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THE PROGNOSTIC ROLE OF MATRIX METALLOPROTEINASES MMP-2 AND -9 AND THEIR TISSUE INHIBITORS TIMP-1 AND -2 IN PRIMARY BREAST CARCINOMA

FACULTY OF MEDICINE,
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Abstract

Breast carcinoma is a heterogeneous disease with a prognosis that varies from excellent to very poor. Traditional tumour parameters and biological factors that are also predictive for treatment response are used in determining breast carcinoma prognosis and selecting appropriate treatment. Gelatinases MMP-2 and MMP-9 have been shown to associate with tumour progression. Their tissue inhibitors TIMP-1 and -2 are multifunctional molecules that have been suggested as prognostic markers in some previous reports.

In the present work, the expression and prognostic value of gelatinases MMP-2 and MMP-9 and their tissue inhibitors TIMP-1 and -2 were assessed in primary breast carcinoma. The material consisted of a total of 416 patients. Tissue expression of TIMP-1 and -2 was analysed in a population of 203 patients using immunohistochemistry. Circulating gelatinases and their inhibitors were studied using ELISA in two different populations of 71 at preoperative state and 213 patients at pre- and postoperative state.

High expression of TIMP-1 immunoreactive protein positively correlated with high histological grade of the tumour and associated with aggressive disease course in grade 2–3 subpopulation. High preoperative plasma TIMP-1 was prognostic for relapse in a modern patient series after a median follow-up time of 18 months. TIMP-1 as a continuous variable was prognostic in Cox regression univariate analysis, and was an independent prognostic variable superior to nodal status in multivariate analysis. High preoperative serum TIMP-1 was an independent prognostic variable for poor disease-specific survival, and TIMP-1 was found to maintain its prognostic value when assessed independently with different ELISA analyses, and was not very sensitive for preanalytical conditions. In addition, low circulating preoperative serum MMP-2 was observed to associate with high stage and positive nodal status in breast carcinoma.

These results indicate that circulating TIMP-1 may be a potential new marker of worsened prognosis in breast carcinoma, although careful validation of assay platforms and identification of the sources of physiological variation are needed before it can be adopted into clinical decision-making.

Keywords: breast carcinoma, ELISA, MMP-2, preanalytical factor, prognostic marker, TIMP-1

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Abbreviations

AEC	aminoethyl carbazole
AIs	aromatase inhibitors
AP-1	activator protein-1
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
CISH	chromogen in situ hybridisation
CMF	cyclophosphamide methotrexate 5-fluorouracil –chemotherapy regimen
DCIS	ductal carcinoma in situ
DSS	disease-specific survival
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMMPRIN	extracellular matrix metalloproteinase inducers
EPO	erythropoietin
ER	oestrogen receptor
ERE	oestrogen responsive element
FAC	5-fluorouracil, doxorubicin, cyclophosphamide –chemotherapy regimen
FEC	5-fluorouracil, epirubicin, cyclophosphamide –chemotherapy regimen
FISH	fluorescent in situ hybridisation
HER	human epidermal growth factor receptor
HR	hazard ratio
HRP	horseradish peroxidase
HRT	hormone replacement therapy
IHC	immunohistochemistry
kDa	kilodalton
LHRH	luteinizing hormone releasing hormone
MAPK	mitosis associated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
OPD	ortophenyl diamine

PAI-1	plasminogen activator inhibitor type 1
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3’-kinase
PR	progesterone receptor
RECK	reversion-inducing cysteine-rich protein with kazal motifs
ROC	receiving operating characteristics
RR	relative risk
SCC	squamous cell carcinoma
SERD	selective oestrogen receptor downregulator
SERM	selective oestrogen receptor modulator
TDLU	terminal duct lobuloalveolar unit
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumour necrosis factor
TNM	Tumour-Node-Metastasis
TS-2	thrombospondin-2
UICC	International Union against Cancer
uPa	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
WHO	world health organization
4-OHE	4-hydroxyoestradiol

List of original articles

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

I Kuvaja P, Talvensaari-Mattila A, Pääkkö P & Turpeenniemi-Hujanen T (2005) The absence of immunoreactivity for Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), but not for TIMP-2, protein is associated with a favorable prognosis in aggressive breast carcinoma. Oncology 68: 196-203

II Kuvaja P, Talvensaari-Mattila A, Pääkkö P & Turpeenniemi-Hujanen T (2006) Low serum level of pro-matrix metalloproteinase 2 correlates with aggressive behavior in breast carcinoma. Hum Pathol 37: 1316-1323

III Kuvaja P, Talvensaari-Mattila A &Turpeenniemi-Hujanen T (2007) The sample type used affects the levels of gelatinases (MMP-2 and -9) and their inhibitors (TIMP-1 and -2) in circulating blood of healthy controls and breast cancer patients. Biomarker Insights 2: 117-127

IV Kuvaja P*, Würtz SØ*, Talvensaari-Mattila A, Brünner N, Pääkkö P & Turpeenniemi-Hujanen T (2007) High serum TIMP-1 correlates with poor prognosis in breast carcinoma – a validation study. Cancer Biomark. In press.

V Kuvaja P, Talvensaari-Mattila A & Turpeenniemi-Hujanen T (2007) High preoperative plasma TIMP-1 is prognostic for early relapse in primary breast carcinoma. Manuscript.

* Equal contribution

Contents

1 Introduction

Breast carcinoma is the most prevalent cancer worldwide, resulting annually in more than one million new breast cancer diagnoses (Parkin *et al.* 2005). In Finland, there were over 4,000 new cases in 2005, and the numbers are expected to continue rising (Finnish Cancer Registry 2006). Ageing of the population can be seen as being responsible for some of the observed rise in the number of diagnoses per year, but other reasons such as changes in the reproductive behaviour have an effect on incidence. Breast carcinoma is a heterogeneous disease with a varying prognosis. Some patients with small local tumours carry an excellent prognosis of over 90% 5-year survival rate, whereas other patients diagnosed with larger tumours, axillary metastases and aggressive biological traits are in a much worse situation. The latter are often found among young patients. Due to the heterogeneous nature of the disease, prognostic markers are needed for the selection of suitable treatment options. The problems today are dual: the treatments for patients with the most aggressively-behaving tumours are not sufficient, and on the other hand, many patients with less aggressive diseases are over-treated and suffer from the known severe side effects of chemo- and radiotherapies.

Several markers that determine breast carcinoma prognosis exist, including traditional prognostic markers relating directly to tumour or patient characteristics such as tumour size, nodal involvement, histological tumour type and differentiation, as well as patient's age at diagnosis. Biological prognostic markers which are predictive for treatments such as hormone-dependency of the tumour and its HER-2 expression are increasing in importance. Novel markers are still needed in order to better identify patients that need the most intensive treatments, and also those who could be spared from the most toxic treatments.

Matrix metalloproteinases (MMPs) are a group of enzymes that regulate the extracellular matrix turnover. Their subgroup of gelatinases MMP-2 and MMP-9 are able to break down components of basement membranes that surround all tissue boundaries. If cancer cells are to spread and metastasize, they must have the ability to cross these tissue barriers. Therefore, the expression of gelatinases has been viewed as important for cancer invasion potential (Liotta *et al.* 1980), and high tumour tissue expression of MMP-2 has been shown to be a marker of worsened prognosis in breast carcinoma (Talvensaari-Mattila *et al.* 1998).

The regulation of MMPs involves their naturally occurring tissue inhibitor molecules, TIMPs. They are small molecules that also possess growth-promoting

capacities and can act as regulators of angiogenesis and programmed cell death. Their role in tumour invasion and spread is therefore controversial (Fassina *et al.* 2000). Tissue expression of TIMP-1 (Schrohl *et al.* 2004) and circulating TIMP-1 (Talvensaari-Mattila & Turpeenniemi-Hujanen 2005a) have recently been suggested as prognostic markers in breast carcinoma.

The purpose of this study was to examine the expression and prognostic potential of the TIMP-1 molecule applying different sample types, tumour tissue, serum and plasma. The role of MMP-2 in circulating form was also studied, and some preanalytical aspects of selecting sample types for measuring circulating gelatinases and their inhibitors were analysed.

2 Review of the literature

2.1 Breast carcinoma

Breast carcinoma is the most abundant malignancy of women in all industrialized countries. Today, the 5-year survival of breast cancer is about 88% (Finnish cancer Registry 2006). The prognosis has been much improved due to mammography screening and rapid development of adjuvant therapies during the last decades, but the nature of the disease is heterogeneous. Small tumours that are local and often found in screening mammograms carry an excellent prognosis, but some carcinoma subtypes behave very aggressively and are potentially metastatic, causing treatment failure and considerable mortality. These more aggressive types often accumulate in younger women, thus causing individual suffering for many.

2.1.1 Epidemiology and aetiology of breast cancer

Worldwide, there are estimated to be 1.15 million new breast cancer diagnoses annually. The incidence rates are higher in developed countries (99.4 per 100,000 in North America) compared with Western Asia, Southern Africa and South America, with the lowest incidence in Central Africa (16.5 per 100,000). (Parkin *et al.* 2005)

In Finland, there were 3,903 new breast cancer diagnoses in 2004, comprising 31.4% of all newly appearing cancers in women in 2004. The incidence per 100,000 was 86.2, and mortality 14.3 per 100,000. (Finnish Cancer Registry 2006)

The incidence of breast cancer varies according to age, rising steeply after age 35 and starting to decrease slightly after 55 years of age in Finland (Parkin *et al.* 2005). The incidence has shown rapid increase in all age groups during the last decades, but the rise is today limited to white women over 50 years of age (Smigal *et al.* 2006). The prognosis of breast carcinoma is generally good, though differences appear. Due to good prognosis and high incidence, breast carcinoma is the most prevalent cancer in the world. (Parkin *et al.* 2005) Several risk factors for breast cancer are known, but are not present in about 50% of the cases (Madigan *et al.* 1995).

Several hormonally linked risk factors for breast cancer development concerning a woman's reproductive life are well established. These reproductive risk factors include early menarche, giving RR (relative risk) of 1-2 to control population, late menopause (RR 2-4), and nulliparity or first childbearing after 35 years of age (MacMahon *et al.* 1970; Pike *et al.* 1983; Hayes 1996; Clavel-Chapelon *et al.* 2002; MacMahon 2006). Higher risk for breast cancer is observed in women with late (>35 years) first childbearing compared with nulliparous women (MacMahon 2006). Early childbearing is known to reduce breast cancer risk below that of nulliparous women (Clavel-Chapelon *et al.* 2002, MacMahon 2006).

Positive family history for breast cancer is a strong risk factor for the disease (Dupont *et al.* 1993, Hayes 1996). Genetic susceptibility is estimated to play a role in about 30% of breast cancer cases (Lichtenstein *et al.* 2000), but known high-risk susceptibility alleles explain only 5-10% of the breast cancer cases that appear (Claus *et al.* 1996). The most important breast cancer susceptibility genes known today are BRCA-1 (Miki *et al.* 1994) and BRCA-2 (Wooster *et al.* 1995). Normally they act as tumour suppressor genes participating in DNA repair and stabilization. Over 200 germline mutations of BRCA-1 associating with cancer susceptibility are known (Yoshida & Miki 2004). When studying breast cancer families, surprisingly low proportions of the cases were linked to BRCA-1 or BRCA-2, suggesting the existence of other susceptibility genes (Vehmanen *et al.* 1997; Ford *et al.* 1998). In Finland, seven founder-mutations are known for both BRCA-1 and BRCA-2 (Vehmanen *et al.* 1997; Huusko *et al.* 1998; Eerola *et al.* 2001). Additional breast cancer susceptibility alleles are continuously introduced (Walsh & King 2007)

Other well-established risk factors for breast cancer are ionizing radiation (Mohan *et al.* 2002; Travis *et al.* 2003), earlier benign breast disease (London *et al.* 1992, Dupont *et al.* 1993) and risk factors giving moderately elevated risk such as alcohol consumption (Hamajima *et al.* 2002) and use of hormone replacement therapy (Rossouw *et al.* 2002; Beral *et al.* 2003). The often observed positive correlation of high education and elevated breast cancer risk can be fairly well explained when adjusted for reproductive and other risk factors (Braaten *et al.* 2004) Some additional risk factors have recently been suggested, such as higher birth weight (Ahlgren *et al.* 2003; Troisi *et al.* 2006) and heavy exposure to cigarettes (Lissowska *et al.* 2006).

