

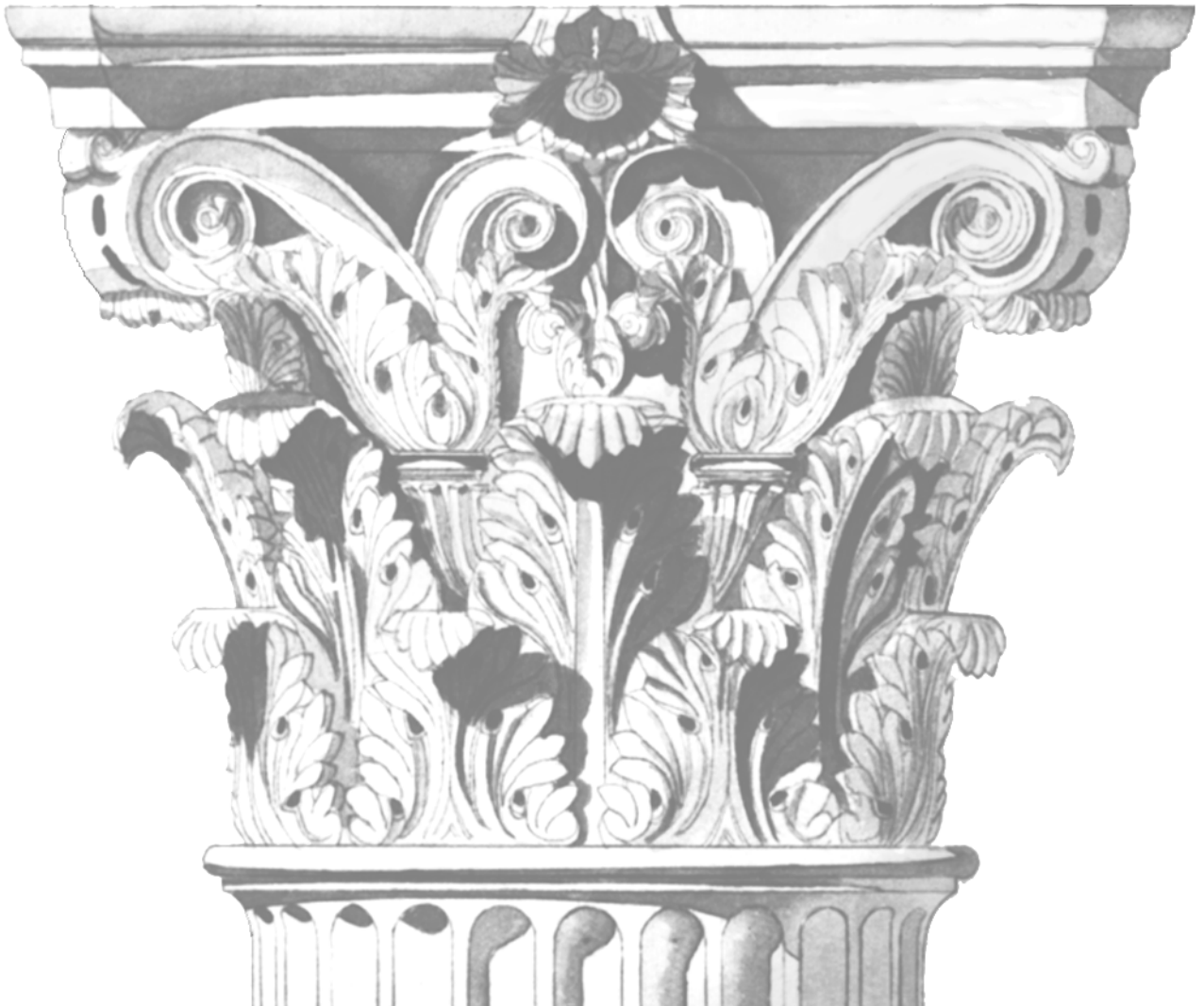
**MMP-2 IMMUNOREACTIVE  
PROTEIN IN BREAST  
CARCINOMA AND  
NEOPLASTIC CERVICAL  
LESIONS**

**ANNE  
TALVENSAARI-MATTILA**

Department of Oncology and Radiotherapy

MMP-2 is a new prognostic factor in breast carcinoma

OULU 1999



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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 7 of the University Hospital of Oulu, on October 15th, 1999, at 12 noon.

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**Anne Talvensaaari-Mattila, MMP-2 immunoreactive protein in breast carcinoma and neoplastic cervical lesions. MMP-2 is a new prognostic factor in breast carcinoma**

Department of Oncology and Radiotherapy, University of Oulu, FIN-90401, Oulu, Finland

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*Abstract*

Tumor invasion and metastasis are the major causes of treatment failure or death for carcinoma patients. Matrix metalloproteinases (MMPs) are zinc dependent endopeptidases implicated in tumor invasion and metastasis. The expression of MMP-2 has been previously linked to invasiveness of carcinoma cells. The MMP-2 immunoreactive protein was studied here in squamous cell carcinoma of the utrine cervix and in adenocarcinoma of the breast by using a specific monoclonal antibody in immunohistochemical stainings. Immunoreactive protein of latent MMP-2 was found to be an early event in neoplastic transformation of the cervix in 60 patients. All cases of early stage cervical carcinoma expressed the latent MMP-2 protein, suggesting that MMP-2 could be a prerequisite for invasive behavior. In early stage cervical carcinoma the high score of MMP-2 expression seemed to be associated with poor histological differentiation and lymph node metastases. The intensivity (score) of the immunoreaction was not, however, associated with clinical behavior of this disease.

New predictive markers would be useful in selecting breast carcinoma patients to different modalities of adjuvant therapy. The MMP-2 protein has been found in breast carcinoma tumor cells in immunohistochemical analyses. MMP-2 has been found to be expressed in breast carcinoma in some preliminary studies, but there are no reports so far that would show a correlation of MMP-2 to survival in breast carcinoma. In the current study comprising 373 patients the expression of MMP-2 protein was found immunohistochemically in primary breast carcinomas. It is shown here for the first time that immunoreactive protein of MMP-2 in primary breast carcinoma is associated with a shortened relapse-free survival (RFS) or relative overall survival (OS). MMP-2 correlated to the risk of failure during the anti-estrogen adjuvant therapy in postmenopausal breast carcinoma patients with axillary lymph node metastasis without a high tumor burden. It was also found here that premenopausal patients with a node positive breast carcinoma showing MMP-2 positivity relapsed early after the primary operation. Young patients (<40 years) with MMP-2 positive tumors had a poor outcome when compared to other node-positive premenopausal breast carcinoma patients. A patient group with a high risk for an early relapse was identified from node-positive, premenopausal breast carcinoma patients.

In conclusion, the present data show for the first time MMP-2 immunoreactive protein to be a prognostic factor in breast carcinoma, indicating further studies to explore the value of this enzyme in clinical decision making.

*Keywords:* Gelatinase A, 72 kD type IV collagenase, metastasis, invasion



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Oulu, September 1999

Anne Talvensaari-Mattila

## Abbreviations

AEC	amino-ethyl-carbazole
ASCO	American Society of Clinical Oncology
BRCA 1, 2	breast cancer 1 gene and 2 gene
BSA	bovine serum albumin
CA-4001	monoclonal antibody
CIN	cervical intraepithelial neoplasia
CMF	cyclophosphamide-methotrexate-fluorouracil
DNA	deoxyribonucleic acid
EBV	Ebstein-Barr virus
FEC	cyclophosphamide-epirubicin-5-fluorouracil
FIGO	International Federation of Gynecology and Obstetrics
FSH	Follicle stimulating hormone
GVA	glycerol-vinyl-alcohol
HPV	human papilloma virus
HSV	herpes simplex virus
IARC	International Agency for Research on Cancer
IgG	immunoglobulin G
mRNA	messenger RNA
MMP	matrix metalloproteinase
MT-MMP	membranetype-matrix metalloproteinase
OS	overall survival
RFS	relapse free survival
RR	relative risk

SCC	squamous cell carcinoma
TIMP-1	tissue inhibitor of metalloproteinase-1
TIMP-2	tissue inhibitor of metalloproteinase-2
TNM	tumor, node, metastasis
UICC	International Union Against Cancer
WHO	World Health Organization

## List of original articles

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

- I Talvensaari-Mattila A, Apaja-Sarkkinen M, Höyhty M, Westerlund A, Puistola U & Turpeenniemi-Hujanen T (1999) Matrix metalloproteinase-2 immunoreactive protein appears early in cervical epithelial dedifferentiation. *Gynecol Oncol.* 72: 306-311
- II Talvensaari-Mattila A, Pääkkö P, Höyhty M, Blanco-Sequeiros G & Turpeenniemi-Hujanen T (1998) Matrix metalloproteinase-2 immunoreactive protein, a marker of aggressiveness in breast carcinoma. *Cancer* 83: 1153-1162.
- III Talvensaari-Mattila A, Pääkkö P, Blanco-Sequeiros G & Turpeenniemi-Hujanen T. Matrix metalloproteinase-2 (MMP-2) discerns well subgroups at risk for a relapse during anti-estrogen adjuvant therapy in postmenopausal patients with node-positive breast carcinoma. Submitted for publication.
- IV Talvensaari-Mattila A, Pääkkö P & Turpeenniemi-Hujanen T. MMP-2 and age < 40 years associate to high risk for early relapse in node-positive premenopausal breast carcinoma patients. *Breast Cancer Res Treat.* In press.

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## 1. Introduction

Matrix metalloproteinases are a family of zinc-dependent endopeptidases. These enzymes are synthesized by connective tissue cells, and are secreted as proenzymes in the latent form and require extracellular activation. MMP-2 (72-kDa type IV collagenase/gelatinase A) is a member of the matrix metalloproteinase family, which is found in several types of normal and malignant tissues. The role of this proteinase is thought to be associated in tissue remodeling and invasion of migrating cells such as leucocytes and malignant cells (Ray & Stetler-Stevenson 1994). MMP-2 expression is linked to invasiveness in several human neoplasias including breast, colon, ovarian, and hepatocellular carcinomas and melanoma, suggesting an interesting role in determining the invasive potential and metastatic capacity of the neoplastic lesions (Liotta *et al.* 1980, Monteagudo *et al.* 1990, D'Errico *et al.* 1991, Campo *et al.* 1992, Väisänen *et al.* 1998). In this work the role of MMP-2 was studied in two different types of carcinoma, squamous cell carcinoma of the uterine cervix and adenocarcinoma of the breast. The pattern of the growth of these carcinomas is different. Cervical carcinoma typically grows *per continuitatem* and metastasizes to regional lymph nodes, whereas breast carcinoma spreads locally. The distant metastases of breast carcinoma often result from a lymphatic or hematogenous spread.

