumors issued by the International Union Against Cancer (UICC) and the WHO classification for the characterization of tumor histopathology. Clinical case characteristics and follow-up data of the patients were obtained retrospectively from the patient records of the Department of Oncology and Radiotherapy at Oulu University hospital. Hormone receptor status was evaluated either by immunohistochemistry or enzyme immuno assay (EIA). In immunohistochemical staining tumors representing >5% positive cells were considered receptor-positive cases. When EIA was used, the cut-off value for estrogen receptor concentration was four or more fmol/cytosolic protein and 10 or more fmol/protein for progesterone receptor concentration.
The prognostic role of p53 was studied in a breast cancer population of 254 cases (I) treated during the years 1982–1986. 63% of the patients were premenopausal. The mean age of the patients was 56 years. A large proportion of the primary tumors were 21–50 mm in size (45%) (T2), ductal type of histology (82%) and poorly differentiated (36%). Positive axillary lymph nodes were seen in 57% of the patients. Hormone receptor status was analyzed either by radioimmunoassay or by immunohistochemistry. 67% of the tumors were estrogen receptor positive and 71% were progesterone receptor positive; 24% of the patients received adjuvant hormonal therapy, cytotoxic agents were used in 15% of the cases. During the follow-up time (range 3–228 months) 121 patients experienced local recurrence or developed a metastatic disease; responses to systemic therapies and survival curves were analyzed in this subpopulation and evaluated according to p53 immunostaining (Tables 3, 4). There were no differences in terms of adjuvant therapies according to p53 status in patients developing metastases, because 18 patients in the p53 positive group received adjuvant hormonal treatment, the number being 14 in p53 negative patients. Fifteen patients received adjuvant chemotherapy in both the p53 positive and negative group.

The prognostic value of c-erbB-2 status was analyzed in a limited subpopulation of 79 patients out of 254 patients (Table 3), where adjuvant therapies were used as follows: in the c-erbB-2 positive group endocrine therapy for 8 patients and cyto-

Table 3. Patient characteristics and analysis for p53, c-erbB-2 and MMP-9.

<table>
<thead>
<tr>
<th>Study</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, total (n)</td>
<td>254</td>
<td>97</td>
<td>168</td>
<td>129</td>
</tr>
<tr>
<td>Premenopausal (%)</td>
<td>63%</td>
<td>0%</td>
<td>0%</td>
<td>20%</td>
</tr>
<tr>
<td>Postmenopausal (%)</td>
<td>37%</td>
<td>100%</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>p53 status determined (n)</td>
<td>254</td>
<td>97</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c-erbB-2 status determined (n)</td>
<td>79</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MMP-9 status determined (n)</td>
<td>-</td>
<td>-</td>
<td>168</td>
<td>129</td>
</tr>
<tr>
<td>Patients developing recurrent BC during follow-up n, (%)</td>
<td>121 (48%)</td>
<td>34 (35%)</td>
<td>56 (33%)</td>
<td>-</td>
</tr>
</tbody>
</table>
toxic therapy for 9 patients, the numbers being 12 and 6 in the c-erbB-2 negative group. Six patients received a combination of both hormonal and cytotoxic therapy in the c-erbB-2 positive group, but combination therapy was not used in the c-erbB-2 negative group. The prognostic relevance of co-expressing p53 and c-erbB-2 was studied in terms of overall survival.

Table 4. Tumour characteristics (I–III).

<table>
<thead>
<tr>
<th>Factor</th>
<th>I study Patients n (total 254)</th>
<th>II study Patients n (total 97)</th>
<th>III study Patients n (total 168)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>78</td>
<td>41</td>
<td>72</td>
</tr>
<tr>
<td>T2</td>
<td>114</td>
<td>50</td>
<td>84</td>
</tr>
<tr>
<td>T3</td>
<td>34</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>T4</td>
<td>15</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>115</td>
<td>91</td>
<td>200</td>
</tr>
<tr>
<td>Positive</td>
<td>123</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>54</td>
<td>89</td>
<td>153</td>
</tr>
<tr>
<td>Stage II</td>
<td>127</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Stage III</td>
<td>29</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Stage IV</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>20</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>II</td>
<td>82</td>
<td>46</td>
<td>16</td>
</tr>
<tr>
<td>III</td>
<td>91</td>
<td>29</td>
<td>51</td>
</tr>
<tr>
<td>Unknown</td>
<td>60</td>
<td>16</td>
<td>34</td>
</tr>
<tr>
<td>ER receptor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>89</td>
<td>22</td>
<td>33</td>
</tr>
<tr>
<td>Positive</td>
<td>119</td>
<td>65</td>
<td>122</td>
</tr>
<tr>
<td>Unknown</td>
<td>46</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>PR receptor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>95</td>
<td>42</td>
<td>68</td>
</tr>
<tr>
<td>Positive</td>
<td>112</td>
<td>44</td>
<td>86</td>
</tr>
<tr>
<td>Unknown</td>
<td>47</td>
<td>11</td>
<td>14</td>
</tr>
</tbody>
</table>

The predictive value of p53 immunopositivity was further examined in 97 post-menopausal women with lymph node positive breast carcinoma receiving 20mg tamoxifen or 40mg toremifene daily for three years after definitive local therapies (II). The mean age of the patients was 63 years. In patients less than one year from menopause postmenopausal status was confirmed by measuring follicle-stimulating hormone levels (>30U/l). The patients were operated during the years 1992–1995; 86 patients underwent mastectomy and axillary evacuation, segmental resection of the breast with axillary dissection was performed in 11 cases. Postoperative irradiation was given in 90 cases. None of the patients received adjuvant chemotherapy. 52% of the patients presented with a T2 tumor, with ductal type of histology
Estrogen receptors were positive in 67% of the patients and negative in 23%, the proportions being 45% and 44% in progesterone receptor analysis, respectively; receptor status was mainly investigated by the immunohistochemical method. Unknown hormone receptor status was exceptional. The median follow-up time in this study was 59 months (Tables 3, 4).