2.1.2 Breast cancer diagnosis and determination of the prognosis

The most common symptom of breast cancer is a palpable lump in the breast (reported by 60% of the patients in Paajanen *et al.* 2006); some patients experience pain or discomfort in the breast or notice abnormal excretion, retraction of the nipple or a skin reaction. In the majority of the cases mammogram and coarse needle biopsy confirm the diagnosis, but other radiological techniques such as ultrasound or MRI of the breasts can be used as an aid. The majority of breast tumours appear in the upper-outer quadrant of the breast. (FBCG 2002a)

In Finland, screening mammograms are conducted on women 50-59 years of age every 2 years, and in some areas also on women aged 60-69 years. Twenty percent of breast cancers are found in screening mammograms. Those that are found are significantly smaller and more rarely spread to the axillary lymph nodes, and carry a better prognosis than tumours of a similar size that are found outside of screening (Joensuu *et al.* 2004; Paajanen *et al.* 2006)

Traditional prognostic factors

Traditional prognostic factors include size of the tumour, axillary lymph node involvement, presence of metastasis (of which the former three are included in staging of breast tumours), histological differentiation of the tumour, patient's age, tumour ER/PR expression and HER-2 status. New biological prognostic factors are continuously being introduced, but there is a lack of consensus as to which of these should be included in general practice. (Bast *et al.* 2001; Bundred 2001; Duffy 2006)

TNM classification and staging of breast carcinomas

Breast tumours are classified according to International Union Against Cancer (UICC) Tumour Node Metastasis (TNM) Classification (Sobin & Wittekind 2002), briefly outlined in Table 1. The TNM scores are combined in clinical staging of breast tumours.

Table 1. TNM classification of breast tumours (modified from UICC TNM classification)

Tumour parameter	Definition
T Primary tumour	
X	Primary tumour not assessable
0	No primary tumour
is	Carcinoma in situ
1	Tumour diameter < 2cm
2	Tumour diameter 2 - 5cm
3	Tumour diameter > 5cm
4	Tumour extending to chest wall or skin infiltration
N Regional lymph nodes	
X	Lymph nodes not assessable
0	No regional lymph node metastasis
1	Ipsilateral, mobile metastasis in regional lymph node(s)
2	Ipsilateral, fixed regional lymph node metastasis or metastasis in internal mammary lymph nodes only
3	Ipsilateral infraclavicular or supraclavicular lymph node metastasis or internal mammary lymph node metastasis with regional lymph node metastasis
M Distant metastasis	
X	Distant metastasis not assessable
0	No distant metastasis
1	Distant metastasis present

Traditionally, tumour size and nodal status are both viewed as prognostic indicators that are independent and additive (Carter *et al.* 1989). Today, nodal status is considered to be the strongest and most important traditional prognostic factor predicting locoregional relapses and survival (Elston *et al.* 1999; Bundred 2001; Beenken *et al.* 2003).

Histological typing and grading

The epithelial tumours of the breast are divided into *in situ* carcinomas and invasive carcinomas. *In situ* carcinomas are defined by the lack of invasion through ductal basement membrane and are thus considered premalignant lesions. They are accompanied by a risk of developing invasive carcinomas if left untreated (Leonard and Swain 2004). *In situ* carcinomas do not develop axillary or distant metastases, and thus they have an excellent prognosis compared with invasive carcinomas (Rosen 1987; Leonard and Swain 2004).

Invasive carcinomas can be divided into several histological subtypes. The majority of invasive carcinomas represent ductal infiltrative carcinoma (77.5%, Blanco 1980). The second most common subtype is lobular carcinoma, followed by the rare subtypes of tubular, mucinous, medullary, papillary, comedo-type carcinomas and Paget's disease (Rosen 1987). A correlation between microscopic appearance and breast tumour malignancy is an old finding. Ductal infiltrative carcinomas have been carrying the worst prognosis (Blanco 1980; Elston *et al.* 1999). The prognosis of lobular carcinomas has been better, (Toikkanen *et al.* 1997; Elston *et al.* 1999) and more rare subtypes are associated with further enhanced prognosis than ductal and lobular carcinomas (Rosen 1987; Elston *et al.* 1999).

Prognostic and predictive markers

Prognostic marker correlates with patients' survival, whereas predictive markers predict efficacy of a therapy form, but not necessarily survival. The current trend is to search for new markers that can predict treatment response. Especially in the case of new drugs the need for biomarkers indicating the likelihood of response is continuously increasing. In clinical practise, the most used predictive marker is the oestrogen receptor (ER) status of the tumour (Duffy 2006).

Oestrogen and progesterone receptors

The majority of breast carcinomas are hormone-dependent for growth. About 75% of breast tumours express the oestrogen receptor ER, of which 50% also express progesterone receptor PR. Less than 10% of breast tumours express only PR, and about 20% are hormone-independent for growth (Osborne *et al.* 2005). Reproductive risk factors for breast cancer (delayed childbearing, nulliparity, early menarche) mainly increase the risk for oestrogen-dependent breast cancer, suggesting a role for oestrogen in breast carcinogenesis (Althuis *et al.* 2004). The effects of oestrogen are mediated via oestrogen receptors (ERs) that exist in two isoforms, ERα and ERβ.

The classical model for oestrogen action is ligand-dependent, where oestrogen binds to nuclear ER, and ER as a dimer binds to oestrogen-responsive elements (EREs) in the promoter regions of target genes causing up- or downregulation of gene transcription. In addition, oestrogen effects can be mediated via ERE-independent genomic mechanisms using other DNA-bound

transcriptional factors. Oestrogen-like effects can also appear without oestrogen through ER activation by other growth factors, such as EGF and HER-2. (Duffy 2006) Genomic oestrogen-like effects are not immediate. Also very rapid oestrogen effects exist and they are believed to mediate through the plasma membrane form of ER. (Duffy 2006).

Over 50% of patients with ER positive breast cancer respond to hormonal therapy. Antioestrogen tamoxifen binds to ER and blocks the mitotic signal of oestrogen. Selective inhibitors of aromatase, the enzyme that converts androgens to oestrogens, have emerged as more potential alternative to tamoxifen.

The isoforms ERα and ERβ are not equally prognostic. Most studies on the prognostic relevance of ER concern the ERα, and it is well established that ER-positive tumours are less aggressive and the patients are more likely to benefit from endocrine treatment of breast cancer, despite the limited prognostic value of ER expression in node-negative breast carcinoma. (Mirza *et al.* 2002; Osborne *et al.* 2005; Duffy 2006).

HER-2

HER-2, also known as c-erbB2 and neu, is a receptor that belongs to the epidermal growth factor receptor (EGFR) family. Activation of these receptors causes activation in MAPK and PI3K-Akt cell signalling pathways, leading to cell growth and survival. Gene amplification of HER-2 in breast carcinoma has been found to correlate with aggressive behaviour, high recurrence rates and poor survival. About 20-25% of invasive breast carcinomas carry HER-2 gene amplification. (Jukkola *et al.* 2003; Joensuu *et al.* 2003; Bernard-Marty *et al.* 2006; Gonzalez-Angulo *et al.* 2006)

Trastuzumab, a monoclonal antibody, was developed and specifically targeted against HER-2 receptor, and today it is used in the adjuvant treatment and in the treatment of metastatic disease. HER-2 serves as a predictive marker for the treatment response for trastuzumab, and is therefore suggested to be determined in all newly diagnosed breast carcinomas (Bast *et al.* 2001). The current recommendation for the determination of HER-2 status of the tumour is first to use immunohistochemistry (ICH), and to confirm positive result with *in situ* hybridization (FISH/CISH) (Gonzalez-Angulo *et al.* 2006).

Novel markers

Several other, promising prognostic factors for breast cancer have been suggested, of which only few are represented in the following. These potential prognostic markers include markers of enhanced proliferation such as Ki67, a cell-cycle regulating protein expressed in proliferating cells (Railo *et al.* 1993; Pietiläinen *et al.* 1996), S-phase fraction, giving an estimate of the proportion of proliferating cells in the tumour (Ferno *et al.* 1992; Michels *et al.* 2000), and mitotic count, either performed by counting mitotic figures per 10x power microscopic field or by expressing the count per square millimetre (Kronqvist *et al.* 1998; Baak *et al.* 2005). These predict that tumours contain a higher proportion of proliferating cells and behave more aggressively. The loss of adhesion marked by loss of E-cadherin has also been suggested to worsen the prognosis of breast carcinoma (Elzagheid *et al.* 2002; Rakha *et al.* 2005)

Another group of prognostic factors consists of gene mutations or amplifications of c-Myc and bcl-2, and tumour suppressor gene p53. C-Myc is a transcriptional regulator and its expression is associated with cell proliferation and survival. Thus the amplification of c-Myc has been associated with worsened prognosis of breast cancer (Al-Kuraya *et al.* 2004; Elzagheid *et al.* 2006). Bcl-2 functions as an anti-apoptotic protein in normal cells. Dysregulation and abnormal function of bcl-2 can lead to tumour progression and chemoresistance, while normal expression of bcl-2 has been associated with favourable prognosis in breast carcinoma (Elzagheid *et al.* 2006). Phosphorylation of bcl-2 has been found to correlate with breast carcinoma progression (Matsuyoshi *et al.* 2006).

The mutation of the tumour suppressor gene P53 is the most frequently found gene mutation in cancers. In breast carcinoma, P53 mutations occur in approximately 30% of the cases (Borresen-Dale 2003). P53 targets include genes that inhibit cell cycle, apoptosis regulators, DNA-repair genes, and inhibitors of angiogenesis, all aiming for maintenance of genetic stability (Gasco *et al.* 2002). P53 mutations have been associated with worsened prognosis of breast carcinomas (Gasco *et al.* 2002; Borresen-Dale 2003; Rahko *et al.* 2003; Olivier *et al.* 2006). However, mutations occur in several exons and are not equally prognostic. In addition, immunohistochemical detection of P53 might reflect accumulation of the wild-type P53 and not a P53 mutation; more reliable results are therefore produced by determining P53 status of the tumour by mutation analysis (Gasco *et al.* 2002; Borresen-Dale 2003). In addition, co-amplification of different oncogenes such as HER-2, c-Myc, P53 and bcl-2 is common, (Beenken

et al. 2001; Al-Kuraya *et al.* 2004; Erdem *et al.* 2005; Rolland *et al.* 2007) and the evaluation of the prognostic value of a single gene mutation is thus difficult. Moreover, genomic instability in general, containing several mutations of known oncogenes, is a marker of worsened prognosis.