In many developing countries cervical carcinoma continues to be the leading cause of carcinoma deaths for women (Parkin *et al.* 1997). In Western countries the incidence of cervical intraepithelial neoplasia (CIN) is increasing, whereas the occurrence of squamous cell carcinoma is decreasing, at least partially due to the screening and early detection of premalignant lesions (Netherlands Cancer Registry 1994). During the last few decades the incidence of cervical carcinoma has been decreasing in Finland (Finnish Cancer Registry 1997). However, a slightly increasing trend in cervical carcinoma has been seen during the last few years in Finland.

Breast carcinoma is the most frequent malignancy in women in most populations (Parkin *et al.* 1997). It constitutes about 20% of all female carcinomas. Breast carcinoma includes a heterogeneous group of tumors with variable prognosis. They are potentially highly malignant tumors due to their capacity to invade locally and metastasize. Although improved access to mammography has increased the incidence of early stage breast carcinoma with a favorable prognosis (Cody 1995), locally advanced breast carcinoma with

unfavorable prognosis still accounts for 10-29% of all breast carcinomas in industrialized countries (Valero *et al.* 1996). The traditional prognostic factors of breast carcinoma include axillary lymph node involvement, the size of the primary tumor, tumor histologic grade, hormone receptor status, and menopausal status of the patients (Harris *et al.* 1997). At the time of the diagnosis 35 % of the breast carcinoma cases have axillary metastases (Shetty & Reiman 1997) and are thus subjected to adjuvant therapies (McGuire & Clark 1992, Gelber *et al.* 1993). In some cases the high risk patients have been suggested to benefit from increased dose intensity (Budman *et al.* 1998) or high dose chemotherapy (Crown & Norton 1995) that is still under debate (Bezwoda 1999, Mouridsen *et al.* 1999, The Scandinavian Breast Cancer Study Group 1999). Despite adjuvant systemic therapy, some early stage breast carcinoma patients still die of metastatic disease.

It is possible that new predictive markers could be useful in selecting the patients for different modalities of adjuvant therapy. Systemic chemotherapy is today widely used as an adjuvant therapy in premenopausal patients with node-positive breast carcinoma. Node positive breast carcinoma is considered to be an aggressive disease with a 69% overall survival 5 years after diagnosis (Finnish Cancer Registry 1997). In most western European countries 40% of breast carcinomas are diagnosed at the age of 65 years or more (Ries *et al.* 1983). Anti-estrogens have been commonly used as an adjuvant therapy in postmenopausal breast carcinoma patients (Osborne 1998). It is, however, likely that some patients in this group could also benefit from other treatment modalities. MMP-2 has in several studies been shown to be expressed in breast carcinoma (Monteagudo *et al.* 1990, Davies *et al.* 1993, Tryggvason *et al.* 1993). Using immunohistochemical methods, the protein has been found in breast carcinoma tumor cells (Daidone *et al.* 1991, Höyhty *et al.* 1994). MMP-2 has been linked to invasiveness in breast carcinoma (Azzam *et al.* 1993, Iwata *et al.* 1996), but the prognostic value of MMP-2 has not been evaluated previously. In the present work the role of latent MMP-2 was studied in cervical intraepithelial neoplasias as well as in cervical and breast carcinomas. The value of MMP-2 protein was then studied further in detail as a prognostic factor in breast carcinoma patients with axillary lymph node metastases.

## **2. Review of the literature**

### **2.1. Cervical neoplasias**

Cervical carcinoma is the most common carcinoma in developing countries and it is also associated with a lower socioeconomic status. Incidence and death rates are highest in Latin America, Africa, India and in Eastern Europe (Herrero 1996, Krul *et al.* 1996, Parkin *et al.* 1997). On the other hand, in Western countries the incidence of cervical intraepithelial neoplasia (CIN) is increasing, whereas the occurrence of squamous cell carcinomas is decreasing, at least partially due to the screening and early detection and treatment of premalignant lesions (Netherlands Cancer Registry 1994). The nationwide mass screening program in Finland has decreased the incidence and mortality of squamous cell carcinoma, whereas the incidence and mortality of cervical adenocarcinoma has not changed during the last thirty years (Nieminen *et al.* 1995). Women younger than 30 years have the highest rate of CIN, whereas invasive carcinoma is mainly diagnosed among women older than 40 years (Lawson *et al.* 1998).

The incidence of cervical carcinoma has been decreasing in Western countries and especially in Finland due to cytological screening tests (PAP smear screening) (Mahlck *et al.* 1994, Nieminen *et al.* 1995). However, the incidence of cervical carcinoma has recently increased in younger women (30-49 years) in Finland (Finnish Cancer Registry 1997), being 6.9 - 7.1 per 100 000. This malignant disease is, however, still extremely common in developing countries (Parkin *et al.* 1999). The majority (80-90%) of cervical carcinomas are squamous cell carcinomas (SCC).

#### ***2.1.1. Cervical intraepithelial neoplasia***

The incidence of noninvasive intraepithelial neoplasia (CIN I, II and III or cervical dysplasia and carcinoma in situ) is not known but it is estimated that approximately 10% of the Papanicolaou tests performed annually in the United States will show some type of abnormality (Kurman *et al.* 1994).

The epithelium covering the cervix contain two cell types: squamous and columnar. The squamous epithelium covers the ectocervix and is continuous with the vaginal epithelium. It is stratified, non-keratinizing epithelium, which is separated from the underlying stroma by basal lamina. Stratification refers to the way in which the epithelium is divided into layers of progressively more mature and flattened cells as the surface is reached. Basal layer cells have a large nuclear cytoplasmic ratio and this is attached to the basal lamina. The parabasal cells form the next few layers. Further maturation and differentiation produces the intermediate cells in which the cytoplasm is more abundant, and the superficial layer of epithelium which is composed of markedly flattened cells. The fully mature and differentiated cell of the squamous epithelium is very flat (Anderson *et al.* 1992).

It is believed that CIN represents stages of morphologic continuum towards invasive carcinoma of the cervix. These changes begin with minimal atypia and progress through stages of more marked abnormalities to invasive squamous cell carcinoma (Johnson *et al.* 1968). CIN or dysplasia are divided into three grades. The squamous epithelium undergoes changes leading to mild abnormalities, i.e. nuclear abnormalities confined to the deeper layers of the epithelium (CIN I). In CIN II the nuclear abnormalities are more marked and extend higher in the epithelium than in CIN I. In CIN III or carcinoma in situ nuclear abnormalities may extend through the entire thickness of the epithelium.

It has been shown that a large proportion of low-grade lesions in cervical cytology will regress spontaneously (Kurman *et al.* 1994, Matsuura *et al.* 1998, Holowaty *et al.* 1999). Holowaty *et al.* (1999) found that at 10 years, over half of the women with mild or moderate dysplasia had not progressed to a more severe lesion. Progression from moderate to severe dysplasia or from mild to moderate dysplasia was highest in the first two years. High-grade CIN lesions are less likely to regress to normal and are more likely to progress to invasive carcinoma (Koss 1989, Melnikow *et al.* 1998). At least 20% of the CIN III cases progress to invasive carcinoma within 10 years. Long-term follow-up studies show that invasive recurrences continue to appear for at least 8 years after treatment for CIN, and the risk remains 4 to 5 times greater than in the background population (Soutter 1998). Some studies have expressed that less aggressive treatment will result in increased rates of invasive cervical carcinoma (Ferenczy 1993, Flannelly G & Kitchener 1995).

In CIN lesions colposcopic examination is important in order to delineate the extent of the lesions to indicate the areas to be biopsied. The majority of the CIN I-III changes are treated with LEEP (the large loop excision of the transformation zone) and also cryotherapy, laservaporization or cone biopsy are used. The choice of treatment is based on the severity and location of the lesion as well as on the availability of different treatment modalities in specific hospitals.

### ***2.1.2. Cervical carcinoma***

Squamous cell cervical carcinoma is often associated with chronic cervicitis, severe dysplasia and carcinoma in situ, usually progressing over 10-20 years (Barron & Richart 1970, Kashigarian & Dunn 1970). Several risk factors for cervical carcinoma have been

identified, such as HPV infection (Kadish *et al.* 1986, zur Hausen 1996, Gomousa-Michael *et al.* 1997, Lombard *et al.* 1998, Kjellberg *et al.* 1999), early age at first intercourse, large number of lifetime sexual partners (Brinton 1992), sexually transmitted diseases (Paavonen *et al.* 1998), and low socioeconomic status (Herrero 1996).

Moleculobiological studies have demonstrated a strong relationship between human papillomavirus (HPV), CIN and invasive cervical carcinoma (Kadish *et al.* 1986, Smotkin & Wettstein 1986, Park *et al.* 1995). HPV DNA (deoxyribonucleic acid) sequences are found in approximately 90% of the cervical carcinomas (Iwasawa *et al.* 1996). HPV types 16 and 18 were declared to be human carcinogens by the International Agency for Research on Cancer (IARC 1995). They are the most important single etiological causes of cervical squamous cell carcinomas (zur Hausen 1996). Types 16, 18, 31, and 33 have been associated with high grade CIN lesions and invasive carcinomas (Crum *et al.* 1985, zur Hausen 1991, Lorincz *et al.* 1992, Ho *et al.* 1998, Roteli-Martins *et al.* 1998, Kjellberg *et al.* 1999), whereas types 6 and 11 usually cause benign warts, but are occasionally associated with invasive lesions. Also other viruses, like the Herpes simplex virus (HSV) and the Epstein-Barr virus (EBV) are linked as cofactors to CIN (Landers *et al.* 1993, van den Brule *et al.* 1995, Payne *et al.* 1995).

In the earliest stage at which invasion can be recognized a small group of cells is seen to penetrate the basement membrane and push into the underlying stroma. Cervical carcinoma grows by direct invasion to surrounding structures and through lymphatic vessels, and only rarely by the hematogenous route. Squamous cell cervical carcinoma has been found to extend into the lower uterine segment and endometrial cavity in 10% to 30% of the patients (Perez *et al.* 1981). Local extension into surrounding tissues results in ureteral compression as well as bladder and rectal involvement. Metastases to regional lymph nodes involve the paracervical, hypogastric and external iliac nodes. As invasion becomes more advanced, the cells become better differentiated than the overlying CIN. (Anderson *et al.* 1992).

The International Federation of Gynecology and Obstetrics (FIGO) has accepted a staging system for cervical carcinomas (Table 1). The FIGO stage is based on careful clinical examination and the results of specific radiologic studies.

The standard treatment for patients with early stage I cervical carcinoma is radical hysterectomy and bilateral pelvic lymph node dissection. In more advanced diseases preoperative radio-, chemo- or chemoradiotherapy before operation can be used. Postoperative external radiation therapy is offered to high risk patients. There are several studies in which adding concurrent chemoradiotherapy (cisplatin or cisplatin and fluorouracil) to radiotherapy significantly improved survival and recurrence free survival among women with stage IB (Keys *et al.* 1999) and with locally advanced cervical carcinoma (Morris *et al.* 1999, Rose *et al.* 1999).