Prevalence and prognostic significance of matrix metalloproteinase 9 immunoreactivity was evaluated in a series of 168 postmenopausal breast cancer patients with lymph node positive disease (III) where the adjuvant endocrine therapy was similar to a previously described p53 study (II) (Table 3). The average age of the patients was 64 years. 58% of the patients underwent mastectomy, breast conservative operation was performed in the rest of the patients. Due to locally advanced disease, every patient had an axillary dissection and all but ten patients received locoregional post-operative radiation therapy. 84 patients (50%) had a T2 primary tumor and 108 (64%) had 1–3 metastatic lymph nodes in the axilla. Ductal type of histology (75%) and moderate differentiation (38%) were most frequently seen in primary tumors. 122 patients (72%) were estrogen receptor positive and 33 (20%) were estrogen receptor negative, the proportions being 51% and 41% in progesterone receptor analysis, respectively (Table 4). Radioimmunoassay was used for receptor status evaluation in 71% of the tumors, immunohistochemical assay being used in the rest of the cases. 65 patients relapsed within the follow-up time that varied from seven to 111 months.

MMP-9 expression was evaluated in a series of 129 breast tissue samples representing a variety of breast tissue histologies from hyperplastic changes to fully developed cancer (IV) (Table 3). 24 samples represented ductal hyperplasia, 22 atypical ductal hyperplasia (ADH) and 18 ductal carcinoma in situ (DCIS). In ductal infiltrative primary breast carcinoma samples 26 cases were of grade I, 20 of grade II and 19 samples of grade III histology (Table 5).
Table 5. Study four breast tissue samples and matrix metalloproteinase-9 immunohistochemical staining result.

<table>
<thead>
<tr>
<th>Histological group</th>
<th>Immunohistochemical staining result for MMP-9 (Samples, number)</th>
<th>negative</th>
<th>weak positive</th>
<th>moderate positive</th>
<th>strong positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasias</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>usual</td>
<td>9</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>atypical</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DCIS</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ductal invasive carcinomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>histological grade I</td>
<td>12</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>grade II</td>
<td>9</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>grade III</td>
<td>12</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hyperplasias, total</td>
<td>19</td>
<td>13</td>
<td>9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DCIS</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Invasive carcinomas, total</td>
<td>33</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Immunohistochemical antibodies used in the studies.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Study</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Specificity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>I,II</td>
<td>Do7</td>
<td>1:1000</td>
<td>monoclonal</td>
<td>Novocastra Laboratories</td>
</tr>
<tr>
<td>HER2</td>
<td>I</td>
<td>NCL-CB11</td>
<td>1:500</td>
<td>monoclonal</td>
<td>Novocastra Laboratories</td>
</tr>
<tr>
<td>MMP-9</td>
<td>IV,IV</td>
<td>GE-231</td>
<td>1:50 (10 µg/ml)</td>
<td>monoclonal</td>
<td>Diabor</td>
</tr>
</tbody>
</table>

4.2 Immunohistochemistry

4.2.1 Antibodies and immunostaining

The immunohistochemical analysis for the evaluation of p53 mutation status in a tumor is based on the detection of abnormally accumulated p53 protein in a cell (Figure 5). A mouse monoclonal antibody against the anti-p53 antibody (Do7) was purchased from Novocastra Laboratories (Newcastle upon Tyne, UK) (I, II). C-erbB-2 gene amplification and/or increased transcription can lead to HER-2 overexpression, and this was examined by using a mouse monoclonal antibody against the anti-c-erbB-2 (NCL-CB11, Novocastra Laboratories) (Figure 6). The MMP-9 immunoreactivity was evaluated by using GE-231 (10µg/ml) as a primary antibody (Diabor Ltd, Oulu, Finland) (Figure 7) (Table 6).
Fig. 5. Ductal invasive grade II carcinoma, positive p53 immunohistochemical staining.

Fig. 6. Ductal invasive grade II carcinoma, positive c-erbB-2 immunohistochemical staining.
Fig. 7. a) Ductal invasive grade II carcinoma, positive MMP-9 immunohistochemical staining.
b) Ductal invasive grade III carcinoma, negative MMP-9 immunohistochemical staining.

Immunohistochemistry was determined from formalin-fixed and paraffin-embedded tissue samples (3 or 4 μm) by using the avidin-biotin peroxidase method according to Hsu et al. (1981) (I–IV). Dewaxed sections were heated in a microwave
oven in 10mM citrate buffer, pH 6.0, for 10 minutes before application of the primary antibody in p53 analysis (I, II). After a 60-minute incubation with the primary antibody at room temperature, a biotinylated secondary anti-mouse antibody (Dakopatts, Copenhagen, Denmark) was applied (dilution 1:200), followed by the avidin-biotin-peroxidase complex (Dakopatts). The color was developed by diaminobenzidine, whereafter the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Careful rinses with phosphate buffered saline (PBS) were done between each step of the procedure. Negative control stainings were carried out by substituting non-immune mouse serum and PBS for the primary antibody. Lung cancer tissue samples known to be p53 mutated were used as positive controls.

The immunopositivity of c-erbB-2 was determined by using a mouse monoclonal antibody (NCL-CB11, Novocastra Laboratories, Newcastle upon Tyne, UK) that recognizes the internal domain of the c-erbB-3 oncogene protein (Corbett et al. 1990) and the avidin-biotin peroxidase method as described above. The dilution for the primary antibody for c-erbB-2 was 1:500 and diaminobenzidine (Dakopatts, Copenhagen, Denmark) was used as a chromogen (I).

MMP-9 sections were incubated at 37°C for at least four hours and deparaffi-nized. After endogenous peroxidase activity was blocked with 0.3% hydrogen pero-oxidase/methanol incubation for 20 minutes, incubation in 10% goat serum for 15 minutes was performed to prevent any non-specific binding. The specimens were incubated with a primary antibody for MMP-9 in a humidity chamber at room temperature for 24 hours. A biotinylated antimouse IgG served as a secondary antibody with an incubation time of 15 minutes (Histostain-bulk kit®, Zymed, San Francisco, Ca, USA), after which peroxidase was introduced with a streptavidin conjugate (Histostain-bulk kit®). The immunohistochemical reaction was then visualized using romulin AEC chromogen substrate (Biocare Medical, CA, USA). The samples were washed in PBS between every step of the staining process. The sections were counterstained with hematoxylin, dehydrated and mounted in Pertex® (Histo-lab, Gothenburg, Sweden). For the negative controls, the primary antibody was replaced by either mouse non-immune serum or phosphate-buffered solution (PBS). Known sections positive for MMP-9 were included and used as positive controls (III, IV).