New microchip techniques allow the search for gene expression profiles that would lead to molecular fingerprinting and more accurate determination of breast carcinoma prognosis. Several gene sets have been suggested (Van't Veer *et al.* 2002; Wang *et al.* 2005), including a 70-gene prognostic signature first described by Van't Veer *et al.* in 2002. This gene set, dividing node-negative patients into low- or high-risk group gave very promising results (van de Vijver *et al.* 2002) that were later re-evaluated and questioned (Eden *et al.* 2004). This set has later been validated with other patient series (Buyse *et al.* 2006), the gene set giving HR 2.3 in a high-risk group for recurrence-free survival. It has been suggested that gene profiling does not give a more accurate result in prognosis determination compared with traditional prognostic markers (Eden *et al.* 2004), but some studies suggest that combining genetic profiling and traditional markers could yield an optimal result for prognosis determination (Eden *et al.* 2004; Sun *et al.* 2007). It has also been suggested that independent prognostic gene profiles that do not overlap are able to distinguish quite well the patients in similar prognostic categories (Fan *et al.* 2006). Today, two commercial kits are available for breast cancer geen expression profiling (Sorlie *et al.* 2003)

2.1.3 Treatment of primary breast cancer

The treatment of breast carcinoma aims primarily for complete surgical removal of the tumour. However, gross tumours are commonly accompanied by occult micrometastases, and effective treatment of breast carcinoma often requires postoperative radiotherapy and/or adjuvant chemo- and/or endocrine therapy that are effective in the treatment of microscopic residual disease. Due to the heterogeneity of the disease, the adjuvant treatment protocol varies according to patients' risk of relapse, and different treatment modalities are offered to those patients who are likely to benefit from them. (Goldhirsch 2005, 2007)

Surgery

The surgical options for the treatment of primary breast carcinoma are either mastectomy or breast-conserving surgery. *In situ* and invasive carcinomas are

both treated surgically, and for both, either of the two surgical options can be chosen. The results of studies comparing mastectomy and breast-conserving surgery accompanied with radiotherapy have shown them as effective when disease-specific and overall survivals are compared (Leidenius 2001; Veronesi *et al.* 2002; Poggi *et al.* 2003; Rutqvist *et al.* 2003). When breast-conserving surgery is used, there is a risk that some occult micrometastases may exist in the remaining breast tissue, which is why achieving tumour-free margins in the resection preparate is crucial (Schwartz *et al.* 2006). Breast-conserving surgery is therefore accompanied by an increased risk for local recurrence of the tumour. These local recurrences are not always signs of advanced diseases, and can often be treated with mastectomy. The use of postoperative radiotherapy reduces the risk for local recurrence considerably (Fisher *et al.* 1998; EBCTCG 2002b; EBCTCG 2005).

The benefits of breast-conserving surgery are better cosmetic results and improved quality of life. For small tumours (<3cm of diameter) breast-conserving surgery is a most suitable option, but there is no definite size limit for its use, as long as there is enough remaining breast tissue to guarantee a satisfactory cosmetic result (Leidenius 2001; Schwartz *et al.* 2006).

Breast-conserving surgery is not usually recommended for patients under thirty-five years of age due to the aggressive nature of breast tumours occurring at a very young age. Furthermore, if there is a multifocal tumour or if the tumour is accompanied by a very large *in situ* component, mastectomy is the recommended option. The use of mastectomy can sometimes spare patients from postoperative radiotherapy, as in the case of non-invasive carcinoma *in situ*, and therefore mastectomy might be a more preferable option in some cases. Patient's personal wishes for either option should be considered when surgery is planned. (Leidenius 2001; Schwartz *et al.* 2006).

Breast surgery, either mastectomy or breast-conserving option, is done simultaneously with axillary staging procedure, usually sentinel-node biopsy when there are no clinically detectable axillary metastases. The sentinel nodes of the ipsilateral axilla are located with either blue dye or radiocolloid. If a breast carcinoma metastasis is found in the pathological analysis of the frozen section, surgery continues with axillary dissection. The accuracy of sentinel-node biopsies is >90%, and they are preferred as a staging procedure due to lesser arm morbidity when compared with axillary dissection. (Leidenius 2005; Mansel *et al.* 2006; Schwartz *et al.* 2006).

Postoperative radiotherapy

Postoperative radiotherapy is usually offered for all patients with invasive breast carcinoma, and also for those with *in situ* carcinoma treated with breast conserving surgery. Postoperative radiotherapy is shown to decrease the risk for ipsilateral breast tumour recurrence in patients with invasive and *in situ* carcinoma (Fisher *et al.* 1998; EBCTCG 2002b; EORTC Breast Cancer Cooperative Group *et al.* 2006). It has been a matter of controversy during the last decades whether postoperative radiotherapy improves overall survival. The latest analyses show that there is evidence that postmastectomy radiotherapy does give an about 10% benefit for overall survival (Cutuli 2000). It has been shown that postoperative radiotherapy enhances breast cancer-related survival (EBCTCG 2002b). Overall survival for other, mainly cardiac diseases has been somewhat decreased, which is thought to originate from radiotherapy-induced cardiac effects that are more apparent in patients over 60 years of age (EBCTCG 2002b). Only about 15% of primarily node-negative patients relapse during the follow-up, but there are no tools to identify the low-risk cases that do not need the postoperative radiotherapy. (Arriagada *et al.* 1999).

Adjuvant endocrine therapy

Oestrogen is the most important growth factor in hormone-receptor positive breats cancer. Oestrogen originates differentially in pre- and postmenopausal women. In premenopausal women, ovaries produce the most oestrogen, whereas in postmenopausal women oestrogen originates from enzymatic activity in e.g. adipose tissues, liver and skeletal muscle.

The first endocrine treatment for breast carcinoma was developed as a form of surgical ovarian ablation over a century ago. Ovarian ablation can be done either surgically or medically by administering luteinizing hormone releasing hormone (LHRH) analogues (goserelin) that stop the ovarian oestrogen production. These treatments have been shown to be effective among premenopausal patients with ER-positive tumours, both in the adjuvant and metastatic setting (Pritchard 2005).

Tamoxifen is a selective oestrogen receptor modulator (SERM) that binds to ER and inhibits oestrogen activation of ER. Tamoxifen has been used for treatment of breast cancer over 30 years, first in the metastatic setting and in the adjuvant treatment of breast carcinoma since the 1990s. Meta-analyses of a 15-

year follow-up of tamoxifen trials show that patients with ER-positive tumours benefit from adjuvant tamoxifen treatment irrespective of age, menopausal status, nodal status or prior chemotherapy administration. Proportional recurrence reduction of about 47% has been seen in ER-expressing tumours. Absolute mortality reductions were 10.9% for node-positive, and 5.6% for node-negative breast cancer (EBCTCG 2005). Five-year administration of tamoxifen has been shown to produce significantly greater reduction in recurrence and breast cancer mortality than shorter courses in meta-analyses (EBCTCG 2001; EBCTCG 2005).

Aromatase inhibitors (AIs) are drugs that inhibit the postmenopausal oestrogen synthesis that occurs through converting adrenal androgens to oestrogen. Third-generation aromatase inhibitors anastrozole, letrozole and exemestane have yielded promising results in terms of recurrence and mortality reductions (Dellapasqua and Castiglione-Gertsch 2005). In a recently published meta-analysis it was shown that third-generation aromatase inhibitors are superior to tamoxifen treatment when postmenopausal, ER-positive breast cancer is treated both in the adjuvant and metastatic setting (Mauri *et al.* 2006).

The current protocol is to offer anti-oestrogen adjuvant treatment for patients with ER-positive tumours (Goldhirsch *et al.* 2007). For premenopausal patients, tamoxifen has been the drug of choice. For postmenopausal patients, a recent report suggests the superiority of AIs. Endocrine therapies produce different side effects than cytotoxic drugs. The major adverse side effect of tamoxifen is the increased risk of thromboembolic events and secondary endometrial cancer (EBCTCG 2001, Rutqvist and Johansson 2007). In premenopausal patients, (but not in postmenopausal patients) the use of tamoxifen associates with the loss of bone mineral density (Powles *et al.* 1996). Also climacteric symptoms are a common side-effect of tamoxifen and AIs, which may cause osteoporosis.

Adjuvant chemotherapy

The use of chemotherapeutic agents that can demolish the possibly remaining micrometastases has dramatically improved the prognosis of breast carcinoma over the last decades. The agents effective in metastatic setting have stepwise been adopted to curative adjuvant treatment (Fossati *et al.* 1998; Nicolini 2006). It has been shown that adjuvant chemotherapy improves the prognosis, irrespective of the hormone-receptor status of the tumour, menopausal status and in both node-positive and node-negative subgroups, of which node-positive and

young patients under 50 years of age gain the highest survival benefit (EBCTCG 2002b; EBCTCG 2005). Today, the general guideline for the use of adjuvant chemotherapy is to include all patients with node-positive disease, or node-negative disease accompanied with other unfavourable prognostic factors, such as young age, large primary tumour (>1cm) or high histological grade, increasing the risk of recurrence within the next ten years to exceed 10% (Finnish Breast Cancer Group 2002b; Goldhirsch *et al.* 2005). Chemotherapy is seldom used among patients >70 years of age due to toxicity of the treatments and lack of knowledge on its efficacy, since only few elderly patients have been included in clinical chemotherapy trials (EBCTCG 2002a).

Polychemotherapy is shown to be more effective than single-agent chemotherapy, and longer than three to six months' duration does not add to the survival benefit (EBCTCG 2002a). The longest experience of the use of polychemotherapy exists for cyclophosphamide (C), methotrexate (M) and fluorouracil (5-FU) combination. This includes an alkylating agent (C) that binds to DNA, causing cross-linkage in the double-strand structure that inhibits DNA synthesis, and antimetabolites (M and 5-FU) that mainly act by inhibiting enzymes required in the DNA synthesis (Ackland *et al.* 2003; Nieto 2003). This treatment has been shown to substantially reduce the risk of relapse and death in a recently published 30-year follow-up cohort study (Bonadonna *et al.* 2005). Anthracyclines (epirubicin and doxorubicin) are antibiotic agents causing cytotoxic effects via inhibition of topoisomerase II enzyme and generation of free radical compounds of oxygen (Gianni *et al.* 2003). Anthracycline-based polychemotherapy (FEC or FAC) has been the treatment of choice in recent years, since it has survival benefit over the use of CMF (EBCTCG 2005**;** Ejlertsen *et al.* 2007). One of the latest developments in the area is the use of taxanes (paclitaxel, docetaxel), agents that stabilize the mitotic spindle. It has been show in the most recent studies that taxanes might add 3-5% and 2-3% to the recurrence-free and overall survival, respectively (Hudis 2005; Bria *et al.* 2006, Joensuu *et al.* 2006).

Since chemotherapeutic agents target all actively dividing cells, the side effects are often dramatic. The transient, common acute side effects of cytotoxic drugs include hair loss, fatigue, nausea, diarrhoea and neutropenic infections. The most significant, permanent side effect is premature menopause (Huovinen & Joensuu 2004). Rare cardiotoxic side effects occur with anthracyclines (Fumoleau *et al.* 2006), and the risk of secondary leukaemia is mildly elevated, especially when high dosages of cytotoxic drugs are used (Praga *et al.* 2005). Recently it has been shown that individually tailored high-dose FEC accompanied with

granulocyte-stimulating growth factors produces a survival benefit over standard FEC, but secondary AML/MDS incidence is increased (Wilking *et al.* 2007). The use of taxane-based adjuvant therapy (docetaxel, cyclophosphamide, 5-fluorouracil) versus FAC has been shown to produce more severe side effects, even though administered with granulocyte growth factors (Brain *et al.* 2005; Martin *et al.* 2006). When doxetaxel was given sequentially after three cycles of standard FEC, a less severe side-effect profile was reported (Roche *et al.* 2006)

Adjuvant monoclonal antibody-based therapies

Monoclonal antibody targeted therapies currently exist against epidermal growth factor receptor (EGFR) family. The use of these drugs inhibits tumour growth and angiogenesis. The first approved monoclonal antibody trastuzumab has been shown to reduce breast cancer recurrence among patients with HER-2 overexpressing tumours (Piccart-Gebhart *et al.* 2005; Romond *et al.* 2005; Joensuu *et al.* 2006; Smith *et al.* 2007), and is currently used in the adjuvant treatment of breast carcinoma for patients with HER-2 overexpressing tumours (Goldhirch *et al.* 2007). Recently it was shown that the use of adjuvant trastuzumab produces an overall survival benefit in HER-2 positive early breast cancer (Smith *et al.* 2007) The most detrimental side effect of trastuzumab is rarely occurring cardiac toxicity (Gonzalez-Angulo *et al.* 2006).