The 5-year overall survival rate in cervical carcinoma is approximately 65-70% (FIGO Annual Report 1998, Suris & Dexeus 1998). Early stage cervical carcinoma has a relatively favorable prognosis, with a 5-year survival rate of 80-95% (FIGO Annual Report 1998). When the pelvic lymph nodes are involved, the 5-year survival rate decreases to 40-50% (Sevin *et al.* 1995, Lin *et al.* 1996).

*Table 1. International Federation of Gynecology and Obstetrics Staging of Carcinoma of the Cervix (1994)*

Stage	Definition
Stage 0	Carcinoma in situ, intraepithelial carcinoma
Stage I	The carcinoma is strictly confined to the cervix
Stage IA	Invasive cancer identified only microscopically. All gross lesions, even with superficial invasion, are stage IB cancers. Invasion is limited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm.
Stage IA1	Measured invasion of stroma no greater than 3 mm in depth and no wider than 7 mm.
Stage IA2	Measured invasion of stroma no greater than 3 mm and no greater than 5 mm in depth and no wider than 7 mm.
Stage IB	Clinical lesions confined to the cervix or preclinical lesions greater than IA.
Stage IB1	Clinical lesions no greater than 4 cm
Stage IB2	Clinical lesions greater than 4 cm
Stage II	The carcinoma extends beyond the cervix, but has not extended onto the pelvic wall; the carcinoma involves the vagina, but not as far as the lower third.
Stage IIA	No obvious parametrial involvement.
Stage IIB	Obvious parametrial involvement.
Stage III	The carcinoma has extended onto the pelvic wall.
Stage IIIA	No extension onto the pelvic wall, but involvement of the lower third of the vagina.
Stage IIIB	Extension onto the pelvic wall or hydronephrosis or nonfunctioning kidney.
Stage IV	The carcinoma has extended beyond the true pelvis or has clinically involved the mucosa of the bladder or rectum.
Stage IVA	Spread of the growth to adjacent organs.
Stage IVB	Spread to distant organs.

The prognosis of cervical carcinoma is dependent on the presence or absence of pelvic lymph node metastasis (Sevin *et al.* 1995, Lin *et al.* 1996), which is the most powerful prognostic factor. Also other prognostic factors such as the size of the primary tumor, tumor grade, depth of invasion, lymphovascular space involvement and tumor extension over the cervix appear to be related to survival. As invasion progresses other histological features need to be taken into account, such as tumor dimension, lymphatic channel involvement and pattern of growth (Anderson *et al.* 1992).

## 2.2. Breast carcinoma

Breast carcinoma is the most frequent malignancy among women in Finland as well as in other Western countries (Finnish Cancer Registry 1997, Parkin *et al.* 1997). It is a pathologically and clinically heterogenous disease with variable prognosis. Breast carcinomas are potentially highly malignant tumors due to their capacity to invade locally and metastasize. The prognosis of breast carcinoma has improved from the 1970s due to better

access to mammography and the development of adjuvant therapy (Cady *et al.* 1996, Finnish Cancer Registry 1997, Edwards *et al.* 1998).

### 2.2.1. Epidemiology

The incidence and prevalence of breast carcinoma have increased during the last decades in Finland and in other Western countries (Kelsey & Horn-Ross 1993, Garfinkel *et al.* 1994, Finnish Cancer Registry 1997, Parkin *et al.* 1997). In Finland over three thousand new breast carcinoma cases appear annually. In women < 40 years 146 and in women > 70 years 883 new breast carcinoma cases appeared in 1995 (Finnish Cancer Registry 1997). The incidence rate per 100 000 person-years has increased from 30 in the 1960s to 67.7 in 1994 (Finnish Cancer Registry 1997). In Western countries the high-risk population age-specific incidence increases rapidly between the ages 45-50, and again after 50 years of age at a slower pace (Stevens *et al.* 1982). The recurrence rate within 10 years is about 20-30% even in node-negative carcinomas due to residual disease or biologically aggressive disease (Macmillan *et al.* 1996).

Causes of breast carcinoma are multifactorial. Several risk factors for breast carcinoma have been found, including early menarche (RR (relative risk) 1-2), late full-term pregnancy (RR 2-4), age < 50 years (RR 4), upper social class (RR 2-4), postmenopausal obesity (RR 2-4) (Hayes 1996a, Trentham-Diez *et al.* 1997) as well as others, such as positive family history (relative risk at least 4 times that of population without the factor) (Egan *et al.* 1998), increased genetic risk (BRCA 1, 2) (Miki *et al.* 1994, Wooster *et al.* 1995), earlier benign breast disease, previous ionizing radiation (Hancock *et al.* 1993) or alcohol consumption (Friedenreich *et al.* 1993, Longnecker *et al.* 1995, Enger *et al.* 1999). Early menopause, natural or surgical, is associated with decreased breast carcinoma risk and the protective risk is strongest in women with surgical menopause before age <40 years (Titus-Ernstoff *et al.* 1998). Also women with heavy physical activity have a lower risk of breast carcinoma (Coogan *et al.* 1997). Approximately 50% of women who develop breast carcinoma have no identifiable risk factors (Madigan 1995).

### 2.2.2. Staging

When the diagnosis of breast carcinoma has been confirmed, the primary tumor must be staged to determine prognosis and optimal treatment. Disease stage, tumor size and axillary node involvement were determined according to the UICC (International Union Against Cancer), TNM classification (Hermanek *et al.* 1992), and the histology of the tumors was reviewed and classified according to the World Health Organization (WHO) classification of breast tumors (Scarff *et al.* 1986). The breast carcinoma staging system is found in Table 2.

The most common type of carcinoma is infiltrative ductal carcinoma (70%) graded into well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3) carcinoma. The grading is based on three microscopic features: tubular differentiation, the degree of nuclear pleomorphism and the number of mitotic figures.

There is a significant correlation between grade and prognosis (Schnitt 1993, Berg & Hutter 1995, Heywang-Köbrunner *et al.* 1997). Lobular carcinoma is the second most common histology group in breast carcinoma, followed by smaller groups such as medullary, mucinous, comedocarcinoma, Paget's disease, papillary, tubular and inflammatory carcinoma (Berg & Hutter 1995).

*Table 2. Clinical staging system for breast carcinoma*

T (Tumor)	Definition
TX	Primary tumor not assessable
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor 2 cm or less in greatest dimension
T2	Tumor more than 2 cm but not more than 5 cm in greatest dimension
T3	Tumor more than 5 cm in greatest dimension
T4	Tumor of any size with direct extension into chest wall or skin edema or skin ulceration or satellite skin nodules confined to the same breast or inflammatory carcinoma
N (Regional lymph nodes)	
NX	regional lymph nodes not assessable
N0	No regional lymph node involvement
N1	Metastasis to movable ipsilateral axillary lymph nodes
N2	Metastasis to ipsilateral axillary lymph nodes fixed to one another or to other structures
N3	Metastasis to ipsilateral internal mammary lymph nodes
M (Metastasis)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present

### **2.2.3. Prognostic factors**

Many prognostic factors have been identified in breast carcinoma. The most commonly used indicators for prognosis include the clinical stage of the disease, tumor size (Chevallier *et al.* 1990, Joensuu & Toikkanen 1991, Galea *et al.* 1992), lymph node metastases (Du-Toit *et al.* 1990, Pisansky *et al.* 1993, Ravdin *et al.* 1994, Barth *et al.* 1997), tumor grade (Bloom & Richardson 1957), estrogen or progesterone receptor status of the primary tumor (Chevallier *et al.* 1990, Castagnetta *et al.* 1992, Strierer *et al.* 1993). In patients with small primary tumors (less than 1 cm) metastatic axillary nodes are present in <10 %-20% of the patients (Carter *et al.* 1989, Chadha *et al.* 1995). In many studies low ER and PR rates are predictive of early recurrence and short overall survival (Fisher *et al.* 1981, Clark *et al.* 1993, Pichon *et al.* 1996). The expression of estrogen and progesterone receptor suggests a high likelihood of response to hormone therapy. They were the only prognostic and predictive tumor markers recommended for routine clinical use in breast carcinoma (ASCO (American Society of Clinical Oncology) Tumor Marker Panel 1996). Grade 3 ductal tumors are associated with poor prognosis and grade 1 tumors with better

outcome (Toikkanen & Joensuu 1990). The metastatic potential of tubular, mucinous, medullary or papillary carcinoma is lower than in ductal carcinoma (Toikkanen & Joensuu 1990, Silverstein *et al.* 1996).

Different biomarkers include aneuploidy, overexpression of the HER-2, cyclin D1, p53 oncogenes, absence of bcl-2 expression, growth factors, Ki-67, S-Phase fraction, Cathepsin, and angiogenesis (Gullick *et al.* 1991, Kallioniemi *et al.* 1991, Isola *et al.* 1992, Weidner *et al.* 1992, Poller *et al.* 1993, Railo *et al.* 1993, Guidi *et al.* 1994, Marks *et al.* 1994, Fox *et al.* 1994, MacGrogan *et al.* 1995, Weinstat- Saslow *et al.* 1995, Hayes *et al.* 1996b, Clark 1998, Dowsett 1998, Sjögren *et al.* 1998) are associated to prognosis. The list of potential prognostic and predictive factors for breast carcinoma is long but the validated prognostic and predictive factors very short (Clark 1999).

The overall 5-year survival rate in Finland in unselected breast carcinoma patient material is 80%. In node-positive breast carcinoma the 5-year overall survival rate is 69% (Finnish Cancer Registry 1997).

#### **2.2.4. Treatment**

Most of the breast carcinoma cases are operated by means of modified mastectomy or with breast-preserving techniques with an axillary evacuation. Postoperative radiotherapy covering the axillary, supraclavicular and internal mammary lymph nodes and the chest wall around the mastectomy scar is commonly used if the carcinoma is large, the axillary lymph nodes are positive or if the operation is breast-preserving (Cuzick *et al.* 1994, Arriagada *et al.* 1995, Harris & Morrow 1996).