When evaluating p53 accumulation, nuclear positivity of tumor cells only was considered significant (I, II) and the results were evaluated from the whole sample as follows: negative = less than 1% of cells staining, weak positivity = 1–10% of cells staining (I, II). Samples were interpreted as moderately positive if 11–20% of
the cells were staining and strongly positive if 21–60% were staining. Very strong positivity called for more than 60% of the cells staining (I). In the antiestrogen study (II) the criterion for strong positivity was slightly different: moderate positivity called for 11–50% of cells staining, while in strongly positive samples more than 50% of cells showed immunoreactivity (II). In both p53 studies statistical analysis was carried out by comparing all positive cases as a single group to negative cases. In the c-erbB-2 analysis sections presenting less than 5% of the neoplastic cells staining were considered to be negative while more extensive staining was considered weak, moderate or strongly positive according to the amount of cells staining.

The cytoplasmic MMP-9 staining was scored in neoplastic cells (III). The staining was interpreted as negative in sections with no positive cells, or less than 1% of all malignant cells showing a positive staining, while 2–50% of the tumor cells appearing as positive indicated moderate overexpression of the MMP-9 protein. Sections presenting more than 50% of the neoplastic cells as positive were considered to have extensive MMP-9 overexpression in the tumor (III). In the fourth study the staining was scored in the area of interest only (hyperplasia/atypical hyperplasia/ductal carcinoma in situ/invasive ductal carcinoma). In a negative specimen less than 1% of the cells were staining, while in a weakly positive specimen 2–25% of the cells showed positive staining. 26–50% of the cells staining positively appeared as moderately positive samples, while sections presenting more than 50% of the cells staining were considered to be strongly positive and having extensive MMP-9 overexpression in the cells. In this study IHC staining was evaluated more accurately by a four-step scoring system. A few samples were stained twice and the IHC staining result seemed to be repeatable (IV).

All immunohistochemical staining interpretation was carried out blinded to clinical data of the patients. P53 (I, II) and c-erbB-2 (I) immunopositivity was analyzed by an experienced pathologist and co-author. The MMP-9 staining result was analyzed by three individual observers: the first author and two co-authors including an experienced pathologist (III, IV).

4.3 Statistical analysis

The prognostic value of immunohistochemical staining of p53, c-erbB-2 and MMP-9 was evaluated by comparing the clinical outcome of patients according to p53 or MMP-9 status (I–III). Disease-free survival was determined as the time in months between primary diagnosis and disease relapse and overall survival as the time in months between diagnosis and the last clinical follow-up or breast-cancer
related death (I–III). Patients representing primarily metastatic disease were not included in survival analyses, and patients dying from any other cause than breast cancer were also censored from the survival data at the time of death. Statistical significance was tested using standard tests: Student t-test, Mann-Whitney, Pearson and Chi square tests (I–III). Analysis was carried out by comparing all p53 or MMP-9 positive cases as a single group to cases with p53 or MMP-9 negative tumors. Survival curves (Kaplan-Meier) were compared using the log rank, Breslow or Tarone-Ware test, and p<0.05 was considered statistically significant. Cox regression analysis and stepwise regression analysis were used to find significant predictors of survival (Uhari & Nieminen 2001) (I–III) and Fisher’s exact test was used to find any significant difference in MMP-9 staining between various histological breast tissue samples (IV).
5 Results

5.1 P53 immunoreactivity in primary breast carcinoma (I, II)

Positive p53 immunostaining was seen in 34% (I) in a larger material (254 patients) consisting of both pre- and postmenopausal patients and in 24% of postmenopausal patients (out of a total of 97 patients) in the adjuvant antiestrogen study (II). P53 accumulation correlated with high malignancy grade, negative progesterone receptor status and ductal type of histology (I). Patients with primarily metastatic breast carcinoma were not included in the survival data or reported in the article, but p53 positivity was seen in 40.7% of those patients. P53 positivity did not correlate with the metastatic site (I).

5.2 P53 accumulation as a prognostic factor (I, II)

Disease-free survival of the patients was strongly affected by p53 immunopositivity (I, II) (Table 7). In a larger material, 57.0% of patients with a p53 positive tumor relapsed during the follow-up compared to 34.4% in the p53 negative group (I). Along with the size of the primary tumor, positive nodal status and higher clinical stage, positive p53 emerged as a strong prognostic factor in an univariate analysis (p=0.000) and was further confirmed to be an independent prognostic factor in a multivariate analysis (p=0.001) (I). The adverse effect of p53 accumulation was seen not only in node-positive patients, but also in node-negative patients where the median DFS was 10.4 years in the p53 negative and 4.4 years in the p53 positive subgroup (p=0.0001) (I). The Nottingham Prognostic Index (NPI) predicted shortened disease-free survival and overall survival of patients (p=0.000). When NPI was included with p53 status in a Cox regression analysis, p53 accumulation still seemed to have prognostic power, the result getting close to statistical significance in terms of disease-free survival (p=0.055) (I). P53 also showed independent prognostic power in postmenopausal patients treated with antiestrogen. P53 predicted shortened DFS in univariate analysis (p=0.0036) as well as in multivariate Cox stepwise regression analysis (p=0.01) (II).
Breast cancer related mortality was increased in p53 positive patients (I, II) (Table 7). The mean survival time was 8.3 years in the p53 positive and 13.3 years in the p53 negative subgroup (p=0.0005) (I). P53 positivity was shown to be a strong prognostic factor not only in univariate analysis but also in multivariate analysis (p=0.0001) along with traditional prognostic factors such as positive nodal status (p=0.0001), large primary tumor (p=0.000), high malignancy grade (p=0.000) and high clinical stage (p=0.0009). The Nottingham Prognostic Index (NPI) predicted overall survival of patients (p=0.000), and when it was analyzed with p53 status in a Cox regression analysis, p53 accumulation still retained its independent prognostic power (p=0.013) (I). P53 positivity indicated significantly shorter overall survival even in metastatic breast carcinoma (p=0.0005) (I).

P53 positivity in postmenopausal patients treated with antiestrogen associated with a statistically significant shorter overall survival time than in p53 negative patients (II) (Table 7). In univariate analysis OS was markedly impaired by clinical stage (p=0.0005), tumor size (p=0.001), positive p53 (p=0.005) negative ER status (p=0.001) and negative PR status (p=0.03). The stage of the disease (p=0.02), ER status (p=0.002) and positive p53 (p=0.005) were found to be independent and statistically significant factors predicting OS in the multivariate analysis (II).