2.2 The process of malignant progression of breast cancer

The majority of breast tumours are adenocarcinomas and derived from mammary epithelial cells. The basic structural unit of breast tissue is a terminal duct lobuloalveolar unit (TDLU). These terminal ducts are lined by a double layer of luminal epithelial cells, containing both ductal and lobular cells. They are surrounded by a layer of myofibroblasts and a basement membrane. (Clarke *et al.* 2002; Simpson *et al.* 2005) The majority of breast tumours are seen to be originating from TDLU units (Clarke *et al.* 2002).

Both luminal cells and myofibroblasts are thought to originate from a pluripotent stem cell (Clarke *et al.* 2002). It has been hypothesized that mutations that accumulate to these stem cells might result in cancer formation (Gudjonsson and Magnusson 2005; Woodward *et al.* 2005).

Sporadic breast cancers are thought to originate via the accumulation of mutations, gene amplifications of oncogenes and mutations in tumour suppressor

genes. A multiple set of genes are involved, such as c-erbB2, C-Myc, P53 and BRCA1 (Dickson *et al.* 2005). Mutations cause allelic imbalance, which leads to loss of heterozygosity and further to malignant transformation (Dickson *et al.* 2005).

The presence of certain types of hyperplasias, mainly atypical ductal or lobular hyperplasias (ADH/ALH) and ductal or lobular *in situ* carcinomas is associated with an increased risk for invasive breast cancer (Allred *et al.* 2001). These hyperplasias and *in situ* carcinomas share similarities in their genetic structure according to grade of differentiation. ADH and low-grade DCIS and LCIS are associated with a low number of genetic alterations, but frequent loss of 16q, whereas high-grade DCIS/LCIS have a more complex set of genetic alterations, more frequently including the amplification of HER-2. (Allred *et al.* 2001; Simpson *et al.* 2005)

The cascade of malignant transformation of breast epithelial cells is still far from clear. The true significance of other types of hyperplasia apart from ADH/ALH in the cascade of malignant progression is not known, and not all precancerous lesions of DCIS/LCIS will ever undergo malignant transformation to invasive carcinoma (Simpson *et al.* 2005).

Oestrogen is crucial for the development of a normal breast, causing proliferation of ductal and lobular cells, and is a major mitogen in hormone-dependent breast cancer cell lines. There are also other factors directing epithelial cell proliferation, including local growth factors, such as TGF-α, EGF, IGFs and FGFs (fibroblast growth factors). Several of these molecules contain an oestrogen-responsive element. These factors drive cell growth and may inhibit apoptosis, and once a malignant transformation has occurred, these factors in the tumour microenvironment might be crucial for aggressiveness and metastatic potential of the tumour. Extracellular matrix proteins such as MMPs have also been suggested as important regulators of cancer progression. (Clarke *et al.* 2002, Dickson *et al.* 2005)

2.3 Matrix metalloproteinases and their inhibitors

2.3.1 Matrix metalloproteinases

Matrix metalloproteinases are classified as matrixin subfamily in a family of zinc-degrading metalloproteinases, sharing conserved structure of a zinc-binding

catalytic site. Today, 25 vertebrate MMPs and 23 human homologues are known to exist. They regulate extracellular matrix (ECM) turnover, and digest all of its structural components with overlapping substrate specificities. MMPs are assigned with numbers, but most of them also have trivial names and are further classified according to domain organization and substrate preference (Table 2). (Sternlicht and Werb 2001; Chakraborti *et al.* 2003; Nagase *et al.* 2006)

Table 2. Matrix metalloproteinase classification and main substrates.

Enzyme subclass MMP	Common name	Some substrates
Collagenases		
MMP-1	Collagenase 1	collagens, gelatin, proteoglycan link protein, aggrecan, versican, tenascin, entactin, MMP-2 and -9
MMP-8	Collagenase 2	collagens, gelatin, aggrecan
MMP-13	Collagenase 3	collagens, gelatin, aggrecan, perlecan, large tenascin-C, fibronectin, osteonectin, MMP-9, PAI-2
MMP-18	Collagenase 4	not known
Gelatinases		
MMP-2	Gelatinase A	collagens, gelatin, elastin, laminin, fibronectin, aggrecan, decorin, proteoglycan link protein, osteonectin, MMP-1, -9, -13
MMP-9	Gelatinase B	collagens, gelatin, elastin, laminin, aggrecan, fibronectin, proteoglycan link protein, osteonectin, entactin, plasminogen
Stromelysins		
MMP-3	Stromelysin 1	collagens, gelatin, aggrecan, versican, perlecan, decorin, proteoglycan link protein, osteonectin, entactin, several MMPs
MMP-10	Stromelysin 2	collagens, gelatin, casein, aggrecan, elastin, MMP-1 and -8
Matrilysins		
MMP-7	Matrilysin 1	collagens IV and X, gelatin, aggrecan, decorin, fibronectin, laminin, entactin, osteonectin, elastin, casein, transferrin
MMP-26	Matrilysin 2	collagen IV, gelatin, fibronectin
MMP-11	Stromelysin 3	casein, laminin, fibronectin, gelatin, collagen IV
Membrane-type MMPs		
MMP-14	MT1-MMP	proMMP-2, collagens I, II, III, casein, elastin, fibronectin, gelatin, laminin, vitronectin, proteoglycans
MMP-15	MT2-MMP	proMMP-2, fibronectin, laminin, entactin, aggrecan, perlecan
MMP-16	MT3-MMP	proMMP-2, collagen III, gelatin, casein, fibronectin
MMP-24	MT5-MMP	proMMP-2
MMP-17	MT4-MMP	proMMP-2
MMP-25	MT6-MMP	proMMP-2
Other		
MMP-12	Macrophage elastase	collagen IV, gelatin, elastin, casein, laminin, fibronectin, vitronectin, enactin
MMP-19		gelatin
MMP-20	Enamelysin	amelogenin
MMP-21		not known
MMP-23	CA-MMP	not known
MMP-27		not known
MMP-28	Epilysin	not known

(reproduced from Sternlicht & Werb 2001; Chakraborti *et al.* 2003; Nagase *et al.* 2006)

The main function of matrix metalloproteinases is the breakdown of ECM, simultaneously causing alterations in the cell-cell and cell-matrix interactions. MMP substrates include molecules that are not ECM components, and altogether, in addition to ECM turnover, MMP actions affect cell migration, cell growth and differentiation, inflammatory responses, apoptosis and neovascularization. (Sternlicht and Werb 2001; Chakraborti *et al.* 2003)

Structure and regulation of MMPs

MMPs are structurally related proteins. All share the same basic structure, consisting of a propeptide, a catalytic metalloproteinase domain, a linker peptide, and a haemopexin domain. The exceptions are MMP-7, structurally the simplest MMP lacking the haemopexin domain, and MMP-23 and -26 that have a cysteine-rich domain and an immunoglobulin-like domain instead of the haemopexin domain. (Fig. 1) (Sternlicht and Werb 2001; Nagase *et al.* 2006)

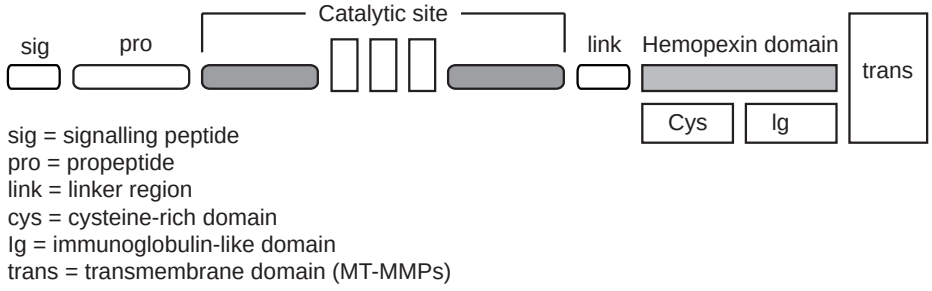

Fig. 1. Structure of matrix metalloproteinases.

Matrix metalloproteinases are expressed in a tissue-specific manner. Their normal expression is generally low, and can be up- or downregulated via cytokine/growth factor dependent and independent pathways. Matrix metalloproteinase regulation involves several different stages, but is primarily produced at transcriptional level. Several cytokines and growth factors, including interleukins, interferons, EGF, VEGF, TNF-α, TGF-β, and extracellular matrix metalloproteinase inducers (EMMPRINs) can enhance MMP expression. Transcriptional activation mechanisms mainly involve induction of c-fos and/or c-jun proto-oncogene families. These proto-oncogene products bind to AP-1 (activator protein-1) as heterodimers, and can induce AP-1-reactive genes. An

AP-1 binding site has been found for each inducible MMP gene, not including MMP-2. (Sternlicht and Werb 2001; Chakraborti *et al.* 2003) (Fig. 2)

Post-transcriptional regulation of MMPs includes stabilization of mRNA for some MMPs (MMP-1, MMP-3 and MMP-13), as well as secretory control. MMPs are synthesized as inactive proenzymes and stored in zymogen granules. When released, they are localized near the plasma membrane where the majority of the extracellular signalling of pericellular proteolysis takes place. MMPs are activated extracellularly via serine proteases or other active MMPs, of which MT-MMPs are transmembraneously located. (Sternlicht and Werb 2001; Chakraborti *et al.* 2003) (Fig. 2)

Another mechanism to regulate MMP activity is enhanced localization of MMPs to the cell surface via cell surface docking receptors. Activated MMP-2 can bind to integrin αvβ3 (Brooks *et al.* 1996), and EMMPRIN molecules can both induce and localize MMP-1 to the cell surface (Guo *et al.* 2000). A similar mechanism has been suggested for MMP-2 (Sier *et al.* 2006). (Fig. 2)

Once activated, the functioning of MMPs is regulated by their naturally occurring inhibitors, TIMPs, which are discussed in more detail in 2.3.2. The activity of MMPs can also be affected by other endogenous inhibitors such as membrane-anchored glycoprotein RECK (reversion-inducing cysteine-rich protein with kazal motifs) (Takahashi *et al.* 1998) and α_2-macroglobulin (Troeberg *et al.* 2002). (Fig. 2)

Lastly, MMP activities are regulated by catabolism and clearance. Most MMPs can bind to α-macroglobulin and become trapped, and the enzyme complex is further endocytosed (Sottrup-Jensen and Birkedal-Hansen 1989). Thrombospondin-2 (TS2) has also been implicated in MMP clearance, since TS2 normally binds latent and active MMP-2, and it has been shown that TS2-deficient mice have a fibroblast adhesion defect that is due to excess MMP-2 (Yang *et al.* 2000). (Fig. 2)