Adjuvant chemo- or hormonal therapy is usually given to breast carcinoma patients who have node-positive primary breast carcinoma or large carcinoma (T3-4), or whose tumors have poor histological differentiation or are hormone- receptor negative (Gelber *et al.* 1993, Goldhirsch *et al.* 1995, Finnish Breast Cancer Group 1999). Adjuvant chemotherapy is usually given to premenopausal patients and adjuvant antiestrogen therapy to postmenopausal patients (Gelber *et al.* 1993, Howell *et al.* 1996, Fossati *et al.* 1998). In premenopausal patients with receptor-positive tumors after adjuvant chemotherapy tamoxifen was recommended for follow-up treatment (Goldhirsch *et al.* 1996). Preoperative chemotherapy is used for subgroups of patients with large tumors (greater than 5 cm) based on the idea of reducing tumor size before surgery, developed earlier in hormonal studies (Fisher *et al.* 1997). New approaches to drug therapy of breast cancer include strategies to increase dose intensity, e.g. dose escalations, dose density, scheduling and the use of cytokines. Also other new modalities such as inhibitors of cancer cell proliferation, exploitation of growth factor receptors, monoclonal antibodies have been used (Norton 1997).

### 2.3. Tumor invasion and metastasis

Tumor invasion and metastasis constitute a major problem in the treatment of carcinoma patients. About 30% of patients with newly diagnosed solid tumors already have clinically detectable metastases. A metastatic colony is a result of a continuous process starting from the early growth of the primary tumor, and detachment of invasive tumor cells from the primary tumor leading to the colonization of other organs (Fidler *et al.* 1978, Fidler & Hart 1982, Weiss 1985, Fidler & Balch 1987). Metastasis is a complex multistage process involving tumor-host interaction which includes adhesion, angiogenesis and proteolysis (Liotta *et al.* 1980, Turpeenniemi-Hujanen *et al.* 1985, Folkman 1986, Khokha *et al.* 1989, Tryggvason *et al.* 1993, Montgomery *et al.* 1994, Chambers & Matrisian 1997). A metastatic tumor cell must leave the primary tumor and invade host tissue. It must enter the circulation, survive in the circulation, arrest at the distant vascular bed and extravasate into organ interstitium and parenchyma. Metastasis is enhanced by a highly selective competition favoring the survival of a minor subpopulation of metastatic cells (Liotta *et al.* 1980, 1991, Fidler & Hart 1982, Hart *et al.* 1989, Vile 1995). The metastatic subpopulation dominates the primary tumor mass early in its growth (Kerbel *et al.* 1990). Fewer than 0.01% of malignant cells entering circulation have been shown to form actual metastases (Fidler & Ellis 1994).

Tumor infiltration is associated with the production of various types of extracellular matrix degrading enzymes, such as serine proteinases, metalloproteinases, cysteine proteinases, threonine proteinases and aspartic proteinases (Brunner *et al.* 1994, Keppler *et al.* 1994, Sloane *et al.* 1994, MacDougall & Matrisian 1995, Birkedal-Hansen 1995, Hewitt & Dano 1996). Also other proteins might be involved in tissue degradation such as cadherins (Takeichi 1991, 1995), the immunoglobulin superfamily (Rucklidge *et al.* 1994), integrins (Hynes 1992, Natali *et al.* 1992, Morino *et al.* 1995, Zhang *et al.* 1995), CD44 (Gansauge *et al.* 1995), tissue inhibitors of metalloproteinases (TIMPs) (Hayakawa 1994, Ogata *et al.* 1995), heparanase (Nakajima *et al.* 1991) and the putative metastasis-suppressor genes NME1 and NME2 (Iizuka *et al.* 1995). MMPs have been widely studied due to their crucial role in tumor cell invasion and metastasis (Chambers & Matrisian 1997, Benaud *et al.* 1998, Cockett *et al.* 1998).

### 2.4. Matrix metalloproteinases (MMPs)

Matrix metalloproteinases form a continuously growing family of zinc-dependent endopeptidases. MMPs are classified into subfamilies based on their substrate preferences. Those include gelatinases, collagenases, stromelysins, elastases, MT-MMPs (membrane type-MMPs) and a group of unnamed members. The members and substrates of MMPs are shown in Table 1.

All MMPs are synthesized in the latent form. They are secreted as proenzymes and require extracellular activation. They can be activated *in vitro* by many mechanisms including organomercurials, chaotropic agents and other proteases (Woessner 1991, Murphy *et al.* 1992, Birkedal-Hansen 1995). MMPs need  $Zn^{2+}$  to be active. MMP activity is regulated at many levels. The messenger RNA (mRNA) is transcriptionally regulated by

biologically active agents such as hormones, oncogenes, growth factor and tumor promoters (Overall *et al.* 1991, Mauviel 1993, Vincenti *et al.* 1994, Birkedal-Hansen 1995, Chambers & Matrisian 1997). The activation processes consist of three different mechanisms: stepwise activation, activation on the cell surface and intracellular activation. In addition, two progelatinases (proMMP-2 and proMMP-9) can bind to endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Nagase 1997).

These enzymes are synthesized and secreted in low amounts by normal cells associated with physiologic tissue remodeling such as wound healing, implantation, trophoblastic invasion and angiogenesis (Puistola *et al.* 1989, Liotta *et al.* 1991, Turpeenniemi-Hujanen *et al.* 1992, Salo *et al.* 1994, Ågren *et al.* 1994, Birkedal-Hansen 1995, Chambers & Matrisian 1997). Increased expression of MMPs is found in various human tumors and cell lines (Liotta *et al.* 1980, Birkedal-Hansen 1995, MacDougall and Matrisian 1995, Chambers & Matrisian 1997). MMPs are often overexpressed in malignant tumors (Stetler-Stevenson *et al.* 1993, Mueller 1996).

MMP activity is regulated at various levels, including regulation of the transcription at the mRNA level, activation from the latent form and by inhibition by the inhibitors of matrix metalloproteinases (TIMPs) (Mauviel 1993, Chambers & Matrisian 1997, Pendas *et al.* 1997).

*Table 3. The matrix metalloproteinase family.*

Subfamily	Name	MMPs	Main substrates
Interstitial	Fibroblast	MMP-1	Fibrillar collagen
Collagenases	Collagenase		
	Neutrophil	MMP-8	Fibrillar collagen
	Collagenase		
	Collagenase-3	MMP-13	Fibrillar collagen
	Collagenase-4	MMP-18	Fibrillar collagen
Gelatinases	Gelatinase A	MMP-2	Gelatin, type IV collagen, fibronectin, elastin, laminin
	GelatinaseB	MMP-9	Gelatin, elastin, fibronectin, vitronectin
Stromelysins	Stromelysin-1	MMP-3	Gelatin, fibronectin, casein, laminin, elastin, MMP-2/ TIMP-2
	Stromelysin-2	MMP-10	same as above
	Stromelysin-3	MMP-11	Fibronectin, laminin, gelatin, aggrecan
	Matrilysin	MMP-7	Fibronectin, vitronectin, laminin, gelatin, aggrecan
Elastases	Metalloelastase	MMP-12	Elastin, gelatin, collagen IV, fibronectin, laminin, vitronectin, proteoglycan
Membrane-type MMPs	MT1-MMP	MMP-14	proMMP-2, procollagenase 3
	MT2-MMP	MMP-15	pro-MMP-2
	MT3-MMP	MMP-16	proMMP-2
	MT4-MMP	MMP-17	unknown
Other MMPs		MMP-19	
	Enamelysin	MMP-20	amelogenin

MMP-2 (Gelatinase A, 72 kDa type IV collagenase) is the most widely distributed enzyme of the MMP family and it was originally described and purified as a basement membrane collagen degrading enzyme activity from a metastatic murine tumor (Liotta *et al.* 1979, Salo *et al.* 1983). MMP-2 is expressed e.g. by fibroblasts, keratinocytes, epithelial cells, monocytes and osteoblasts (Birkedal-Hansen 1995).

Gelatinases A and B (72 kD /MMP-2 and 92 kD /MMP-9), which also degrade type IV collagen, are members of this proteinase family, and the secretion of type IV collagen degrading activity has been found to be correlated with invasiveness and metastatic capacity in cultured tumor cells (Liotta *et al.* 1980, Turpeenniemi-Hujanen *et al.* 1985, Nakajima *et al.* 1987).

MMP-2 expression is linked to invasiveness in several human neoplasias including breast, colon, ovarian, lung, prostate, urothelial, hepatocellular carcinomas and melanoma. The MMP-2 protein has been localized immunohistochemically to tumor cells, suggesting an interesting role in determining the invasive potential and metastatic capacity of the neoplastic lesions (Liotta *et al.* 1980, Monteagudo *et al.* 1990, D'Errico *et al.* 1991, Campo *et al.* 1992, Naruo *et al.* 1993, Montironi *et al.* 1996, Kodate *et al.* 1997, Väisänen *et al.* 1998). mRNA for MMP-2 has been found in stromal cells, the reason for which is not clearly understood, but it seems to be a host response to the presence of invasive malignant cells (Pyke *et al.* 1992,1993, Autio-Harminen *et al.* 1992, 1993, Poulson *et al.* 1993).

MMP-2 activation differs from the other MMPs because it is differently regulated at the transcriptional level. It has been demonstrated that MMP-2 activation can take place in the cell surface of fibroblasts (Ward *et al.* 1994).

Membrane-type MMP-1 (MT-MMP-1, MMP-14) activates 72 kDa progelatinase on the cell surface (Will *et al.* 1996) and coexpression of the two MMPs is detected in various carcinomas (Nomura *et al.* 1995, Okada *et al.* 1995, Polette *et al.* 1996). MT-MMP-1 has also intrinsic proteolytic activity against e.g. gelatin, elastin laminin, fibronectin or types I, II and III collagen (Pei & Weiss 1996, Ohuchi *et al.* 1997).

MMPs can be regulated by the tissue inhibitor of metalloproteinases (TIMP). TIMPs play a major role in the regulation of MMP activity. The TIMP family members include TIMP-1, -2, -3 and -4 (Silbiger *et al.* 1994, Greene *et al.* 1996). TIMPs form binary complexes with active MMPs inhibiting the activity of the enzyme, or they appear in a complex of TIMP and progelatinase regulating the activation of the latent enzyme (Howard *et al.* 1991a, Goldberg *et al.* 1992). TIMP-2 is most effective in inhibiting MMP-2 activity (Howard *et al.* 1991b, Birkedal-Hansen *et al.* 1993, Corcoran *et al.* 1996, Imai *et al.* 1996, Will *et al.* 1996). By establishing a trimolecular complex, consisting of MT1-MMP/TIMP-2/ progelatinase A, the components are concentrated on the cell surface. This complex acts as a concentration mechanism that is crucial for the efficiency of activation (Atkinson *et al.* 1995, Cao *et al.* 1995, Sato *et al.* 1996). The role of TIMPs in suppressing tumor growth has been demonstrated in malignant tumors overexpressing one of the TIMPs (Khokha 1994, Bian *et al.* 1996, Imren *et al.* 1996). TIMPs have also biological activities not related to MMP inhibition: TIMP-1 and -2 are antiangiogenic and possess growth factor-like activity toward a number of cell types. TIMP-2 inhibits microvascular endothelial cell proliferation associated with angiogenesis (Murphy *et al.* 1993). The balance between the levels of activated MMPs and the free form of TIMPs determines the net activity of MMPs.