### 5.3 Co-expressing p53 and c-erbB-2 – the prognostic relevance (I)

Patients with simultaneous p53 and c-erbB-2 IHC overexpression represented an extremely poor prognostic subgroup with a mean overall survival of only 2.6 years, while patients with p53 and c-erbB-2 negative tumors had a significantly better outcome with a mean survival time of 11 years (p=0.0003) (I). OS was 5.5 years among patients with p53+/c-erbB-2- tumor status and 6.2 years among those with p53-/c-erbB-2+ tumor status (I).

### Table 7. Five-year breast cancer specific survival in patients according to p53 immunohistochemical staining in a primary tumour (I, II).

<table>
<thead>
<tr>
<th>Study</th>
<th>5-year disease-free survival (DFS)</th>
<th>5-year DFS</th>
<th>5-years overall survival (OS)</th>
<th>5-year OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P53 positive</td>
<td>P53 negative</td>
<td>P53 positive</td>
<td>P53 negative</td>
</tr>
<tr>
<td>I</td>
<td>36%</td>
<td>68%</td>
<td>51%</td>
<td>85%</td>
</tr>
<tr>
<td>II</td>
<td>42%</td>
<td>72%</td>
<td>50%</td>
<td>80%</td>
</tr>
</tbody>
</table>
5.4 P53 and response to antracycline-based chemotherapy and hormonal agents in metastatic breast cancer (I)

Disease recurrence was diagnosed in 121 out of 254 patients in the first study population and responsiveness to systemic therapies was analyzed in correlation to p53 status among them (I) (Table 3). 25 patients out of 121 received anthracycline-based therapy due to recurrent disease, the most frequent regimen being FEC combination (cyclophosphamide-epirubisin-5-fluorouracil). Four patients (27%) out of 15 in the p53 positive group receiving anthracycline-based chemotherapy responded to the treatment, and 11 patients (73%) progressed during the treatment. In the p53 negative group 10 patients (66%) out of 15 showed clinical benefit (CR, PR or SD) to anthracycline therapy while the minority (33%) progressed. In recurrent disease there was no evident correlation between p53 status and responsiveness to hormonal agents. Of a total of 67 patients receiving endocrine regimens 32% with normal p53 status responded to therapy (CR, PR) while 43% progressed (PD) during therapy, the proportions being 40% and 50%, respectively, in the p53 positive group. Tamoxifen and progestins were the most frequently used therapeutic agents (I).

5.5 MMP-9 immunoreactivity in primary invasive breast cancer (III, IV)

When MMP-9 was studied in postmenopausal node-positive patients treated with adjuvant antiestrogen therapy immunopositivity was seen in 63% of the primary tumors (III). Positive staining did not correlate significantly with other tumor characteristics such as tumor size, malignancy grade, tumor load in the axilla or hormone receptor status. When MMP-9 immunostaining was evaluated in terms of malignancy grade, moderate or strong immunoreactivity was detected in 74% of grade I, 72% of grade II and 56% of grade III invasive carcinomas, so that a slightly descending trend was seen with higher malignancy grade (III) (Table 8). In the fourth study 32 samples out of 65 invasive breast carcinoma cases showed various degrees of MMP-9 positivity in IHC (49%) (IV). Moderate or strong positivity was, however, rarely seen in invasive carcinoma samples; indeed, in grade III carcinoma none of the sections showed strong MMP-9 immunoreactivity (IV) (Table 5).
Table 8. MMP-9 immunohistochemical staining result in invasive breast carcinoma samples (III, IV).

<table>
<thead>
<tr>
<th>Study</th>
<th>MMP-9 negative n/%</th>
<th>MMP-9 weak/moderate positive, n/%</th>
<th>MMP-9 strong positive n/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>54 (34.4%)</td>
<td>45 (28.7%)</td>
<td>58 (37.0%)</td>
</tr>
<tr>
<td>IV</td>
<td>33 (50.8%)</td>
<td>24 (36.9%)</td>
<td>8 (12.3%)</td>
</tr>
</tbody>
</table>

5.6 MMP-9 immunostaining in premalignant breast disease (IV)

Negative MMP-9 staining was the most frequent finding (9 samples out of 24) seen in hyperplastic cases (37.5%) and 10 out of 22 atypically hyperplastic samples were negative in MMP-9 staining (45.5%). However, some (weak, moderate or strong) MMP-9 positivity was found in 62.5% of the hyperplastic cases and in 54% in the atypically hyperplastic group (Table 5).

DCIS’s immunoprofile was distinctive when compared to any other histological group. MMP-9 positivity was most frequently seen in DCIS, where 15 (83%) out of 18 samples showed positive staining, and strong immunoreactivity predominated: in 56% of the cases DCIS cells were staining intensively; the result was statistically significant in both Fisher’s exact test (p=0.005) and Chi square test (p=0.001). Finally, when negative and weak positive samples were analyzed together as a single group and compared to the samples staining extensively, strong immunopositivity in DCIS samples remained distinctive compared to other histological groups. Only three DCIS samples were MMP-9 negative (16%) (Table 5, Figure 8). The result was statistically significant in both Fisher’s exact test (p=0.004) and Chi square test (p=0.003).

The age of the patients was not significantly reflected in the immunoreactivity in the entire population: 55% of the younger women (described as 49 or younger) were MMP-9 positive, the proportion being only slightly greater (61%) in the older age group.
5.7 MMP-9 positivity as a prognostic factor in breast carcinoma (III)

In 168 postmenopausal patients treated with adjuvant antiestrogen therapy, primary tumor size, clinical stage and estrogen receptor status predicted strongly both disease-free and overall survival, but there were no statistically significant differences in terms of survival between patients with MMP-9 positive or negative tumors when studied in the entire study population. However, in receptor negative cases, there was a tendency for compromised survival among MMP-9 positive patients. In estrogen receptor negative patients the mean disease-free survival was 3.5 years in MMP-9 positive patients while patients with negative MMP-9 status had a better outcome with a mean survival time of 5.3 years. When evaluating five-year survival rates between these subgroups, there was a difference in favor of MMP-9 negative patients (63%) compared to MMP-9 positive patients (37%), the result getting close to statistical significance (p=0.06). Patients with progesterone receptor negative tumors seemed to have a greater risk of disease relapse if having MMP-9 expression in their primary tumor, but the difference was not statistically significant.
6 Discussion

6.1 Prognostic value of p53 immunopositivity

The prognostic relevance of p53 accumulation was investigated in two studies (I–II) within various patient populations. The first study consisted of both pre- and postmenopausal women with either node-negative (45%) or node-positive (48%) breast cancer, treated mainly in the 1980s. Adjuvant therapies were infrequently used: 15% received chemotherapy and 24% an endocrine regimen. In the second study, postmenopausal node-positive patients treated with adjuvant antiestrogen therapy for three years during 1990s were investigated (II).