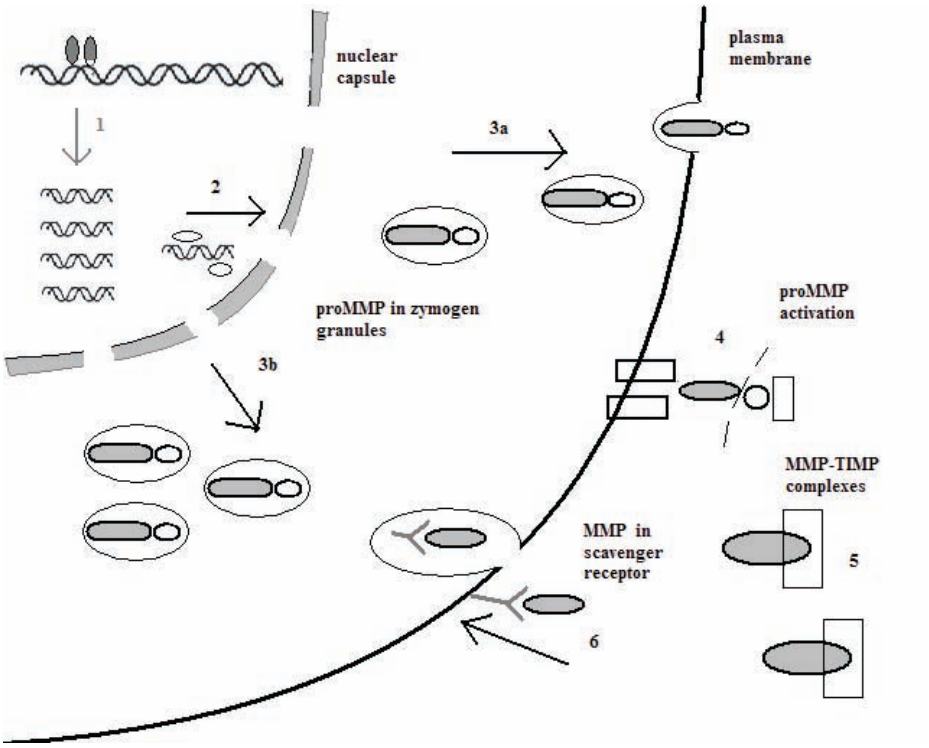

Fig. 2. MMP turnover. 1) transcriptional regulation, 2) mRNA processing, 3) secretion, a) constitutive, b) regulated , 4) activation, 5) inhibition, 6) clearance

ECM turnover and MMP activities are tightly regulated, and any disturbance in this balance might lead to common non-physiological processes, due to either accumulation or degradation of ECM. MMPs have been implicated in several processes that require ECM remodelling, including physiological states such as wound healing (Davis and Saunders 2006), and in various diseases, such as rheumatoid arthritis and osteoarthritis (Prince 2005; Burrage *et al.* 2006), asthma and other pulmonary diseases (Elkington and Friedland 2006; Gueders *et al.* 2006), in diseases of the glomerulus (Lelongt *et al.* 2001), various cardiovascular diseases (Nagase *et al.* 2006), autoimmune diseases (Ram *et al.* 2006) and oral diseases (Sorsa *et al.* 2004). MMPs are also implicated in malignant transformations, including solid tumours (Turpeenniemi-Hujanen 2005) and haematological malignancies (Janowska-Wieczorek *et al.* 2000).

Physiological actions of MMPs

ECM degradation has been thought to be the main function of MMPs, resulting in facilitation of cell migration, release of different signalling molecules, and changes in cell-matrix interactions. Besides this, MMP substrates include various non-ECM components, including growth factor precursors, growth factor receptors, adhesion receptors, cytokines and cytokine receptors (Stamenkovich 2003).

Physiologically, different MMPs have been implicated in development and differentiation, including morphogenesis of the kidney, salivary and mammary gland, postpartum uterine involution, bone morphogenesis, embryo implantation, and wound healing (Sternlicht and Werb 2001; Stamenkovich 2003). These processes require controlled neovascularization and programmed cell death, which relate closely to suggested MMP actions in cancer progression.

MMPs can affect angiogenesis in at least two ways. Either they release growth factors in the proteolysis of extracellular matrix, or liberate or unmask pro- or antiangiogenic sites within the ECM (Handsley and Edwards 2005). It has been shown that endothelial cells express several MMPs and TIMPs *in vitro* (Hanemaaijer *et al.* 1993; Chan *et al.* 1998; Nuttall *et al.* 2003), and their expression has been shown to relate to physiological angiogenic processes, such as cyclic expression in the endometrium during menstrual cycle and during decidualization (Freitas *et al.* 1999; Nuttall and Kennedy 1999). MMP inhibition studies have also shown that angiogenesis is partly dependent on MMP activity, since synthetic MMP inhibitors have been shown to block angiogenic activity *in vitro* and *in vivo* in tumour models (Naglich *et al.* 2001; Kaliski *et al.* 2005).

MMPs have also been shown to play pivotal roles in programmed cell death, in which various mechanisms have been proposed. Through ECM proteolysis, large fragments called matrikines are produced, including angiostatin that is able to stimulate apoptosis. MMP substrates also include non-matrix proteins, such as proneurothophins and cytokines that are also involved in the regulation of programmed cell death. Other possible mechanisms include association of MMPs to cell surface receptors, and apoptosis is enhanced or inhibited through receptor shedding. (Egeblad and Werb 2002; Mannello *et al.* 2005).

Gelatinases A and B: structure and activation

Gelatinases A (MMP-2) and B (MMP-9) share the basic structural organization of metalloproteinases, and both have a three-repeat fibronectin type II motif in their metalloproteinase domain. Pro-MMP-2, a 72-kDa glycoprotein, is constitutively expressed in virtually all cell types at a low level, whereas the expression of pro-MMP-9, a 92-kDa protein is physiologically limited to osteoclasts, macrophages, trophoblasts, hippocampal neurons, and migrating keratinocytes in a healing wound. MMP-9 expression is transcriptionally regulated, and its expression can thus be increased multifold. MMP-9 is activated by cleavage of the propeptide tail, and this can be achieved by several already activated MMPs or serine proteases. (Sternlicht and Werb 2001; Chakraborti *et al.* 2003; Nagase *et al.* 2006)

MMP-2 is activated through a multistep process requiring the presence of TIMP-2 and MT1-MMP simultaneously at the cell surface. First, the N-terminal of the TIMP-2 molecule binds to MT1-MMP, and its C-terminal acts as a receptor for proMMP-2. Then an adjacent MT1-MMP molecule cleaves the propeptide part of MMP-2, and another MMP-2 molecule is required to detach the activated MMP-2 (Strongin *et al.* 1995). The transcriptional expression of MMP-2, TIMP-2, and MT1-MMP is co-regulated, sharing specific similarities in their promoter regions (Lohi *et al.* 2000)

Gelatinases as regulators of angiogenesis and apoptosis

Gelatinases MMP-2 and -9 are able to degrade all basement membrane components, including its main component, type IV collagen. Therefore they have been implicated as proangiogenic proteins in tumour models. Evidence exists from studies where MMP-2 and -9 activities were blocked by either anti-MMP-2 vaccination (Su *et al.* 2003) or upregulation of expression of RECK (endogenous inhibitor of gelatinases) (Takahashi *et al.* 1998), both resulting in antiangiogenic activities. In MMP-2 knockout mice, for example, a suppression of tumour-induced angiogenesis has been shown, suggesting a central role for tumour-induced stromal production of MMP-2 in tumour neovascularization (Itoh *et al.* 1998; Takahashi *et al.* 2002). MMP-9 can favour angiogenesis by increasing the bioavailability of VEGF or by activating TGF-β, both leading to promotion of angiogenic activity (Yu and Stamenkovic 2000; Bergers *et al.* 2000).

The proteolytical activity of gelatinases can also have anti-angiogenic activities by releasing inhibitors of angiogenesis in the proteolysis. MMP-2 is

able to cleave plasminogen, producing angiostatin that is a potent inhibitor of angiogenesis (O'Reilly *et al.* 1999). Thus the net effect of gelatinases on the angiogenesis might be pro- or antitumorigenic, depending on which enzyme cascades are activated.

A similar dual role of regulating apoptosis has been suggested for gelatinases, some of which are physiological, some occur during malignant processes. Both gelatinases and other MMPs have been suggested to increase or decrease apoptosis, depending on the cell types studied and their environmental conditions (Mannello *et al.* 2005). In a mammary tumour regression model the activities of MMP-2 and -9 were found to increase in parallel with a peak in apoptotic activity. This was hypothesized to be due to MMP-induced proteolysis causing loss of contact with the basement membranes, leading to increased apoptotic signalling. (Simian *et al.* 2006.) A similar result has been obtained in a melanoma model (Pereira *et al.* 2005).

Hormonal regulation of gelatinases

Gelatinases have also been implicated in studies examining hormonal regulation of breast or endometrial cancer. *In vitro* models using transfection of ER or PR receptors have suggested that transfecting MDA-MB231 human breast cancer cell line with ERβ receptor could increase proliferation and invasion, and a parallel rise in MMP-9 levels has been observed (Hou *et al.* 2004). A similar observation of a rise in invasion potential together with MMP-9 has been made in Ishikawa human endometrial adenocarcinoma cells after transfection with ERα and oestrogen treatment (Mizumoto *et al.* 2002). When an MDA-MB231 cell line was transfected with PR receptor and then subjected to progesterone, a decrease in invasion and MMP-9 production was seen simultaneously (Sumida *et al.* 2004). A similar observation of a simultaneous decrease of invasion potential and MMP-9 levels was made on Ishikawa cells after transfection with the PRB receptor (Saito *et al.* 2004).

Studies on oestrogen metabolism have implicated that breast carcinoma favours cumulation of 2- and 4-hydroxyoestradiol (2- and – 4OHE), and these metabolites are, through production of free radical compounds of oxygen, capable of activating MMPs. *In vitro* it has been shown that 4-OHE can activate proMMP-2, and simultaneously the invasion of MDA-MB231 cells is enhanced up to 3-fold (Paguette *et al.* 2003; Paguette *et al.* 2005). In contrast, oestrogen has been shown to reduce MMP-2 and –9 production in a T47D human breast cancer

cell line at physiological, but not at growth-stimulating concentrations (Philips and McFadden 2004). It has also been suggested that raloxifen is able to inhibit gelatinase production in MCF-7 human breast cancer cell line (Wolczynski *et al.* 2001). *In vivo* microdialysis technique of MCF-7 ER and PR positive tumours in a mouse model has suggested that tamoxifen together with oestradiol stimulates MMP-2 and -9 production, and following this, stromal production of endostatin is increased, leading to a decrease in neovascularization of the tumour (Nilsson and Dabrosin 2006).

Some evidence on circulating MMP-levels in women using different types of HRT has implicated some type of hormonal regulation of gelatinases. In a study by Lewandowski *et al.* (2006) patients were given tibolone, transdermal oestradiol, conjugated equine oestrogens (CEE) or placebo. The CEE group had higher circulating MMP-2 and -9 and the tibolone group had lower MMP-9 levels than the placebo group.

Gelatinases A and B in solid tumours

Traditionally, gelatinases have been viewed as proteinases that break down basement membrane components and therefore assist tumour cells in invasion and metastasis (Liotta *et al.* 1980; Turpeenniemi-Hujanen *et al.* 1985). Since then, a connection between gelatinase expression, ECM breakdown and tumour invasion has been evident in numerous studies.

Several studies have shown that increased gelatinase expression relates to cancer cell invasiveness. Gelatinase activity has been localized to the leading edge of the primary tumour, indicating enhanced cell motility and invasion (Lynch and Matrisian 2002; Björklund and Koivunen 2005). On the other hand, cancer cells have been shown to be able to induce gelatinase expression as a host cell response in co-culture studies and in vivo (Westerlund *et al.* 1997; Mook *et al.* 2004; Stuelten *et al.* 2005). Escape of metastatic cells, intravasation and extravasation are rate-limiting steps of metastasis formation. The significance of gelatinases in this metastatic cascade is not clear (Deryugina and Quigley 2006).

In breast carcinoma, *in vitro* models have demonstated more aggressive breast cancer cell lines to have enhanced gelatinase expression in concordance with increased invasion in mouse mammary tumours (Tester *et al.* 2000) and human breast cancer cell lines (Bachmeier *et al.* 2001; Hagemann *et al.* 2004). In addition, it has been shown that culturing normal myoepithelial cells with breast cancer cell lines reduces their invasive potential, in concordance with a reduction

of gelatinase expression in both cancer cells and adjacent stromal fibroblasts (Jones *et al.* 2003). Decreased gelatinase expression has also been observed in parallel with increasing cell density in breast cancer cell lines (Bachmeier *et al.* 2005).