### ***2.4.1. MMP-2 in cervical neoplasias***

There are only few studies that demonstrate MMP-2 in cervical neoplasias. MMP-2 expression has been found to be an essential prognostic factor in the early stage of cervical carcinoma (Nuovo 1997). Nuovo *et al.* (1995) suggest that the balance of MMP-9 and MMP-2 to TIMP-1 and TIMP-2 expression is an essential factor in the aggressiveness of cervical carcinoma. MMP-2 expression was found in early stage squamous cell carcinoma of the uterine cervix and a significant relationship was observed between MMP-2 and lymphatic spread of the disease (Garzetti *et al.* 1996a). An association between the MMP-2 immunoreactive protein and histological grade of early stage cervical carcinomas was also found (Garzetti *et al.* 1996 a, b). Davidson *et al.* (1999a) found that the presence of both MMP-2 and TIMP-2 mRNA in tumor cells correlated with advanced stage and with poor survival. The presence of MMP-2 immunoreactivity was significantly more frequent in tumor cells of invasive carcinoma when compared with MMP-2 mRNA signal. In another study neither MMP-9 protein expression nor an intense signal for MMP-9 mRNA was associated with poor survival (Davidson *et al.* 1999b).

### ***2.4.2. MMP-2 in breast carcinoma***

MMP-2 has been detected very early in breast carcinoma but not in normal, resting breast tissue (Poulsom *et al.* 1993), and its expression becomes more consistent with increased tumor grade (Polette *et al.* 1993). MMP-2 has been shown in several studies to be expressed in breast carcinoma (Monteagudo *et al.* 1990, Davies *et al.* 1993, Polette *et al.* 1993, Poulsom *et al.* 1993, Tryggvason *et al.* 1993, Polette *et al.* 1994, Iwata *et al.* 1996). The protein has been localized in tumor cells (Daidone *et al.* 1991, Höyhty *et al.* 1994) and also stromal cells (Okada *et al.* 1995) in breast carcinoma by immunohistochemistry. The mRNA has been detected in stromal fibroblasts (Davies *et al.* 1993, Wolf *et al.* 1993, Soini *et al.* 1994) and in fibroblasts around invasive epithelial cells (Poulsom *et al.* 1993, Polette *et al.* 1994). An association between MMP-2 positive cells and either invasive carcinoma cells or lymph node metastasis has also been found in breast carcinoma, although expression of the enzyme is not strictly confined to neoplastic cells. This supports the role of the enzyme in tumor invasion and metastasis, suggesting that tumor cells are the essential source of the enzyme in these processes (Monteagudo *et al.* 1990). Activation of MMP-2 is found to be significantly higher in the carcinomas with lymph node or distant metastasis compared to carcinomas without metastasis (Ueno *et al.* 1997). Inactive MMP-2 was found in 67 % of cancer tissues and in 54 % of normal tissue (Lee *et al.* 1996).

### **3. Purpose of the present study**

Cervical carcinoma is the most common gynecologic carcinoma in developing countries, and breast carcinoma is the most important neoplastic disease in women in industrialized countries. Matrix metalloproteinases (MMPs) are zinc dependent endopeptidases implicated in tumor invasion and metastasis. The expression of MMP-2 has been found in dysplasias and in both early stage and locally advanced cervical carcinomas and in breast carcinoma. There are no previous studies to show MMP-2 as a prognostic factor in breast carcinoma. The MMP-2 immunoreactive protein was studied here in two important and clinically different carcinoma types, squamous cell carcinoma of the uterine cervix and adenocarcinoma of the breast.

The specific aims of the study were

1. to study whether the appearance of MMP-2 is connected to the early or late events during the progression of neoplastic cervical lesions
2. to study the value of MMP-2 as a prognostic factor in breast carcinoma
3. to study the MMP-2 protein as a prognostic factor in node-positive postmenopausal breast carcinoma patients
4. to study the MMP-2 protein as a prognostic factor in node-positive premenopausal patients

## 4. Materials and methods

### 4.1. Patients and tissue samples

The histological material of breast and cervical lesions was obtained during the routine therapeutical procedures required by the primary disease. Formalin-fixed, paraffin-embedded cervical tissue samples from the primary tumors of 60 patients who had undergone surgery at the Department of Obstetrics and Gynecology, Oulu University Hospital during 1981- 1995 were used for the study. The samples analyzed were taken from the histologically proven vital parts of the lesions. The histological evaluation comprised 22 FIGO stage IB-IIA squamous cervical carcinomas and 38 cervical intraepithelial neoplasias (CIN I-III).

Thirty-six women with a CIN I-III lesion underwent laserconization, one was treated by laservaporization and one with cryotherapy (I). All patients with cervical carcinoma were treated with preoperative intracavitary brachytherapy followed by a radical hysterectomy and lymph node dissection. Postoperative radiation was used in 21 patients. Additional platinum-based chemotherapy was given if pelvic lymph node metastases were found in the operation. HPV was evaluated cytomorphologically from routine stainings without using the in situ hybridization technique (I).

Breast tissue samples were from the primary tumors of 373 patients who had been operated during the years 1981-1995 in Northern Finland. The formalin-fixed, paraffin embedded blocks were obtained from the files of the Departments of Pathology, Oulu University Hospital and the Central Hospitals of Kajaani, Kemi, Kokkola and Rovaniemi.

Stage, tumor size and axillary node involvement of breast carcinoma were determined according to the UICC TNM classification (Hermanek *et al.* 1992). The tumors were classified according to the World Health Organization's International Classification of Breast Tumors (Scarff *et al.* 1986). The ductal carcinomas were graded by evaluating tubule formation, nuclear pleomorphism, and the mitotic rate according to the criteria of Bloom and Richardson (1957).

In study II breast carcinoma patients were 26-85 years of age. postmenopausal breast carcinoma patients with histologically positive axillary lymph nodes were included in study III. The menopausal status was examined by testing the serum FSH (Follicle stimulating hormone). The FSH was > 30 U/l in all patients. The patients were 48-83 years of

age and the median age was 63. Premenopausal breast carcinoma patients with histologically positive axillary lymph nodes were included in study IV. The patients were 26-49 years of age and the median age was 41 (IV, Table 4).

Ductal infiltrating carcinoma was the most frequent histological type: 138/169 (II), 79/96 (III) or 89/108 (IV). A small tumor sample taken during the operation was used for routine steroid receptor assays. Both estrogen and progesterone receptor charcoal assays were performed in 141/169 cases (II), 61/96 cases (III) and 80/108 cases (IV).

Mastectomy with axillary evacuation was the primary treatment in 168 cases diagnosed in 1981-1987, one case remained inoperable (II). Adjuvant antiestrogen therapy had been used in 38 cases, most of them with a stage II or III disease, and adjuvant CMF (cyclophosphamide-methotrexate-fluorouracil) chemotherapy was used in 8 cases (II). Patients with metastatic disease (M1) were operated (except one) and local radiotherapy was given. Additionally, they received antiestrogen therapy (10 patients) or chemotherapy (2 patients). Recurrences in patients with receptor-positive tumors were treated primarily with hormonal therapy (II).

Postmenopausal patients with breast carcinoma diagnosed between 1992-1995 were treated with an adjuvant antiestrogen therapy, tamoxifen in 44 cases and toremifene in 56 (III). The surgical operation in most of these cases (n=85) was mastectomy with an axillary evacuation. Lumpectomy with an axillary evacuation was performed in 11 cases with a primary tumor less than 2 cm in diameter. Premenopausal patients with a breast carcinoma diagnosed between 1990-1995 were treated with an adjuvant chemotherapy, CMF (cyclophosphamide-methotrexate-5-fluorouracil) in 96 cases and FEC (5-fluorouracil-epirubicin-cyclophosphamide) in 12 cases (IV). The surgical operation in most of the cases was mastectomy with an axillary evacuation. Lumpectomy with an axillary evacuation was performed in 19 cases with a primary tumor less than 2 cm in diameter.

All patients without distant metastases and with histologically positive axillary lymph nodes, regardless of the number of nodes or size of the primary tumor, received postoperative radiotherapy covering the axillary, supraclavicular and internal mammary lymph nodes and the chest wall around the mastectomy scar.

The follow-up information of the patients was collected during the years 1981- 1995 (I), 1981-1987 (II), 1992-1995 (III) and 1990-1995 (IV). The medium follow-up time was 92 months in study II. The minimum follow-up time (III) was 2.5 years (median 40.4 months). In study IV the minimum follow-up time was 2.8 years (mean 57.1 months).

## 4.2. Antibodies

The mouse monoclonal antibody (code: CA-4001, Diabor LTD, Oulu, Finland) to MMP-2 (Marquies *et al.* 1992) was used as the primary antibody (1.5 µg/ml in 0.01 M phosphate buffer, 0.9 % NaCl, pH 7.5 ), with 1 % bovine serum albumin (BSA). The antibody recognizes the amino terminal end of the latent MMP-2 and gives a positive immunoreaction with pro-MMP-2 both as a free enzyme and when in complex with TIMP-2.

### **4.3. Immunohistochemistry**

#### ***4.3.1. Immunohistochemical staining***

The histologic material fixed in 10% formalin and embedded in paraffin was cut into 4µm slides and they were incubated for 12 h at 37 °C, dewaxed in a histological clearing agent, Histo-Clear (National Diagnostics, Atlanta, GA, USA), and hydrated. The specimens were treated with 0.4 % pepsin (Sigma, St Louis, MO, USA) for 20 min at 37 °C. The avidin-biotin-immunoperoxidase technique was used according to Hsu *et al.* (1981). Endogenous peroxidase activity was blocked by incubating the slides in 3 % hydrogen peroxide in absolute methanol for 15 min, and non-specific binding was blocked with 10% goat serum for 15 min.

The specimens were incubated for 60 min at room temperature in a humidity chamber, and immunohistological staining was continued by using a Histostain-bulk kit (Zymed, San Francisco, CA, USA) according to the manufacturer's instructions. Biotinylated anti-mouse IgG served as a second antibody, and the peroxidase was introduced using a streptavidin conjugate. The slides were washed thoroughly with phosphate buffered saline between each stage in the procedure. The antibody reaction was visualized using a fresh substrate solution containing an aminoethyl carbazole substrate kit (AEC, Sigma). The sections were counter-stained with hematoxylin, dehydrated and mounted in glycerol-vinyl-alcohol (GVA mount, Zymed). For the negative controls the primary antibody was replaced with mouse non-immune IgG. For the positive controls we used MMP-2 positive specimens of breast carcinoma.