P53 accumulation was detected in 34% (I) and 24% (II) of the cases, which is in line with other reports (Davidoff et al. 1991, Reed et al. 2000, Borresen-Dale 2003, Malamou-Mitsi et al. 2006). In the first study, breast cancer recurrence was diagnosed in 48%, and in the second study population in 35% of the patients during follow-up. Fewer relapses in the second study may perhaps reflect the overall beneficial progress in breast cancer specific survival in Finland as well as the clinical efficacy of adjuvant antiestrogen. Despite the differences in patient populations, p53 accumulation emerged as a powerful adverse prognostic factor in both studies, the results being statistically highly significant in terms of disease-free and overall survival, providing new evidence of the independent prognostic power of p53 in breast carcinoma, where previous data have shown controversial results (Lipponen et al. 1993, Ferrero et al. 2000, Olivier et al. 2006). The positive association between altered p53 status and high malignancy grade has been reported previously (Pietilainen et al. 1995, Ferrero et al. 2000, Olivier et al. 2006). P53 positive immunostaining associated with other aggressive primary tumor characteristics such as ER negativity (I), but altered p53 status was still found to be an independent indicator of a compromised clinical outcome in multivariate analysis. This study also provides new evidence of the prognostic relevance of p53 accumulation in node-negative breast cancer patients (I), which has previously been a particularly controversial issue (Silvestrini et al. 1996, Chappuis et al. 1999, Ferrero et al. 2000, Reed et al. 2000).

A subgroup of patients developing metastatic disease was analyzed separately in terms of p53 status. Positive IHC for p53 indicated shortened survival, the result being statistically highly significant (I), indicating the aggressive course of p53 positive disease in the metastatic phase of the disease as well. Previous literature includes only a few studies supporting this finding (Berns et al. 2003, Chang et al.
In a recent large material investigating prognostic factors in patients with breast cancer related liver metastases, low IHC expression for p53 indicated favorable survival (Eichbaum et al. 2006). No correlation has been shown in other studies (Niskanen et al. 1997, Sezgin et al. 2005).

The controversy in the large body of study data investigating the prognostic power of p53 has given rise to many questions. First of all, the variety in p53 function diagnostics could in part explain the diversity in the results reported. Mutation analysis by gene sequencing technique provides detailed information on distinctive mutations that may have varying prognostic relevance (Runnebaum et al. 1991, Bergh et al. 1995). However, a mutated p53 protein may lose its normal function completely or only partly. In addition, a mutant p53 with abnormally high apoptotic activity has been described (Saller et al. 1999). There is no doubt that p53 DNA sequencing is an important and delicate method, but false positive results may occur due to contamination of tumor samples, while false negative findings are possible if the mutation is located in a position that is disadvantageous for a proper primer (Sjögren et al. 1996). Immunohistochemistry for the analysis of p53 mutation analysis has been criticized, and IHC clearly has its limitations as a mutation detector. In Sjögren’s study immunohistochemistry failed to detect 33% of p53 gene mutations detected by sequencing. Up to 30% of immunohistochemically p53 positive tumors were detected in tumors with a wild-type p53 gene, suggesting a false positive finding in IHC (Sjögren et al. 1996). Bearing in mind the complex post-translational regulation of p53 protein IHC, positive staining in a tumor with wild-type p53 gene may not always indicate a “false” positive immunohistochemical result as p53 accumulation in a cell could serve as an indicator of aberrant p53 function. However, a false negative IHC result might be of a more relevant concern, because gross deletions in the p53 gene may produce truncated p53 protein unable to be detected by immunohistochemistry (Bergh 1999). In addition, immunohistochemical staining results are interpreted subjectively; the importance of experience of the pathologist interpreting the findings must therefore be emphasized. Stenmark-Askmalm suggested that an optimal technique for p53 diagnostics is a combination of IHC and gene sequencing, as they provide complementary data on p53 function (Stenmark-Askmalm et al. 2004). The definition of positive immunohistochemical staining may also reflect on the heterogeneity seen in studies evaluating p53 prognostic relevance as well as antibodies used; however, the Do7 clone used in the present studies has established its position in p53 diagnostics (Bonsing et al. 1997, Kroger et al. 2006).
Secondly, the heterogeneity of patient populations may have an impact on the results achieved from p53 studies. Data concerning the genetic changes in pre-malignant breast disease support the idea that p53 mutation is an early step in breast carcinogenesis (Allred et al. 2001, Done et al. 2001), and p53 protein expression has also shown concordance between a primary tumor and metastatic regional lymph node (Tsutsui et al. 2002, Arun et al. 2003). Previous clinical studies have emphasized the adverse prognostic power particularly in node-positive disease. One could speculate that in the case of locally limited disease definitive local therapies have better curative potential in biologically aggressive disease as well, while the lack of a normally functioning p53 pathway might become evident in node-positive patients in whom any remaining subclinical micrometastases may eventually lead to metastatic breast carcinoma. An ideal study setting for the evaluation of the prognostic power of p53 would comprise a large study population treated only with local therapies. On the other hand, various adjuvant therapies are offered to most patients (Pestalozzi et al. 2005), which may alter the prognostic relevance of a single prognostic factor. Only patients with a very favorable prognosis (a small primary tumor with low malignancy grade, positive hormone receptor status and negative for HER-2) are nowadays excluded from medical adjuvant therapies. The sole prognostic importance of a single tumor characteristic may thus not be easy to study in the future. On the other hand, this emphasizes the need for finding new predictive factors in primary breast cancer.