In vivo models have shown that transfection of proMMP-2 into an MDA-MB-231 cell line that lacks MMP-2 expression but expresses MT1-MMP increases the invasive potential. When inoculated into nude mice, the transfected clones grew faster and produced more distant metastases when they were injected intravenously (Tester *et al.* 2004). An experimental rat breast cancer brain metastasis model has shown enhanced expression of gelatinases and MMP-2 activity in brain neoplastic cells compared with normal brain tissue (Mendes *et al.* 2005).

Tumour tissue expression of gelatinases as prognostic markers

Several clinical studies have demonstrated a correlation of tissue gelatinase expression and a more aggressive phenotype and worsened survival in cancer types such as melanoma (Väisänen *et al.* 1998, 1999), head and neck SCC (Franchi *et al.* 2002; Ruokolainen *et al.* 2005b), urothelial and renal cell carcinomas (Kamiya *et al.* 2003; Vasala *et al.* 2003, Kawamura *et al.* 2004), endometrial (Aglund *et al.* 2004; Honkavuori *et al.* 2007) and ovarian (Garzetti *et al.* 1995, Westerlund *et al.* 1999) carcinomas. Additionally, the significance of gelatinase expression might vary according to tumour stage, as it seems to be the case with MMP-9. Contradictory evidence exists concerning the favourable or unfavourable effect of MMP-9 on the survival in breast carcinoma (Rahko *et al.* 2004; Scorilas *et al.* 2001), and in melanoma, enhanced MMP-9 expression is found in early, but not later stages of tumour formation (van der Oord *et al.* 1997). A similar early effect has been suggested for MMP-2 in melanoma formation (Väisänen *et al.* 1996).

In breast carcinoma, MMP-2 and MMP-9 have been shown to correlate with tumour progression and survival. In studies using clinical material, MMP-2 has been implicated as a strong predictor of survival, originally postulated in a study by Talvensaari-Mattila *et al.* (1998), and later confirmed in larger series (Talvensaari-Mattila *et al.* 2003; Sivula *et al.* 2005). In a subgroup of postmenopausal, node-positive patients, high tumour tissue MMP-2 has been implicated as a predictive marker of endocrine treatment failure (Talvensaari-Mattila *et al.* 2001), and in a subgroup of young patients, age less than 40 years

and tumour MMP-2 immunoreactivity were found to be the most powerful prognostic tools (Talvensaari-Mattila *et al.* 1999). In a node-negative subgroup, MMP-2 appears as a less effective prognostic factor (Hirvonen *et al.* 2003).

Clinical studies using tumour specimens have so far presented inconclusive data on the significance of MMP-9. It has been suggested that MMP-9 has only modest prognostic value in breast carcinoma in a study by Rahko *et al.* (2004). Worsened survival was presented only in a patient subpopulation with ER-negative and MMP-9 positive tumours. Another study suggested that MMP-9 is an independent favourable prognostic factor in a subpopulation of node-negative patients (Scorilas *et al.* 2001). Recently it has been suggested that MMP-9 expression is enhanced in malignant tumours compared with benign breast tumours (Jinga *et al.* 2006). However, the prognostic significance might vary according to the origin of the MMP-9 protein. In a study by Pellikainen *et al.* (2004) it was shown that positive MMP-9 immunoreactivity in the adjacent stromal cells of breast tumours predicts shorter recurrence-free survival, whereas carcinoma cell MMP-9 positivity was associated with a more favourable survival (Pellikainen *et al.* 2004).

Circulating gelatinases as prognostic markers

The potential of circulating gelatinases as prognostic markers in solid tumours has been under evaluation during the last few years. Several studies have reported generally higher levels of circulating gelatinases in patients compared with healthy controls (Sheen-Chen *et al.* 2001; LaRocca *et al.* 2004; Staack *et al.* 2006)

Recently Somiari *et al.* (2006) suggested that plasma gelatinase concentration could be used to distinguish patients with a benign breast disease from those who have a high risk of developing breast cancer, or from breast cancer patients. In breast carcinoma, some studies exist that have found a correlation between serum gelatinases and clinico-pathological parameters. One study on 80 preoperative serum samples indicated a correlation between serum MMP-2 and -9 and c-erbB2 expression of the tumour, and an inverse correlation to ER and nuclear grade of the tumour (La Rocca *et al.* 2004). In another small study of 57 breast cancer patients a correlation was reported between high preoperative serum MMP-2 and advanced disease stage (Sheen-Chen *et al.* 2001). The studies on the relevance of circulating gelatinases as prognostic markers for survival are few. One study reported elevated postoperative serum MMP-2 levels to correlate with adverse

prognosis (Leppä *et al.* 2004). Two studies have reported on the significance of MMP-9 on breast carcinoma prognosis. One suggested that elevation of plasma MMP-9 in the follow-up could predict systemic relapses earlier than traditional detection methods (Ranuncolo *et al.* 2003). Another suggested that low serum MMP-9 indicates shortened relapse-free survival in breast carcinoma (Talvensaari-Mattila and Turpeenniemi-Hujanen 2005b). Recently, a study of 81 patients failed to show any prognostic potential on the circulating gelatinases, but found a correlation of HER-2 overexpression and an increase in plasma MMP-2 activity (Decock *et al.* 2005)

Few studies exist on the clinical relevance of circulating gelatinases in other malignancies. In colorectal cancer no correlation of preoperative serum free MMP-2 level to clinical stage was found, but the patients in the highest quartile of serum MMP-2 had shorter survival in comparison with patients with lower levels of MMP-2 (Oberg *et al.* 2000). In another study on colorectal carcinoma, a positive correlation of proMMP-2 plasma levels with lymph node status was found. However, here tumour-class (T) demonstrated an inverse correlation with proMMP-2, so that lower T-class tumors had elevated levels of proMMP-2 in comparison with T4 tumours. In this work, no correlation with survival was found (Langenskjöld *et al.* 2005). In lung cancer a correlation with high stage and high serum proMMP-2 was demonstrated, but this work failed to show any correlation with survival (Sasaki *et al.* 2002). In urothelial carcinomas a correlation with elevated proMMP-2 levels in patients' serum or plasma and tumour recurrence has been shown (Gohji *et al.* 1996; Staack *et al.* 2006).

In head and neck SCC high preoperative serum proMMP-9 was shown to associate with worsened relapse-free and overall survival (Ruokolainen *et al.* 2005b). In lung carcinoma, different subtypes were shown to have different plasma MMP-9 levels, so that adenocarcinoma patients had lower levels than did patients with SCC or large cell carcinoma (Iizasa *et al.* 1999).

Several preanalytical factors have been discussed in measuring metalloproteinase concentrations in blood components, the majority of which concern the analysis of proMMP-9. It has been shown in several studies that the serum has higher levels of MMP-9 than do plasma samples. This has been shown by both ELISA and gelatin zymography (Jung 2005; Jung *et al.* 2001; Gerlach *et al.* 2005; Gerlach *et al.* 2007; Makowski *et al*. 2003; Mannello 2003a; Mannello *et al.* 2003b).

The use of a proper sample type in measuring MMP-9 from blood is discussed in many studies, and some suggest that citrate plasma might be the

safest option (Mannello 2003a; Mannello *et al.* 2003b), suggesting that serum should not be used at all in these measurements. Platelets physiologically contain MMP-9, and it has been shown that platelet aggregation during clotting can lead to increased release of MMP-9 (Sheu *et al.* 2004). It has been suggested that coagulation accelerators used in serum tubes could enhance this process (Jung *et al.* 2001; Jung 2005)

Detectable levels of proMMP-9 have been shown to decrease in citrate plasma samples over time (Rouy *et al.* 2005), but the activity of MMP-9 measured by gelatin zymography has been shown not to be affected by storage temperature -20 or -70º, or by repeated freezing-thawing (Souza-Tarla *et al.* 2005).

MMP-2 is less affected by different preanalytical factors than does MMP-9, but several factors still exist that can alter the MMP-2 concentration in blood. The use of EDTA in blood samples might alter the measured MMP-2 levels, since EDTA is able to chelate Zn2+, possibly leading to lower measured proMMP-2 levels (Imafuku *et al.* 2002), and in some studies (Jung *et al.* 1998; Mannello *et al.* 2003b) lower levels of proMMP-2 immunoreactive protein have been observed in EDTA plasma. It has also been shown that during aggregation, platelets release MMP-2 in its latent form (Sawicki *et al.* 1997), as well as other components of the proMMP2/MT1-MMP/TIMP-2 system (Kazes *et al.* 2000).

2.3.2 Tissue inhibitors of metalloproteinases

Structure, regulation and functions

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs, consisting of 184-194 amino acids. Four mammalian TIMPs are known to exist. The N-terminal end of TIMP is responsible for the MMP inhibitory activity by binding to the haemopexin domain of MMPs. TIMPs are capable of inhibiting most MMPs, but TIMP-1 is a poor inhibitor of MT1-MMPs and MMP-19. (Fassina *et al.* 2000; Nagase *et al.* 2006).

The physiological role of TIMPs is complex and includes a wide range of functions. TIMP-1 and -2 are expressed in several different tissues, whereas the expression of TIMP-3 and -4 is more tissue-specific. TIMP-3 expression has been found mainly in the kidney, lung and brain in mouse studies (Leco *et al.* 1994), and it has been suggested that it has a critical role in eye development (Weber *et*

al. 1994). TIMP-3 expression is rare in human tumours (Fassina *et al.* 2000). TIMP-4 has been shown to be expressed in heart, and low levels have been observed in kidneys, placenta, colon and testes (Greene *et al.* 1996). TIMP-1 and -2, in addition to their MMP inhibitory activities, have displayed growth factor-like capacities (Hayakawa *et al.* 1992; 1994).

TIMP-1 is located in the X-chromosome and is encoded as a 0.9kb mRNA. The protein consists of 184 amino acids with 12 cysteine residues that form six disulfide bonds. TIMP-1 is a soluble, glycosylated protein, and its molecular mass ranges from 28.4 to 34 kDa, depending on the glycosylation. TIMP-1 is mainly involved in the regulation of proMMP-9, being able to bind to the haemopexin domain and slowing down its activities. (Fassina *et al.* 2000; Nagase *et al.* 2006)

TIMP-2 localizes to chromosome 17 and it is a soluble, unglycosylated protein, whose molecular mass is about 21 kDa. TIMP-2 can bind to the haemopexin domain of proMMP-2 and modulate both its activation and inhibition (Fassina *et al.* 2000; Nagase *et al.* 2006). Low concentrations of TIMP-2 have been associated with MMP-2 activation, and high concentrations with inhibition of MMP-2 (Kinoshita *et al.* 1998; Kurschat *et al.* 1999).

The expression of TIMP-1 is controlled at several levels including transcription, mRNA stability, degradation and endocytosis. For example phorbol esters, interleukin 1beta, TGFbeta and retinoids have been shown to up-regulate TIMP-1 expression. The TIMP-1 gene promoter region contains AP-1, SP-1, Ets sites as well as a TPA-responsive element. TIMP-2, in contrast, is constitutively expressed, and can be downregulated by TGF-beta (Fassina *et al.* 2000; Nagase *et al.* 2006). The localization, structure and main functions are summarized in Table 3.

Table 3. The structure, expression profiles and main functions of TIMPs.