#### ***4.3.2. Evaluation of the MMP-2 immunostaining***

A section was considered negative or positive according to the absence or presence of positive staining of the dysplastic / neoplastic cells (I). The staining was scored as follows: no positive cells or less than 20% of the dysplastic / neoplastic cells staining positive (MMP-2 score 1 or +); 20-50% of the neoplastic cells positive (MMP-2 score 2 or ++); >50% of the neoplastic cells positive (MMP-2 score 3 or +++).

Immunostaining for MMP-2 was scored by three independent observers, and only cases giving repeatable scores in immunostaining were included in the data. The clinical data were collected and analyzed after evaluation of the immunostaining scores for a given case. The scoring of the immunoreaction and collecting the clinical data were performed independently without knowledge of each other.

### **4.4. Statistical analysis**

The score for MMP-2 immunoreactivity was compared with other prognostic variables by the Chi-Square method. P-values < 0.05 were considered statistically significant (I-IV).

Survival was defined as the time elapsing from the primary operation to the date of death. The recurrence-free survival was determined as time in months from the date of diagnosis to the date of metastasis.

Survival rates were analyzed by the Kaplan-Meier method (Kaplan & Meier 1958) for up to ten years of follow-up (II) or up to 5 years of follow-up (III-IV). Differences between the subgroups were compared by means of a log-rank test (Mantel 1966) for up to five years. The effect of MMP-2 positivity on survival was analyzed in various subgroups representing the major prognostic variables recognized in breast carcinoma. The multivariate analysis was tested with Cox's proportional hazard model (Cox 1972), the models used being fixed and stepwise (II). BMDP statistical software (University of California Press, Berkeley, CA, USA) was used (Dixon *et al.* 1990).

## **5. Results**

### **5.1. MMP-2 expression in cervical dysplasia (I)**

Positive cytoplasmic MMP-2 immunostaining was found in all CIN I and CIN III lesions and in 90% of the CIN II lesions. A strong cytoplasmic immunostaining (MMP-2 score 3) was found in 21% of the CIN cases and a weak cytoplasmic staining (MMP-2 score 1) in 34% of the cases (I). The most intensive positive staining for MMP-2 protein was typically localized in the periphery of the epithelial cells in cervical intraepithelial neoplasia (CIN) lesions (see I: Fig. 1A). The MMP-2 positivity was found in both deep and outer layers of the transformed epithelium in the CIN-lesions.

The score of the immunostaining was not significantly different between CIN I and CIN III, nor were there any differences in the score of MMP-2 immunostaining between CIN lesions and early squamocellular carcinomas.

Light microscopic changes caused by human papillomavirus (HPV) were found in 92% of the CIN I and in 70% of the CIN II lesions, but only in 33% of the CIN III lesions. No association between the morphological changes associated with HPV and MMP-2 expression was found.

### **5.2. MMP-2 expression in cervical carcinoma (I)**

MMP-2 expression in squamocellular carcinomas of uterine cervix typically displayed a diffuse cytoplasmic staining. In the early stage cervical carcinomas (Stage IB - 2A ), the expression of MMP-2 immunoreactive protein was found in all cases. Strong positive staining (MMP-2 score 3) was found in 23% of the cases and a weak cytoplasmic staining (score 1) in 41% of the cases (I).

Positive correlation was found between a marked overexpression (MMP-2 score 2-3) of the MMP-2 immunoreactive protein and high histological grade (2-3) of early stage cervical carcinomas ( $X^2=5.0$ ,  $p<0.05$ ). The expression of MMP-2 was negative or low (MMP-2 score 1) in all patients with grade I carcinomas, but only three patients were included in this group (I).

There was no correlation between the MMP-2 immunoreactivity and the primary stage, or the clinical course of the cervical carcinoma. Four out of seven primary tumors with lymph node involvement at the time of the diagnosis presented high scores (2-3) of immunoreaction for MMP-2 (see I: Table 2). Three patients out of five with score 3 MMP-2 expression in primary tumor presented lymph node metastases at the time of the diagnosis. All available lymph node metastases were also examined for MMP-2 protein expression. In all these cases with both lymph node metastasis and high score (3) for MMP-2 expression in primary tumor the lymph node metastases were also positive for MMP-2. Four out of five patients with local recidive or distant metastases later during the follow-up had a low MMP-2 score in their primary tumors. Four patients (three with MMP-2 score 1 and one with MMP-2 score 3) out of the five patients with recidive or metastasis (see I: Table 2) died during the time of the follow-up (I).

### **5.3. MMP-2 immunoreactive protein in breast carcinoma (II,III,IV)**

The immunoreactive protein in cancer cells was localized in the cytoplasm. There was some variation in the amount of MMP-2 positive cases in different breast carcinoma patient groups in this study (Table 4). Expression of MMP-2 immunoreactive protein was found in 84 % (n=142/169) of the primary tumors of the nonselected breast carcinoma cases (II), when it was 69 % (n=67/96, III) or 76 % (n=82/108, IV) in node positive breast carcinoma patient groups. In the combined material the expression of MMP-2 immunoreactive protein is shown in Table 5. Strong positive staining (>50 % of the tumor cells positive; +++) was found in 22 % of the cases in study II, while it was 36 % (III) or 48 % (IV) in the node positive cases. Weak to moderate positivity ( $\leq$ 50 % of the tumor cells positive) was found in 62 % (II), 33 % (III) and 28 % (IV) of the cases. Negative staining was found in 16 % (II) to 31 % (III) of the primary tumors of breast carcinoma.

No correlation was found between histological subtype and MMP-2 positivity (II,III, IV), but a correlation between advanced stage and MMP-2 positivity (II) was found in patients younger than 55 years of age ( $\chi^2=4.5$ ,  $p=0.02$ ). No association was found between the expression of MMP-2 and the size of the breast carcinoma or nodal involvement (n=169, II), although 93% of the N2 or M1 cases were positive for MMP-2. Also, a larger proportion of grade III ductal infiltrating carcinomas (n=52/58, II) were positive compared to grade I tumors (n=14/19, II), although the difference did not reach statistical significance (II, IV). Also no statistically significant association was found (II, IV) between the expression of MMP-2 and estrogen or progesterone receptor status. Only 20% of the N0 (II) or grade I (III,IV) cases were positive for MMP-2 (Table 5).

A statistically significant correlation between MMP-2 positivity and distant metastasis ( $p=0.03$ ) during the course of the disease was found (IV). All patients (n=4) with grade I ductal infiltrating primary breast carcinoma (IV) were recurrence-free during the follow-up regardless of the MMP-2 status. In study III all grade I patients (n=12) were recurrence-free and alive during the 5 years of follow-up. In study II one of 19 patients with grade I ductal infiltrating carcinoma died during follow-up and the tumor was positive for MMP-2.

Table 4. Clinical features of the different breast carcinoma patient groups in this study.

Subgroups	II	III	IV	total
All (n)	169	96	108	373
Age years	26-85	48-83	26-49	26-85
T1-2	137	90	89	316
N0	87	0	0	87
N+	82	96	108	286
M1	14	0	0	14
ER and/or PR				
positive	95/141	56/61	73/80	224/282
Grade III	58	29	45	132
MMP-2 positive	142	67	82	291
strong positive	38	34	52	124

Table 5. The expression of MMP-2 immunoreactive protein in breast carcinoma

Patient groups	Patient number	MMP-2 positive	MMP-2 strongly positive
	(n)	n (%)	n (%)
All	373	291 (78)	124 (33)
Age	26-85 y		
T1-2	316	246 (78)	115 (36)
T3-4	57	42 (74)	21 (37)
N 0	87	73 (84)	17 (20)
N 1-2	286	219 (77)	106 (37)
M 0	359	279 (78)	120 (33)
M 1	14	13 (93)	4 (29)
Histology:			
Ductal infiltrating			
Grade I	35	23 (66)	7 (20)
Grade II-III	287	228 (79)	103 (36)
Estrogen receptor			
Positive	222	168 (76)	66 (30)
Negative	60	50 (83)	16 (27)
Unknown	91		
Progesterone receptor			
Positive	207	196 (95)	63 (30)
Negative	75	61 (81)	22 (29)
Unknown	91		

## 5.4. The prognostic value of MMP-2 immunoreactive protein in breast carcinoma

Strong MMP-2 immunoreactivity (+++) was linked to a shortened survival time in nonselected cases of breast carcinoma regardless of age, tumor size, nodal involvement, stage, grade or receptor status of the tumor (II). A statistically significant correlation was found between overall survival and MMP-2 immunostaining. The Kaplan-Meier analysis showed that the survival of patients with MMP-2 negative primary tumors was 88% after 5 years of follow-up in this material, while it was 56% in patients having highly (+++) MMP-2 positive tumors and 73% in those having tumors displaying a weak or moderate (+/++) positivity (II, Table 6).

Table 6. The summary of MMP-2 expression and survival in spesific studies.

Patient group	Years of diagnosis	Kaplan-Meier analysis	
		RFS	OS
		MMP-2 neg/pos	MMP-2 neg/pos
Study II	1981-1987	-	88% / 56%
Study III	1992-19958	3% / 67%	93 % / 73%
Study IV	1990-1995	85% / 65%	83% / 78 %
Total	1981-1995	74% / 62%	82% / 72%

In study II MMP-2 immunoreactive protein served as a prognostic factor in nonselected primary breast carcinoma. MMP-2 immunoreactivity was linked to shortened survival time (II) in the postmenopausal breast carcinoma patients regardless of the size of the primary tumor, stage, grade or receptor status. In all subgroups a higher relapse-rate and a lower survival rate appeared in patients with MMP-2 positive primary tumors. Positive immunoreaction for MMP-2 in breast carcinoma samples in study III was associated with a more frequent relapse rate and a lower relative survival rate also in postmenopausal node-positive patients (III). The recurrence-free survival rate was 71% at 5 years of the follow-up and the overall relative survival rate was 78%. Only 67% of the patients with an MMP-2 positive primary tumor lived relapse-free for 5 years, when 83% of those with an MMP-2 negative tumor presented without recurrence at that time. The difference was statistically significant in a log-rank analysis ( $p < 0.05$ ). 73% of the patients who were MMP-2 positive were alive at 5 years, compared to 93% of the patients with an MMP-2 negative primary tumor (Table 6).

Positive immunoreaction for MMP-2 in premenopausal patients with node-positive breast carcinoma (IV) was associated with a more frequent relapse rate and a lower survival rate in Kaplan-Meier analysis. RFS was 65 % during the 5-year follow-up in MMP-2 positive cases, while it was 85 % in MMP-2 negative cases. This difference did not quite achieve statistical significance in log rank analysis (Table 6,  $p = 0.07$ ). The difference between the survival rates in MMP-2 negative and positive premenopausal breast carcinoma patients was not as distinct as in nonselected patients or in postmenopausal breast carcinoma patients. 78 % of the patients with an MMP-2 positive breast carcinoma sur-

vived for 5 years, compared to 83 % of those with an MMP-2 negative primary tumor (Table 6).