In the present study the concomitant presence of p53 and HER-2 in breast cancer influenced dramatically the outcome of the disease and worsened the overall survival of patients (I). Although the number of patients was limited, the result still emerged as a statistically highly significant finding. This is in line with some earlier reports (Nakopoulou et al. 1996, Beenken et al. 2001). From a molecular-biologic point of view the hypothesis that a malignant cell with a simultaneous aberrant growth-inhibiting system and growth-inducing system necessarily produced a highly malignant genotype leading to excessive growth potential and subsequent poor survival of patients is very logical. P53 accumulation alone had a stronger effect on OS than c-erbB-2 positivity alone, which is in line with a previous study (Beenken et al. 2001). The average OS was more than five times longer in cases with wild-type p53 and negative c-erbB-2 compared to patients with tumors of simultaneous positive p53 and c-erbB-2. It can also be concluded that patients with a p53 positive tumor should be regarded as high-risk patients during clinical follow-up (II).
In the future, further studies are needed to evaluate more accurately p53 function and prognosis in breast cancer, and additional tools are probably needed for this purpose. MDM2 evaluation (Turbin et al. 2006) or determination of TP53BP2 gene expression encoding p53 binding protein (Cobleigh et al. 2005) would provide additional data of p53 function when analyzed together with p53 status. Particularly because p53 mutations occur at a lower frequency in breast carcinoma than in other common solid tumors, p53 dependent apoptosis in breast cancer are very likely to be affected by other mechanisms in addition to p53 gene mutations; for example changes in p53 target genes have scarcely been studied in this respect (Gasco et al. 2002, Lacroix et al. 2006). On the other hand, recent in vitro studies support the idea of therapeutic manipulation of p53 (Bai & Zhu 2006).

6.2 P53 as a predictive marker

The first study (I) suggests that positive p53 immunostaining could be a predictor of anthracycline-based chemotherapy resistance in MBC. Among 30 patients receiving anthracycline-based chemotherapy due to disease recurrence, treatment failure was frequently seen in the p53 positive group (73%) compared to the p53 negative group (33%). This could partly explain the shortened survival in patients suffering from p53 positive disease. The literature concerning the p53 predictive importance in MBC is limited. Mutated p53 is associated with poor anthracycline response in patients receiving weekly doxorubicin (Aas et al. 1996), but this has not been confirmed in other immunohistochemical studies (Niskanen et al. 1997, Sjöström et al. 1998, Hamilton et al. 2000). Despite the optimism based on preclinical data, even taxane-based therapies do not seem to offer the solution to p53 positive MBC (Sjöström et al. 2000, Van Poznak et al. 2002). However, there are reports showing compromised efficacy of anthracycline regimens in the adjuvant setting (Clahsen et al. 1998, Bottini et al. 2000, Mieog et al. 2006). Thor et al. suggested that patients with co-expressing c-erbB-2 and p53 positive tumors benefit from high-dose anthracycline regimens (Thor et al. 1998) and that p53 alteration benefited patients receiving high-dose adjuvant therapy (Kroger et al. 2006). Again, opposite data exist, too: Rozan et al. did not see any connection between p53 status and clinical benefit of adjuvant FAC therapy (Rozan et al. 1998).

Preclinical data reveal controversial results concerning the status of p53 as a marker for sensitivity to chemotherapeutics, which is the case in clinical studies as well. Methodological diversity and various patient populations do not allow making final and exclusive conclusions of the predictive value of p53. First of all: there are
no large prospective trials where a large number of patients is balanced for common primary tumor characteristics and the clinical efficacy of systemic therapies assessed in a subgroup of patients with various p53 statuses (Borresen-Dale 2003). Some randomized trials reporting predictive value of p53 have originally been planned to evaluate different treatment strategies rather than the importance of altered p53 status (Clahsen 1998, Bottini et al. 2000, Malamou-Mitsi et al. 2006). An optimal trial investigating the predictive power of p53 would provide comparative patient groups in terms of other tumor characteristics and treatments used. However, despite the great need for finding new predictive factors, this would be rather difficult to carry out. At least it would demand a multicenter project to assemble large study populations.

So far we are not able to demonstrate the exact mechanism and connection of traditional chemotherapeutics and p53 function; for example, there are no p53 targeted regimens available in clinical work comparable to antiestrogen therapy in estrogen receptor positive breast carcinoma. As estrogen and p53 involve transcription and regulation of very large amounts of genes, it is very likely that some genes are regulated by both mechanisms; therefore one could hypothesize that a defect or aberrant function of p53 could also result in changes in estrogen mediated DNA transcription and antiestrogen efficacy. However, anthracyclines have a toxic effect by topoisomerase II inhibition and free radical formation causing DNA damage, the latter being a possible link to a p53-based apoptotic pathway (Chu & De vita 2003). Polychemotherapy regimens may include both p53-dependent and -independent pathways, which could dilute the p53-related effects (Bergh 1999). On the other hand, the importance of p53 for chemosensitivity has been supported by the fact that p53 is not mutated in the most curable malignomas such as some hematopoietic and germ cell tumors. Inevitably p53 has a dual and complex role in chemosensitivity, because normally functioning p53 is able to increase apoptosis leading to a therapeutic effect, but it can also arrest growth and thereby increase drug resistance (Sjöström & Bergh 2001). In the future p53 will probably emerge as a target for drugs restoring the altered p53 regulatory mechanism in malignant cells (Lacroix et al. 2006). In breast cancer, intratumoral administration of adenoviral vector containing normally functioning p53 gene has been tested in a neoadjuvant setting together with chemotherapy, the results suggesting clinical activity for this kind of treatment approach (Cristofanilli et al. 2006).

The clinical outcome of postmenopausal patients receiving adjuvant antiestrogen therapy was investigated, and in this study population p53 accumulation clearly predicted worse outcome (II). This is in line with Silvestrini’s observations (Silvest-
rini et al. 1996), and similar results have lately been reported in stage I to III breast carcinoma as well (Linke et al. 2006). However, no correlation was seen in a large Danish population (Knoop et al. 2001). In MBC the literature shows controversial findings in terms of the benefit of tamoxifen treatment according to p53 status (Elledge et al. 1997, Berns et al. 2000, Berns et al. 2003). Study I did not provide new evidence of the predictive value of p53 concerning the efficacy of hormonal therapy in MBC; however, a recent study investigating first-line antiestrogen therapy in MBC suggested a positive correlation between p53 immunopositivity and a poor response to tamoxifen (Kai et al. 2006, Yamashita et al. 2006). It is not easy to separate the adverse prognostic and predictive impact of p53 from retrospective studies. However, this has not been discussed in the literature very much.