Protein feature	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Chromosomal localization	Xp	17q	22q	3p
mRNA (kb)	0.9	3.5 – 1.0	5.0	1.4
Molecular mass (kDa)	28.5	21	22 and 27	22
Protein localization	soluble	soluble	matrix-associated	soluble
Protein expression	inducible	constitutive	inducible	inducible
Associates with	proMMP-9	proMMP-2	proMMP-2/-9	proMMP-2
Growth signalling	↑	↑	not known	↑ or ↓
Angiogenesis regulation	↑ or ↓	↓	↓	↓
Apoptosis regulation	↓	↓	↑	↓

(reproduced from Jiang *et al.* 2002; Lambert *et al.* 2004; Stetler-Stevenson & Seo 2005)

Roles of TIMPs in cell-growth signalling, angiogenesis and apoptosis

TIMP-1 was first found as a protein with erythropoetin (EPO)-potentiating effect on erythrocyte progenitor cells (Hayakawa *et al.* 1992). Later TIMP-1 was discovered to produce growth-stimulating effects on a wide range of cells, including keratinocytes, chondrocytes, fibroblasts, epithelial and endothelial cells, as weel as lymphoid and myeloid cells (Lambert *et al.* 2004). TIMP-2 has been implicated in the proliferation of fibroblasts, fibrosarcoma cells, and osteosarcoma cells (Lambert *et al.* 2004). These growth-promoting activities have been suggested to occur independently of the MMP inhibition. It has been shown that alkylated forms of TIMP-1 and -2 that lack the MMP inhibitory capacity are still able to promote growth signalling (Hayakawa *et al.* 1994). Conflictingly, it has also been proposed that the proliferative activity of TIMP-1 is dependent on MMP inhibitory activity in breast carcinoma cell line MDA-MB-435, since synthetic MMP inhibitors have produced similar proliferative effects as TIMP-1 (Porter *et al.* 2004). In contrast, TIMP -1 and -2 have also been described to inhibit the proliferation of carcinoma cells (Miyake *et al.* 1999; Guedez *et al.* 2001)

Angiogenesis regulation was first connected to TIMPs in a study utilizing chick embryo yolk-sac membranes and induced angiogenesis that TIMP-1 and -2 were able to inhibit (Takigawa *et al.* 1990). It has been suggested that TIMPs can suppress angiogenesis in at least two ways: by inhibiting MMP-mediated angiogenesis promotion, or by direct suppression of endothelial cell proliferation. The latter mechanism has been proposed for TIMP-2. (Stetler-Stevenson and Seo 2005) Also TIMP-3 and -4 are implicated as angiogenesis regulators (Handsley and Edwards 2005)

A growing body of evidence suggests that TIMPs are apoptosis regulators. TIMP-1 has been implicated in many studies suggesting that TIMP-1 mediated inhibition of apoptosis occurs independently of MMP inhibition (Guedez *et al.* 1998; Mannello and Gazzanelli 2001; Lambert *et al.* 2004). TIMP-2 and TIMP-4 have also been shown to suppress apoptosis, whereas TIMP-3 has been shown to enhance apoptotic activity (Mannello and Gazzanelli 2001; Lambert *et al.* 2004).

The studies on TIMP-1 regulating apoptosis in breast epithelial or breast carcinoma cells have shown TIMP-1 to inhibit intrinsic apoptotic cell death, involving FAK, PI-3, and MAPK signalling pathways (Liu *et al.* 2003, Lee *et al.* 2003), and to protect breast epithelial cells from extrinsic TRAIL-induced apoptotic pathway (Liu *et al.* 2005). Some studies have depicted bcl-2

involvement in TIMP-1-mediated apoptosis inhibition (Li *et al.* 1999, Guo *et al.* 2006). The detailed mechanism of apoptosis inhibition through TIMP-1 is still not fully understood. Some studies have described it to be independent of MMP-inhibition (Liu *et al.* 2003, Guo *et al.* 2006), while some describe it as occurring via MMP-inhibition (Murphy *et al.* 2002).

TIMPs as prognostic markers in solid tumours

The first suggestions on TIMP activities were strictly based on the proposition that they inhibit MMP activities and therefore restrict tumour growth and invasion. Several studies have clearly demonstrated this relationship (Albini *et al.* 1991; Khokha 1994; Wang *et al.* 1997; Valente *et al.* 1998; Baker *et al.* 1999)

Synthetic MMP inhibitors were designed based on this hypothesis. First generation molecules had a poor bioavailability, but second- and third generation molecules marimastat, prinomastat, tanomastat and neovastat have been tested in phase III trials of advanced pancreatic, gastric, non-small cell lung cancer and in glioblastoma, ovarian, and prostate cancer. They have been tested as single agent versus placebo, and also with chemotherapeutic agents such as gemcitabine, carboplatin, and cisplatin. Most trials have failed to show any benefit, and some have been stopped at side-effects. In some trials, even poorer survival among MMP-inhibitor treated patients were seen. (Coussens *et al.* 2002) In some animal models these synthetic inhibitors have promoted liver metastases (Kruger *et al.* 2001). The speculation on the causes of the failure of MMP inhibitors in clinical trials is ongoing, and some suggest that the usage of these molecules as a single agent is not effective and they should be tested in combination with chemotherapeutic agents. These trials are ongoing. Blockage of some MMP activities might also lead to tumour progression or other very detrimental side effects, of which severe musculoskeletal side effects have been often reported. (Overall and Kleifeldt 2006a, 2006b)

However, today the multiple nature of TIMPs is acknowledged, and a summary of different TIMP activities might finally determine whether the net effect of TIMPs is pro- or antitumorigenic.

The prognostic value of TIMPs in human tumours has produced surprising results. In many solid tumours tissue expression of TIMP-1 has correlated with a more aggressive phenotype and worsened survival. These studies include urothelial (Kallakury *et al.* 2001) and lung carcinomas (Gouyer *et al.* 2005). In breast carcinoma, TIMP-1 is implicated in many studies showing correlation of

enhanced tumour tissue TIMP-1 expression to worsened survival (Hansen Ree *et al.* 1997; McCarthy *et al.* 1999; Nakopoulou *et al.* 2002b). These results have recently been confirmed in a large study population comprising of nearly 3,000 patients (Schrohl *et al.* 2004). In that study, TIMP-1 was evaluated from tumour tissue extracts of primary breast tumours using ELISA analysis. It was shown that high tumour content of TIMP-1 was associated with shortened recurrence-free- and overall survival, and TIMP-1 was an independent prognostic variable for recurrence (Schrohl *et al.* 2004). It has also been suggested that the uncomplexed fraction of TIMP-1 further improves the prognostic value obtained by TIMP-1 assays (Würtz *et al.* 2005a). Conflictingly, TIMP-1 has also been reported to correlate inversely with survival in breast carcinoma (Inoue *et al.* 2000; Nakopoulou *et al.* 2003).

Like TIMP-1, TIMP-2-studies have given conflicting results when evaluated as a prognostic factor in breast carcinoma. Some studies have found a correlation between high tumour tissue TIMP-2 expression and a shortened survival (Visscher *et al.* 1994; Remacle *et al.* 2000), but in a study by Nakopoulou *et al.* (2002a) an inverse correlation of TIMP-2 and survival was found in breast carcinoma.

TIMP-3 and -4 have also been suggested as prognostic factors. Reduced TIMP-3 expression has recently been suggested to have an unfavourable impact on breast carcinoma prognosis (Mylona *et al.* 2006). However, the results concerning TIMP-3 or -4 in clinical material are still few.

TIMPs have also been proposed as predictive markers. Recently it has been hypothesized that the capability of TIMP-1 to inhibit apoptosis might lead to tumour resistance to cytotoxic drugs that work by inducing apoptosis, and some evidence has supported this (Schrohl *et al.* 2006, Davidsen *et al.* 2006). A recent report suggests that elevated serum TIMP-1 predicts decreased response for hormone therapy in metastatic breast cancer (Lipton *et al.* 2007). Additionally, tumour tissue TIMP-3 has been suggested as predictive marker of endocrine therapy success in breast carcinoma (Span *et al.* 2004).

Circulating TIMPs as cancer biomarkers

Circulating TIMPs have been under intensive research during the last few years. It has been suggested that patients with cancer have generally higher levels of circulating TIMP-1 than healthy controls (Holten-Andersen *et al.* 1999). In breast, colorectal and head and neck carcinomas, circulating preoperative TIMP-1 has

been suggested as a prognostic marker (Holten-Andersen *et al.* 2000; Talvensaari-Mattila and Turpeenniemi-Hujanen 2005a; Ruokolainen *et al.* 2005a), and in colorectal cancer, also postoperative plasma TIMP-1 has been associated with survival (Holten-Andersen *et al.* 2006). In ovarian cancer, it was recently suggested that preoperative serum TIMP-1 could be predictive of the surgical result, so that high TIMP-1 was associated with larger residual tumours (Rauvala *et al.* 2006).

Circulating TIMP-2 has been proposed as a predictor of survival in bladder cancer (Staack *et al.* 2006; Vasala and Turpeenniemi-Hujanen 2007). Here, elevated serum TIMP-2 levels were found to associate with favourable survival. However, the data on the significance of circulating TIMP-2 are still few.

However, as for gelatinases, many preanalytical pitfalls exist when determining TIMP concentrations from circulation. Platelets and neutrophils physiologically contain TIMP-1, and during aggregation, the release of TIMP-1 could cause artefactially elevated TIMP-1 levels in the serum (Holten-Andersen *et al.* 2002; Sheu *et al.* 2004). However, plasma has been suggested to be used in measuring TIMP-1 in the blood. It has also been suggested that TIMP-1 levels are affected by the use of different blood coagulation activators or anticoagulants (Jung 2005). TIMP-1 appears to be less affected by long storage or repeated freezing and thawing than for example MMP-9 (Holten-Andersen *et al.* 2003; Rouy *et al.* 2005).

3 Aims of the present study

Breast cancer is a heterogeneous disease, and its prognosis varies accordingly from excellent to very poor. There are several prognostic factors on which the clinical decision-making is based. Today, the role of biological prognostic factors is increasing, and it will probably soon exceed that of the traditional prognostic markers. According to current treatment recommendations, the majority of the patients are offered adjuvant chemotherapy that can be very toxic and costly. Only about 15% of the node-negative patients benefit from adjuvant chemotherapy, but no markers exist at yet that would help identify these patients. On the other hand, some patient groups experience early systemic relapses and have a very poor prognosis, and they should be more efficiently identified.

In earlier studies, the significance of high tumour expression of a basement-membrane degrading enzyme, MMP-2, as a marker of worsened prognosis in breast carcinoma has been clearly shown. In addition, the significance of TIMP-1 as a prognostic marker as been suggested, but results have been conflicting.

The specific aims of this study were:

1. To evaluate the significance of the tissue expression of tissue inhibitors of metalloproteinases (TIMPs) -1 and -2 as prognostic markers in primary breast carcinoma.
2. To examine the prognostic and predictive value of plasma TIMP-1 in a modern breast cancer population in a prospective follow-up study.
3. To validate the result on the prognostic value of circulating serum TIMP-1 and to compare it with tumour tissue expression.
4. To evaluate different forms of the circulating serum MMP-2 and their correlations with disease course.
5. To investigate the relationship of the gelatinases and their inhibitors in different blood components and sample types in order to avoid misinterpretation of the results on the circulating metalloproteinases and their inhibitors.

4 Materials and methods

4.1 Patients

The patient population consists of a total of 416 women diagnosed with primary breast carcinoma in the Northern Ostrobothnia Hospital district during 1989-2006. Three separate subpopulations of this material exist, the first consisting of tissue material of 132 primarily node-positive patients diagnosed during 1990-1995, analysed in original article I. The second subpopulation consists of 71 patients with primary breast carcinoma diagnosed during 1989-1990, of whom both serum and tissue materials have been analysed in original articles II and IV. The third subpopulation consists of a prospective consecutive series of 213 primary breast carcinoma patients diagnosed in 2003-2006 in Oulu University Hospital, whose serum and plasma samples are studied in original articles III and V.

Additionally, two different control populations are used in original articles II and III, of which the first one used in article II consisted of 27 healthy women volunteers whose samples were kept in similar conditions as patient serum samples in II. The second one consisted of 26 volunteers, male and female, who gave 4 different blood samples for the analyses conducted in III.