When combining all breast carcinoma cases (II, III, IV, n=373), a statistically significant correlation between MMP-2 positivity and (RFS) relapse free survival ( $p=0.03$ ) as well as (OS) overall survival ( $p=0.03$ ) was found. In this combined material the 5-year RFS was 62 % in patients with MMP-2 immunoreactive protein positive breast carcinomas, compared to 74 % in the MMP-2 negative patient group. A similar correspondence was found with overall survival and MMP-2 positivity, 72 % versus 82 %, respectively (Fig 1 A, B, Table 6).

MMP-2 positivity correlated significantly with shortened RFS (III, IV) and overall survival (II,III) in patients with small tumors (T1-T2)N0 or N+. The Kaplan-Meier analysis showed that the recurrence-free survival rate of the patients with an MMP-2 negative small primary tumor (T1-T2) in postmenopausal patients with nodal involvement was 88%, while it was 69% in the corresponding MMP-2 positive patient group (III). 97% of the patients with an MMP-2 negative breast carcinoma survived for 5 years in this patient group, compared to 74% of those with an MMP-2 positive primary tumor (III). MMP-2 positivity correlated statistically significantly with the unfavorable outcome in this patient group (III,  $p=0.04$ ).

Similarly, in the premenopausal, node-positive breast carcinoma patients the Kaplan-Meier analysis showed that RFS of the patients with an MMP-2 negative, small primary tumor (T1-2)N+ (IV) was 87%, when it was only 65 % in the MMP-2 positive cases. This difference was statistically significant. (95% CI: 1.0-28.3). 75% of the small primary tumors were MMP-2 positive. The median 5-year survival rate of these patients was 80%, when it was 90% in the MMP-2 negative cases. The three patient groups with T1-2 primary tumors (II-IV) were combined in order to confirm the prognostic value of MMP-2 expression in patients with a small primary tumor. MMP-2 positive cases relapsed earlier than those with MMP-2 negative tumors, with 5-year RFS rates of 68 % versus 80 % ( $p=0.03$ ). The differences in the OS rates, 80 % versus 90 %, were also significant ( $p=0.01$ , Fig 1 C, D).

Sixty-one out of 282 breast carcinoma patients were negative for estrogen receptors and 75 for progesterone receptors. MMP-2 positivity was an unfavorable prognostic factor also in these patient subgroups. None of the patients having an MMP-2 negative, estrogen (n=10) or progesterone (n=14) receptor negative primary tumor died during the 5 years of follow-up. As a contrast 60% (n=50,  $p=0.04$ ) of the patients with an MMP-2 positive estrogen receptor negative and 58% (n=61,  $p=0.007$ ) progesterone receptor negative patients were alive at that time (Fig 2 A, B).

When combining age and both MMP-2 and node positivity (IV) the Kaplan-Meier analysis showed that the RFS in younger patients (<40 years) with an MMP-2 positive primary tumor was only 50% while in both the premenopausal patients over 40 years and in the MMP-2 negative patients the RFS was 74%. The most distinct difference in RFS between these two patient groups, 28%, appeared after 3 years of follow-up and the difference seen in the Kaplan-Meier analysis was also highly significant in log rank analysis ( $p=0.007$ ).

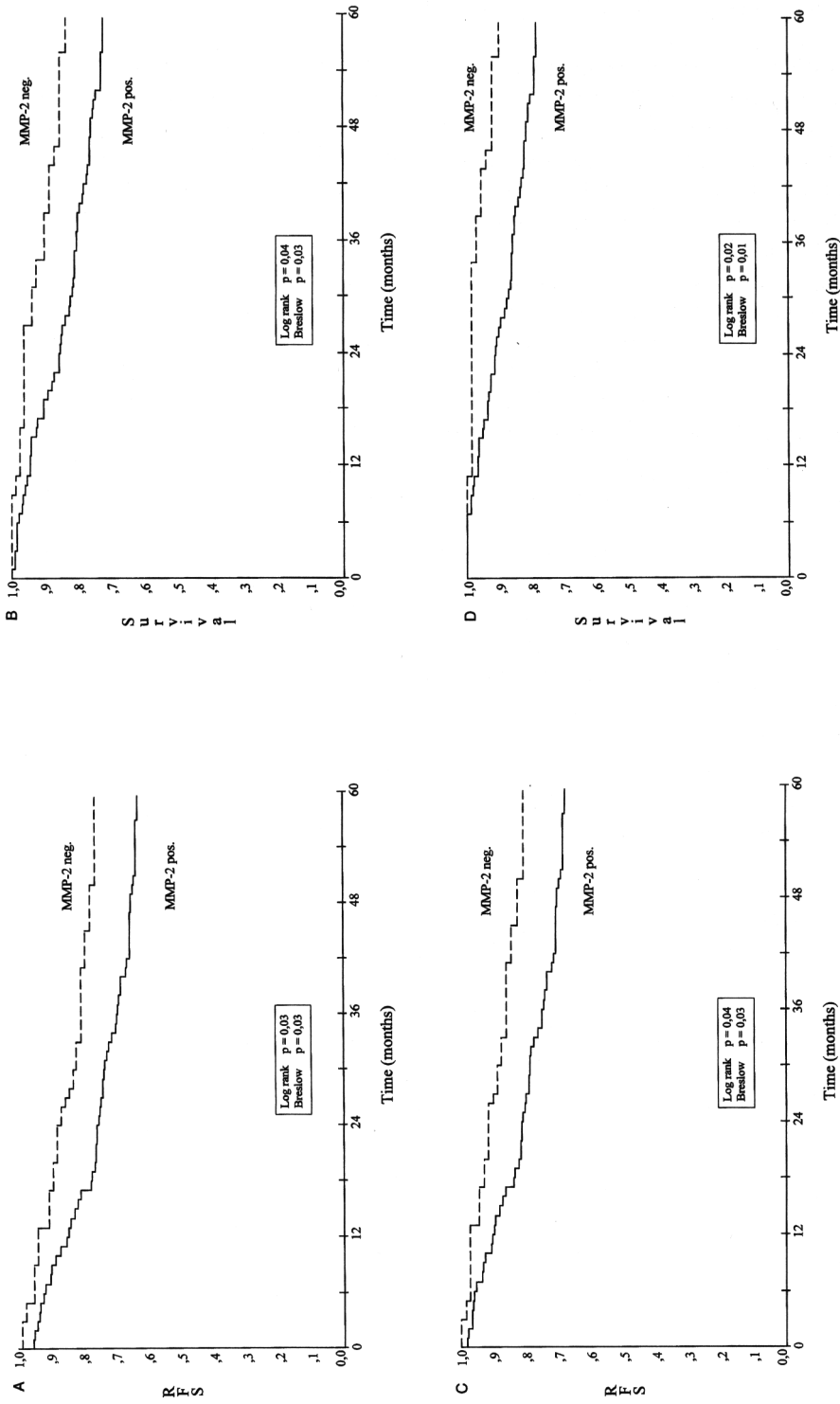
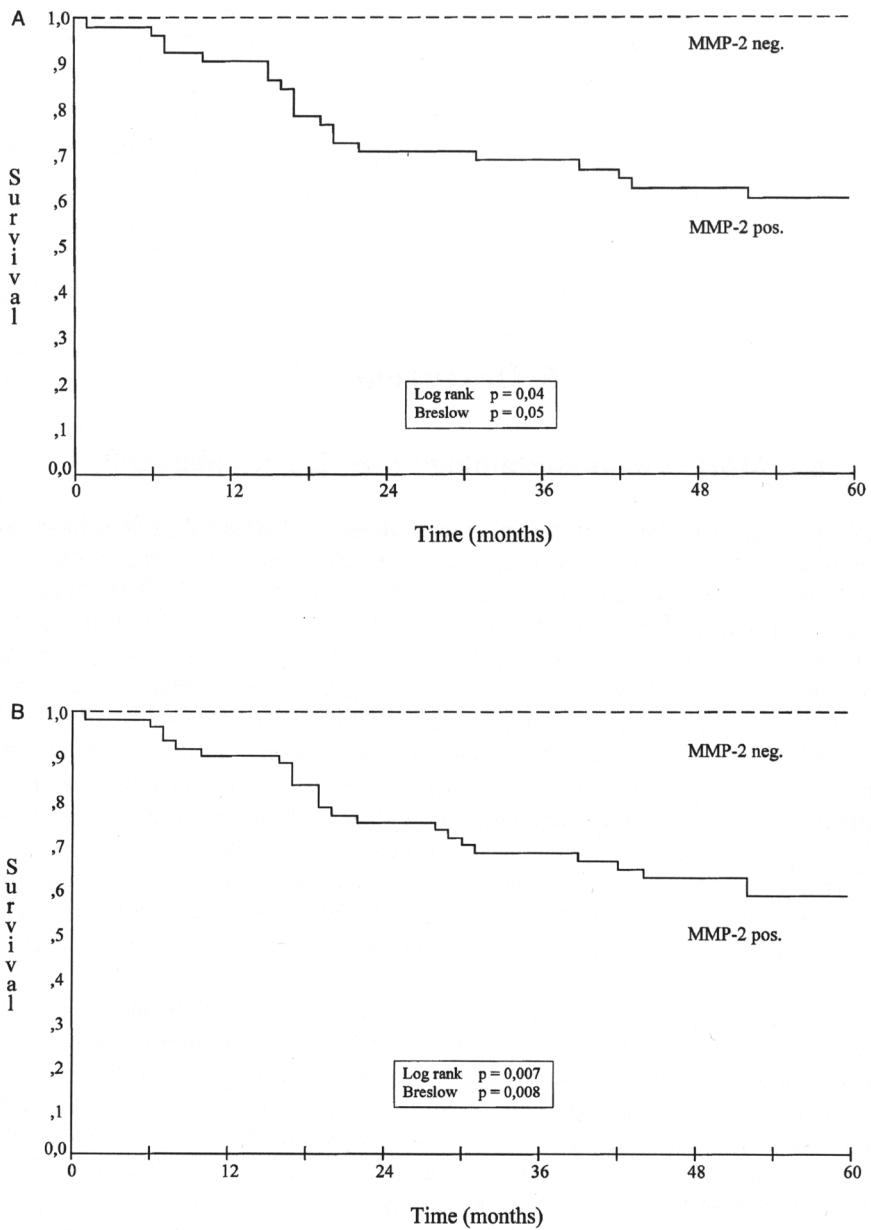


Fig. 1. The 5-year relapse-free survival and overall survival rates of the breast carcinoma patients according to MMP-2 immunoreactivity of the primary tumor in different patient groups. A, B: all patients; C,D: T1-T2 tumors.