Despite methodological limitations, the present results justify the statement that antiestrogen therapy seems to be an insufficient adjuvant regimen in postmenopausal patients with axillary node metastasis and a p53 positive primary tumor (II).

6.3 MMP-9 expression in breast carcinoma and in premalignant lesions of breast

MMP-9 positivity was seen in 61% of postmenopausal breast carcinoma patients (III) and in 49% of invasive samples in a study examining different breast histologies (IV). This is generally in line with previous reports (Li et al. 2004, Kim et al. 2006b). In studies using IHC for the evaluation of MMP-9 expression, around 50% of breast carcinomas have expressed high MMP-9 immunopositivity (Scorilas et al. 2001, Pellikainen et al. 2004). MMP-9 is usually detected from infiltrative breast lesions, but the degree of immunopositivity varies also because of the diverse interpretation and scoring systems used in immunohistochemical staining.

When matrix metalloproteinase-9 immunoreactivity was investigated in different breast tissue samples ranging from benign lesions to clinically evident infiltrative carcinomas (IV), a distinctive pattern of MMP-9 staining was seen in ductal carcinoma in situ samples, where the majority of DCIS cells were staining intensively. In addition, hyperplastic and invasive carcinomas were most often negative for MMP-9, and MMP-9 staining intensity decreased along with malignancy grade. The MMP-9 immunoprofile in this type of setting is rarely investigated, but the present results are in line with a study of Zhao et al., where expression levels of MMP-9 along with MMP-26, TIMP-2 and TIMP-4 by immunohistochemistry were higher in DCIS than in normal breast, hyperplastic tissue or invasive cancer specimens (Zhao et al. 2004). When MMP-9 expression has been evaluated by mRNA
in situ hybridization, MMP-9 activity is reported to be greater in DCIS than in infiltrative cancer, not reaching statistical significance, however (Kim et al. 2006b).

In other malignancies MMP-9 levels have usually been higher in invasive tumors compared to benign tissue. This is the case especially in prostate cancer (Zeng et al. 2003, Zhang et al. 2004) and bladder cancer where MMP-9 activity increases with tumor grade (Davies et al. 1993). On the other hand, a correlation between MMP-9 immunoreactivity and tumor malignancy grade has been a controversial issue in other malignancies: a positive connection has been shown in malignant gliomas (Wang et al. 2003), non-small cell lung cancer (Leinonen et al. 2006) and endometrial cancer (Aglund et al. 2004), or epithelial ovarian cancer (Sillanpää et al. 2006) or chondrosarcoma (Sugita et al. 2004).

6.4 MMP-9 and neoplastic process in breast tissue

It is possible that after crucial genetic damage and cell transformation, interaction between a tumor and its microenvironment becomes more restrictive in promoting malignant progression, such as the infiltration that eventually leads to a clinically evident tumor. The degradation of ECM is very important in this context, and gelatinase activity is therefore of clinical interest. Based on the present preliminary findings and the reviewed literature, it can be postulated that the overexpression of MMP-9 is an early phenomenon in breast carcinogenesis, already existing in the preinvasive stage of the malignant progress. MMP-9 activity could be essential for the malignant progress especially in the preinvasive stage, while other factors may play a more important role after basement membrane breakdown and cancer invasion (Zhao et al. 2004). In line with this concept it is logical that strongest MMP-9 protein expression was seen in preinvasive DCIS and there was a clear descending trend in strong immunoreactivity in invasive carcinoma.

However, the final biologic effect of MMP-9 and other MMPs may reflect the interaction between tumor and ECM rather than expression of a single factor (DeClerck et al. 2004). Especially studies reporting different prognostic effects of MMP-9 in stromal and tumor cells suggest that MMP-9 has a dual role in tumor progression (Pelikainen et al. 2004, Sillanpää et al. 2006). MMP-9 activity might be indirectly involved in the regulation of the metastatic tumor phenotype since angiogenic switch and increased host immune responses may dominate in directing the clinical course of malignancy. Preclinical studies also show that the role of MMPs in carcinogenesis in general is very complex, and even anti-metastatic acti-
vities have been described (Chambers & Matrisian 1997, Duffy & McCarthy 1998, Deryugina & Quigley 2006).

Many matrix metalloproteinases are usually linked to malignant progression in breast tissue. MMP-2 activity seems to increase from hyperplastic samples to DCIS while the highest activities have been seen in invasive cancer (Lee et al. 1996). mRNA levels of MMP-2 also seem to gradually increase from non-invasive to invasive cancers (Brummer et al. 1999), and collagenase-3 (MMP-13) has been shown to associate particularly with microinvasive lesions in DCIS samples when studied by immunohistochemistry (Nielsen et al. 2001).

The immunohistological profile of MMP-9 in premalignant breast disease (IV) shows some similarities to changes in p53 and c-erbB-2. While p53 alterations are not seen in atypically hyperplastic cells and HER2 positivity/amplification is rare, both markers show frequent alterations in high grade in situ carcinomas (Done et al. 2001, Latta et al. 2002, Ottesen 2003). When applying the MMP-9 immunoprofile to a genetic progression model, the present results may mirror a dynamic process and flexibility in gene expression in relation to tumor microenvironment in carcinogenesis rather than accumulative, stable genetic changes.

6.5 MMP-9 as a factor for prognosis

The prognostic relevance of MMP-9 in breast carcinoma has rarely been studied, while more convincing data suggest that MMP-2 is an indicator of poor survival (Talvensaari-Mattila et al. 1998, Talvensaari-Mattila et al. 2003, Duffy et al. 2000, Leppä et al. 2004). The aim of study three was to examine the possible prognostic impact of MMP-9 immunoreactivity in node-positive postmenopausal breast cancer patients treated with an adjuvant antiestrogen therapy, and a tendency for compromised disease-free survival in a subgroup of hormone receptor-negative patients expressing MMP-9 in their primary tumor was observed. However, MMP-9 immunopositivity did not predict survival in the patients with a hormone receptor-positive primary tumor. Scorilas et al. found MMP-9 positivity to be an indicator of a favorable prognosis in node-negative patients, but this was not seen in node-positive patients, the latter finding being comparable with the present results (Scorilas et al. 2001). However, in a Chinese population DFS was clearly worsened by MMP-9 positivity in node-negative patients (Li et al. 2004).

The varying results may be partly explained by different study methods. It has been shown that MMP-9 expression in carcinoma cells and surrounding stroma cells may have different prognostic relevance. In a Finnish material strong MMP-9
immunoreactivity in breast cancer cells predicted favorable outcome of the patients, while MMP-9 expression in stroma associated with disease recurrence (Pellikainen et al. 2004). On the other hand, MMP-9 expression in stromal cells has had no prognostic value in some other studies (Remacle et al. 1998, Tetu et al. 1998, Li et al. 2004). In studies evaluating MMP-9 activity in blood samples low MMP-9 has associated with worse outcome (Ranuncolo et al. 2003, Talvensaari-Mattila & Turpeenniemi-Hujanen 2005), but this has not been confirmed elsewhere (Remacle et al. 1998, Leppä et al. 2004). Previous prognostic studies reveal inconclusive results, probably due to heterogeneous patient materials. Particularly in MMP studies the stage of the disease seems to be important: MMP-9 activation in a small primary tumor may result in different biological effects and prognostic relevance than in a case of massive metastatic disease (Deryugina & Quigley 2006).

Adjuvant therapies have impact on prognosis, too. Patients with decreasing plasma MMP-9 activity during adjuvant therapy have been shown to have better survival compared to those whose plasma activity showed enhancement in correlation with a lack of response (Ranuncolo et al. 2003). All patients in study three had been treated with an adjuvant antiestrogen therapy due to axillary node involvement, which is known to be effective in patients with a hormone receptor-positive primary tumor. It is thus logical that MMP-9 seemed to be associated with unfavorable prognosis only in patients negative for hormone receptors who did not receive such effective anticancer therapy. Preclinical data also show a connection between estrogen-mediated signals in a cell and MMP expression. Estradiol signal mediates EGFR transactivation along with activation of both MMP-9 and MMP-2 in vitro (Razandi et al. 2003). Letrozole has been shown to be a potent in vitro inhibitor of cell proliferation and it has been reported to decrease both MMP-9 and MMP-2 expression in breast cancer cells (Mitropoulou et al. 2003). Wolczynski et al. investigated breast cancer cells in vitro, and found raloxifene to have a dose-dependent effect on collagen metabolism and MMP expression, suggesting that these mechanisms may explain raloxifene’s role in the prevention of breast cancer development (Wolczynski et al. 2001).

6.6 MMP-9 immunoreactivity and implications for a clinical use

In the third study postmenopausal, node-positive patients with MMP-9 positive and ER negative tumors experienced compromised disease-free survival, the difference being 26% in the 5-year follow-up, which would be clinically important if confirmed in a larger material. This raises a question of whether MMP positivity could
indicate the benefit of hormonal treatments in ER positive cases, because matrix metalloproteinases, including MMP-9, are partially hormonally regulated. Tamoxifen reduces breast carcinoma recurrences in DCIS patients (Fisher et al. 1995). Therefore one could also speculate whether in the future a patient with DCIS expressing strong MMP activity could be a candidate for hormonal chemoprevention. It would also be interesting to find out whether aromatase inhibitors lower the risk effect of MMP-9 positivity similarly as, or in some groups even better than antiestrogens. So far, matrix metalloproteinase inhibitors have not provided a promising new treatment strategy; marimastat has failed to show any efficacy in metastatic breast cancer (Sparano et al. 2004) and musculoskeletal toxicity restricts the tolerability of this regimen (Sparano et al. 2004, Miller et al. 2002).

Finally, when the immunohistochemical profile of MMP-9 in different breast histologies was investigated, MMP-9 expression was seen to be very different in atypical ductal hyperplasias compared to DCIS samples. Intensive MMP-9 staining was rare in ADH cells while negative MMP-9 staining predominated, the results being quite opposite in DCIS cells. By classic morphology the histological distinction between atypical ductal hyperplasia and low grade ductal carcinoma in situ is a demanding one, and there is no general agreement as to whether quantitative criteria should be used to separate ADH from low-grade DCIS (Tavassoli & Devilee 2003). At the same time, mammography screening increases the detection of preinvasive DCIS lesions (Ernster & Barclay 1997). If these very preliminary findings are confirmed in a larger clinical material, we would be able to evaluate further whether the immunoreactivity of MMP-9 could serve as a diagnostic tool in differential diagnosis between ADH and low-grade DCIS.

The data presented reveal remarkable but transient MMP-9 immunoreactivity in ductal carcinoma in situ, suggesting its active role in breast carcinogenesis. However, the clinical relevance of MMP-9 in DCIS should be examined in a prospective clinical study and in a larger material considering its relationship to other biological factors such as DCIS grade, metalloproteinase tissue inhibitor activity and stromal reactions (Jinga et al. 2006).
Conclusions

In the present study, tumor suppressor gene p53, oncogene c-erbB-2 and matrix metalloproteinase-9 were explored as prognostic and predictive factors in breast cancer patients by immunohistochemical methods. The MMP-9 immunoreactive protein was also evaluated in premalignant lesions of the breast in order to investigate MMP-9 positivity in different phases of breast carcinogenesis. The specific conclusions of this study are:

1. P53 positivity correlates strongly with poor breast cancer specific survival of patients, and especially the co-expression of p53 and c-erbB-2 in a primary tumor associates with a great risk of disease relapse and compromised survival.

2. Positive p53 immunohistochemical analysis of a primary tumor also correlates with compromised survival in the metastatic stage of breast carcinoma.

3. The results suggest p53 immunopositivity being an indicator of anthracycline resistance in metastatic breast carcinoma. However, the finding needs to be validated in a larger material.

4. Antiestrogen therapy seems to be insufficient adjuvant therapy in postmenopausal patients with a p53 positive primary tumor.

5. In postmenopausal patients treated with an adjuvant antiestrogen therapy MMP-9 immunopositivity in a primary tumor does not markedly predict breast cancer specific survival, but disease relapses seem to occur more often in a subgroup of patients with MMP-9 positive and receptor negative hormone primary tumor, the result getting close to a statistical significance.

6. Ductal carcinoma in situ of the breast expresses stronger MMP-9 immunopositivity than hyperplastic or invasive breast carcinoma lesions, suggesting active role of MMP-9 in the premalignant phase of breast carcinogenesis.
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