**Fig. 2.** The 5-year overall survival rates the breast carcinoma patients according to MMP-2 immunoreactivity of the primary tumor in A: estrogen and B: progesterone receptor negative cases.

## 6. Discussion

### 6.1. MMP-2 immunostaining in cervical neoplasias (I)

Type IV collagen degrading activity and the expression of MMP-2 protein have been linked previously to invasive behavior of carcinoma cells when compared to normal cells in *in vitro* studies (Turpeenniemi-Hujanen *et al.* 1985, Höyhty *et al.* 1990, Hujanen & Turpeenniemi-Hujanen 1991, Hujanen *et al.* 1994, Nuovo 1997). The expression of MMP-2 immunoreactive protein has been found previously in dysplasias and in early stage or locally advanced cervical carcinomas (Garzetti *et al.* 1996a, Garzetti *et al.* 1996b). In this study the MMP-2 protein was found to be expressed also in early stage of cervical dedifferentiation before the dysplastic cells become invasive. The MMP-2 enzyme activity is regulated in a complex manner, including the function of inhibitors and activators. The observations reported here on the early appearance of the MMP-2 protein during malignant transformation are in line with previous data found in human melanocytic tumors (Väisänen *et al.* 1996). It is possible that the presence of MMP-2 in carcinoma still plays a role in metastasis since the MMP-2 protein has been shown to be associated with hematogenous metastasis and survival in human melanoma (Väisänen *et al.* 1998).

In contrast to melanoma (Väisänen *et al.* 1996), in the current study all cervical squamous cell carcinomas were found to be positive for MMP-2. The high MMP-2 expression was not associated with metastasis. There was a correlation between the poor histological differentiation and the high score of MMP-2 expression. Also, most patients presenting primary lymph node metastases displayed a high score for MMP-2 in the primary tumor, but the number of those patients was limited in this study. Davidson *et al.* (1999a) recently studied MMP-2 and TIMP-2 expression in cervical intraepithelial neoplasias (CIN II-III) and in squamous cell carcinomas of the uterine cervix. Intense staining for MMP-2 immunoreactive protein was found in three out of ten CIN cases and a weak staining in seven out of ten cases. These results thus confirm our finding that MMP-2 protein can be found in cervical intraepithelial neoplasia. mRNA for MMP-2 was found in carcinoma cells but not in normal cells or dysplasias. MMP-2 expression was found to be correlated with advanced stage and poor survival. In our study the MMP-2 protein did not, however, correlate with the outcome in cervical carcinoma. It is possible that the dis-

crepancy between these data and those found in our study is due to limited patient material in both studies. The present study does not exclude the possibility that MMP-2 could be an important factor in metastasis of cervical squamous cell carcinoma. Activators or inhibitors of the enzyme are most likely also involved. EGF, TGF-B, PDGF and many oncogenes have been found to increase the expression of MMPs in several cell types (Overall *et al.* 1991, Birkedal-Hansen 1995). Also Gilles *et al.*, for example, have shown in 1996 that the expression of MT-MMP (membrane-type metalloproteinase), an enzyme that is a potential MMP-2 activator, is associated with invasive cervical carcinoma and lymph node metastases (Gilles *et al.* 1996).

## 6.2. MMP-2 expression in breast carcinoma (II, III, IV)

The expression of MMP-2 immunoreactive protein has in previous in vitro studies been associated with invasive and metastatic tumors (Liotta *et al.* 1980, Garbisa *et al.* 1987, Nakajima *et al.* 1987, Bernhard *et al.* 1990). In this study intracytoplasmic expression of MMP-2 was found in 84 % (II), 69 % (III) and 76 % (IV) of the primary tumors of breast carcinoma. The amount of positive cases is in line with previously published data (Monteagudo *et al.* 1990, Daidone *et al.* 1991, Visscher *et al.* 1994, Lee *et al.* 1996). The amount of extensive MMP-2 positivity (>50% of the tumor cells positive; +++) varied in this study considerably between the different breast carcinoma patient groups. Extensive MMP-2 expression was found in 22 % (II), 36 % (III) or 48 % (IV) of primary tumors in breast carcinoma patients. In premenopausal node-positive breast carcinoma patients (IV) the proportion of cells expressing strong MMP-2 positivity was two times that found in nonselected patient material (II). It is possible that the node-positivity might be correlated to the incidence of strong expression of MMP-2 protein in breast carcinoma, although MMP-2 positivity did not in itself correlate with stage of carcinoma in postmenopausal patients (III).

## 6.3. The prognostic value of MMP-2 in breast carcinoma (II-IV)

This study is the first to show that the expression of MMP-2 is associated with a higher relapse rate and a lower overall survival rate in breast carcinoma (II). In the pooled patient material consisting of all the 373 cases the 5-year RFS was 62 % in patients with MMP-2 immunoreactive protein positive breast carcinoma (n=291), compared to 74 % in the MMP-2 negative patient group (n=82), and the 5-year overall survival was 72 % versus 82 %. These differences were statistically significant, suggesting that the MMP-2 immunoreactive protein is worthy of careful evaluation as a possible marker for biologic aggressiveness in breast carcinoma.

The Kaplan-Meier analysis showed a favorable overall survival rate for patients with MMP-2 negative primary tumors, being 88% (II), 93% (III) or 83% (IV) after 5 years of follow-up, when compared to 73% (III) or 78% (IV) in node-positive breast carcinoma patients with an MMP-2 positive tumor, or 56% in the cases diagnosed during 1981-1987 displaying high MMP-2 positivity (II). The difference in survival in premenopausal,

node-positive breast carcinoma patients between MMP-2 positive and negative cases was 5 % (IV). This difference was not statistically significant. It is possible that adjuvant chemotherapy could diminish the influence of MMP-2. It has to be taken into account that the median follow-up time in this study was only 57.1 months, and changes during further follow-up time may be possible. These results suggest that MMP-2 positivity alone could still retain its prognostic value despite the adjuvant therapies in node-positive breast carcinoma patients, and it could be useful in identifying patients for trials searching for new strategies for adjuvant therapy in breast carcinoma.

Node positivity is a known risk factor in breast carcinoma (Gallager 1984, Carter *et al.* 1989). In this material the number of the node-positive cases is overrepresented because of the selection of the patient material for two studies concerning node-positive patients (III,IV). The recurrence-free survival rates were 83 % (III) and 85 % (IV) at 5 years in MMP-2 negative cases compared to 67% (III) and 65% (IV) in MMP-2 positive cases with node positive breast carcinoma. The difference was statistically significant in the patient group that was treated with antiestrogen adjuvant therapy (III). In patients treated with adjuvant chemotherapy the difference in RFS in Kaplan-Meier analysis was distinct (20%), although it did not quite reach statistical significance ( $p=0.07$ ). These data suggest that MMP-2 could serve as an indicator for selecting patients with a favorable prognosis among those with axilla involvement. MMP-2 negativity could also serve as a biological indicator for favorable prognosis when the axilla status is unknown.

Another important risk factor in breast carcinoma is hormone receptor negativity. It is interesting that none of the patients with an MMP-2 negative, estrogen or progesterone receptor negative primary tumor died during the 5 years of follow-up. On the other hand, only 60 % of patients displaying MMP-2 positivity and estrogen receptor negativity and 58 % of those with MMP-2 positivity and progesterone receptor negativity were alive at that time. These differences were statistically highly significant, suggesting that these patient groups might need more attention in further studies.

RFS of the patients with an MMP-2 negative, small primary tumor with axillary lymph node metastases T1-2N+ was favorable (>85%) when compared to MMP-2 positive cases (<70%) (III, IV). The difference was seen also in the relative overall 5-year survival rate of these patients (>90% in MMP-2 negative vs <80% in MMP-2 positive cases (III, IV)). The three patient groups with T1-2 primary tumors were combined in order to confirm the prognostic value of MMP-2 expression in patients with a small primary tumor. MMP-2 positive cases relapsed more often than those with MMP-2 negative tumors, with a 5-year RFS rate of 68 % versus 80 %. The OS rates of MMP-2 positive and negative cases were 80 % and 90 %, respectively. These differences were statistically significant, suggesting that MMP-2 negativity together with limited T could be associated with a favorable prognosis even in N+, provided that there is not a massive metastasis in axilla (the number of affected nodes less than 50% of the examined nodes).

A strong (+++) MMP-2 positivity was associated with poor prognosis in node-positive breast carcinoma diagnosed during 1981-1987, the 5-year survival rate being only 28% (II). Also moderate (+/++) MMP-2 positivity was linked to an unfavorable prognosis in node-positive patients (50% 5 year OS, II). In postmenopausal, node-positive breast carcinoma, diagnosed during 1992-1995, the 5-year survival rate of patients displaying MMP-2 positivity in the primary tumor was 73% after 3 years of antiestrogen therapy. In premenopausal MMP-2 positive patients with axillary lymph node metastasis, diagnosed

during 1990-1995, the 5-year survival rate was 78%. This improvement in survival figures in node-positive patients might be partly due to improved adjuvant therapies in breast carcinoma. It has to be taken into consideration, however, that the breast carcinoma patients in unselected material were diagnosed during 1981-1987, while the patients treated with adjuvant therapies were diagnosed during 1991-1995. It is thus likely that the better survival rate in patients displaying MMP-2 positivity treated with adjuvant therapy is also partly due to overall improvement in the treatment of breast carcinoma.

## 7. Conclusions

In this study the expression of MMP-2 immunoreactive protein was found in cervical dysplasias, carcinomas and breast carcinoma. No correlation was found between the enzyme and metastasis in cervical carcinoma. However, MMP-2 was a prognostic factor in breast carcinoma.

MMP-2 negativity was associated with a favorable prognosis (>90% 5-year survival) especially in patients with T1-2 primary tumors when there has no massive axillary lymph node metastasis or distant metastases. In hormone receptor negative breast carcinoma MMP-2 negativity correlated with a good prognosis. This patient group is worthy of further studies. These results also suggest that MMP-2 could be useful in studies selecting breast carcinoma patients for adjuvant therapies or for new treatment innovations.

The specific conclusions of this study are

1. The expression of latent (non activated) MMP-2 appeared to be an early event in cervical neoplastic transformation. The great majority of dysplasias and all cases of early stage cervical carcinomas expressed the latent MMP-2 protein. Although the intensity (score) of the immunoreaction was not associated with clinical behavior it is possible that MMP-2 could be a prerequisite for invasive behavior.
2. In this study it is shown for the first time that the immunoreactive protein for MMP-2 correlates with shortened survival in primary breast carcinoma. It is independent of other important prognostic factors, i.e. stage, nodal status, receptor status and age.
3. In postmenopausal breast carcinoma patients with low tumor burden and node-positive disease MMP-2 is an important prognostic factor.
4. In young (<40 years) premenopausal patients node positive tumors showing MMP-2 positivity predicted a relapse in half of the patients.

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