Table 4. Study populations.

Subpopulation	Nr of patients	Study setting	Materials used	Included in original article
1	132	retrospective	tissue	I
2	71	retrospective	tissue and serum	II, IV
3	213	prospective	serum and plasma	III, V
total	416			

The serum/plasma material consists of two separate series, 2 and 3, the former including 71 patients and their preoperative serum samples and the latter including 231 patients, with pre- and postoperative and follow-up serum and plasma samples. Population 3 comprises a study population of 80 patients in which serum and plasma are analysed and compared in original article III.

The tissue material consists of 199 patients with primary breast carcinoma, including subpopulations 1 and 2.

4.1.1 Demographic data according to patient subpopulations

Study population 1 consisted of 132 patients with lymph-node positive primary breast carcinoma diagnosed in the Northern Ostrobothnia Hospital district during 1990-1995. The mean age at diagnosis was 51 years (range 28-83). Seventy-five (56.8%) patients were premenopausal and 57 (43.2%) postmenopausal. The majority of the cases, 109 (82.6%) represented ductal carcinoma, 16 lobular, 2 tubular, 1 anaplastic, 1 mucinous and 1 comedo-type carcinomas. Histological grading was performed in 119 cases (Bloom and Richardson 1957). Nine patients (7.5%) represented histological differentiation grade 1, 61 (51.3%) grade 2, and 49 (34.5%) grade 3. The patients represented stages 2A-3B diseases. Hormone receptor status of the tumour was determined in 95 cases with dextran-coated charcoal assay, in which 73 tumours (76.8%) demonstrated positivity for ER and 65 (68.5%) for PR. Patients were treated with mastectomy and axillary dissection, followed by either chemotherapy for premenopausal, or hormonal therapy for postmenopausal women. The chemotherapy used was classical CMF or FEC and the hormonal therapy was tamoxifen or toremifen. The minimum follow-up time was 60 months, with a median of 76 months (range 60-152). Fifty-four patients (40.9%) developed distant metastasis and 39 died of the disease. Paraffin-embedded tissue material that was obtained in the primary operation was collected from the pathology archives, and clinical data from patient records.

Study population 2 consisted of 71 women with primary breast carcinoma diagnosed in the Oulu University Hospital during 1989-1990. The median age at diagnosis was 55 years (range 25-87). Menopausal status was available for 65 patients, of which 21 (32.3%) were premenopausal and 44 (67.7%) postmenopausal. Histological grade was available for 17 patients. Thirty-four (47.9%) patients represented stage 1 and 37 (52.1%) advanced stage (2A-3B) diseases. Fifty-three (74.6%) patients were node-negative and 18 (25.4%) node-positive. Hormone receptor status was determined for 52 cases by dextran-coated charcoal assay. Thirty-five tumours (67.3%) were positive for ER and 31 (59.6%) for PR. Most patients were treated solely with mastectomy and axillary dissection, none of the patients received adjuvant chemotherapy, and only eight patients received adjuvant tamoxifen for 3 years after operation. The follow-up time for each patient was 120 months at the minimum. Twenty-six patients (36.6%) relapsed and 16 died of the disease. Paraffin-embedded tissue material from the primary operation was obtained from pathology archives, blood samples

were collected preoperatively, and patient records were used to collect clinical data.

Study population 3 consisted of 213 patients with newly diagnosed primary breast carcinoma in the Oulu University Hospital district during March 2003-April 2006. Patient selection system was consecutive, when volunteering. The study design aimed to collect venous blood samples in different phases of disease management and follow-up. The first sample was taken prior to operation, the second post-operatively before adjuvant chemo and/or radiotherapies. The third sample was taken after completion of adjuvant therapies, after which additional samples were taken simultaneously with clinical follow-up visits, approximately once every 4 or 6 months. Preoperative blood samples were available for 195 patients and postoperative samples for 180 patients. Follow-up samples taken 1 year after completion of adjuvant therapies were available for 26 patients.

Patients' median age was 56 years (range 28-87). Menopausal status was known for 200 patients, of which 67 (33.5%) were premenopausal and 133 (66.5%) postmenopausal. The patients' median age at menarche was 13 years, and at menopause 50 years. On average half of the postmenopausal women (50.4%) had used HRT, 19.5% less than 5 years and 30.8% more than 5 years. The majority of the cases represented ductal (75.9%) or lobular (16.0%) infiltrative carcinomas. Other histological subtypes represented were 7 DCIS, 4 tubular, 4 mucinous and 2 papillar carcinomas. Ductal and lobular carcinomas were graded according to Bloom and Richardson. Twenty-eight (13.8%) represented grade 1, 92 (45.3%) grade 2 and 83 (40.9%) grade 3. Eighty-eight (41.3%) patients had stage 1 disease, 79 (37.1%) were stage 2A, 40 (18.8%) were stage 2B, and 9 (4.2%) were stage 3A or B. The majority of the carcinomas displayed positive immunoreactivity for ER (80.6%) and PR (66.0%). Twenty-one carcinomas (10.2%) showed positive HER-2 immunostaining, and the gene amplification was later verified by chromogen *in situ* hybridization (CISH).

The surgical procedure used was mastectomy in 58.9% of the cases and breast-conserving surgery in 41.1% of the cases. Sentinel node biopsy was used as a staging procedure in all cases. Postoperative radiotherapy was administered in 91.7% of the cases. Ninety-two patients were treated with adjuvant chemotherapy; 46.7% received anthracycline-based, 46.7% taxane-based, and 6.5% classical CMF chemotherapy. Adjuvant endocrine therapy was used in 92 cases, of which 53 (57.6%) received tamoxifen, 33 (35.9%) aromatase inhibitors and 6 (6.5%) some other hormonal treatment. Adjuvant trastuzumab was administered in 4 cases. The median follow-up time was 18 months (range 1-42).

Eleven patients (5.2%) experienced recurrence of the disease during the follow-up.

Two control populations were used in original articles II and III. The controls included healthy volunteers, mainly laboratory staff. These control groups were not age-matched.

4.1.2 Collection of blood samples

In patient subpopulation 2, preoperative serum samples were obtained by collecting venous blood into glass tubes with no artificial coagulation activators, allowed to coagulate for 30 minutes at room temperature, centrifuged at 3000rpm for 10 minutes, after which serum was separated, frozen and stored at -20ºC until assayed. Control samples were collected accordingly.

In patient subpopulation 3, two different blood samples were taken, one in a serum tube containing platelet activator, and one in a plasma tube containing K_2EDTA. Venous blood samples were taken and allowed to clot thoroughly for 30 minutes before centrifugation, centrifuged at 3000rpm for 10 minutes, after which serum/plasma was aspirated into polypropylene micro tubes (Sarstedt, Nurnberg, Germany) and stored at -75ºC until assayed. Preoperative samples were taken prior to operation on a same day.

Control samples used in study III were collected into four different test tubes, 2 serum and 2 plasma samples. The serum samples were collected into one glass tube that contained no artificial coagulation activator, and into another plastic serum tube with silicone-coated interior and gel, with added artificial coagulation activator, corresponding to patient serum sample. Plasma samples were collected into one lithium-heparin plastic tube, and into another plastic tube containing K_2EDTA, corresponding to patient samples. The patient samples were collected during March 2003 – April 2006, and control samples during September 2005.

4.2 Immunohistochemistry

4.2.1 Staining protocol

The breast tissue samples from the operation were fixed in formalin and embedded in paraffin. 4µm sections of a representative tumour area of the paraffin-embedded specimen blocks were first incubated for 24 hours in 37°C,

deparaffinized in a clearing agent, Histo-Clear (National Diagnostics, Atlanta, Georgia, USA), and washed in declining alcohol series. Samples were incubated for 20 minutes in 0.3% H_2O_2 absolute methanol for blocking the endogenous peroxidase activity. Monoclonal anti-human mouse IgG-class TIMP-1 or TIMP-2 antibody (R&D Systems, Minneapolis, MN, USA) was diluted to the concentration of 16μg/ml in a diluent that contained blocking serum for non-specific binding (Dako Cytomation, ChemMate^{TM}, DK-2600, Glostrup, Denmark) prior to application on each sample. The incubation was carried out for 12h in a humidity chamber. The staining was then continued with a standard streptavidin-biotin-peroxidase Histostain bulk-kit (Zymed Laboratories Inc., South San Francisco, CA, USA). Aminoethyl carbazole (Romulin AEC chromogen kit, Biocare Medical, Walnut Creek, CA, USA) was used as a substrate for the peroxidase. Previously known positive control samples were used in each set of staining. In negative controls PBS replaced the antibody.

4.2.2 Evaluation of samples

The samples were evaluated with a microscope using 10x, 20x and 40x objectives by two observers blinded for the clinical data, and the results were verified by a pathologist. The samples were considered negative or positive according to the absence or presence of immunoreaction for TIMP-1 or TIMP-2 protein in the cytoplasm of the tumour cells. Positive cases were scored 1-3 according to the extent of the staining. Score 1 represented positive staining in 5-25% of the cells, score 2 in 26-50%, and score 3 in over 50% of the tumor cells, respectively. (I)

4.3 Enzyme-linked immunoassay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) was used to detect the circulating TIMP-1, TIMP-2, proMMP-9, proMMP-2 levels, active MMP-2 levels, and proMMP2-TIMP2 complex levels from serum and plasma samples. Circulating proteins were detected by coating 8-well E.I.A/R.I.A strips for microtiter plates (Corning Inc., Corning, NY, USA) with monoclonal capture antibodies. Diluted serum or plasma samples were then added, followed by polyclonal detection antibodies. Rabbit anti-chicken horseradish peroxidase enzyme (Chemicon International, Temecula, CA, USA) served as the enzyme-labelled antiglobulin, with enzyme substrate o-phenylenediamine dihydrochloride (OPD), (Sigma, Steinheim, Germany). The absorbances were read at 492 nm

wavelength by Anthos Reader 2001. The principle of the ELISA assays done is illustrated in Fig. 3.

A commercial assay kit (Human Biotrak Elisa system for detecting MMP-2 by Amersham Biosciences, Buckinghamshire, England) was used to detect the total and active MMP-2 levels. The assay for total proMMP-2 (RPN 2617) recognized both free proMMP-2 and proMMP2-TIMP2 complexes, whereas the assay used in measuring the active MMP-2 (RPN 2631) recognized only free active forms of MMP-2. The assay was conducted following the manufacturer's instructions.

Each sample was run in duplicate in order to minimize intra-assay variation. The absorbance values for standard samples and the standard curves constructed for each assay were compared and used to minimize the interassay variation. The sensitivity of the assays was 1ng/ml for TIMP-1 and MMP-9, and 2ng/ml for TIMP-2 and the proMMP-2/TIMP-2 complex. Sensitivities for the total proMMP-2 and active MMP-2 were 0.37ng/ml and 190pg/ml, respectively.

Table 5. Monoclonal and polyclonal antibodies used for ELISA analyses

Detected protein	Capture antibody (monoclonal)	Detection antibody (polyclonal)
TIMP-1	anti-TIMP-1 (DB120)*	anti-TIMP-1*
TIMP-2	anti-TIMP-2 (T2-101)*	anti-TIMP-2 (DB-205)*
MMP-9	antiMMP-9 (Ge-213)*	anti-MMP-9 (DB-209)*
proMMP2-TIMP2 complex	anti-TIMP-2 (T2-101)*	anti-MMP-2 (DB-202)*
total proMMP-2**	anti-MMP-2 (RPN 2617)	peroxidase labelled Fab 1 antibody to MMP-2
active MMP-2**	anti-MMP-2 (RPN 2631)	peroxidase labelled Fab1 antibody to MMP-2

*from SBA Sciences, Oulu, Finland

**commercial assay kit by Amersham Biotrak, Buckinghamshire, UK

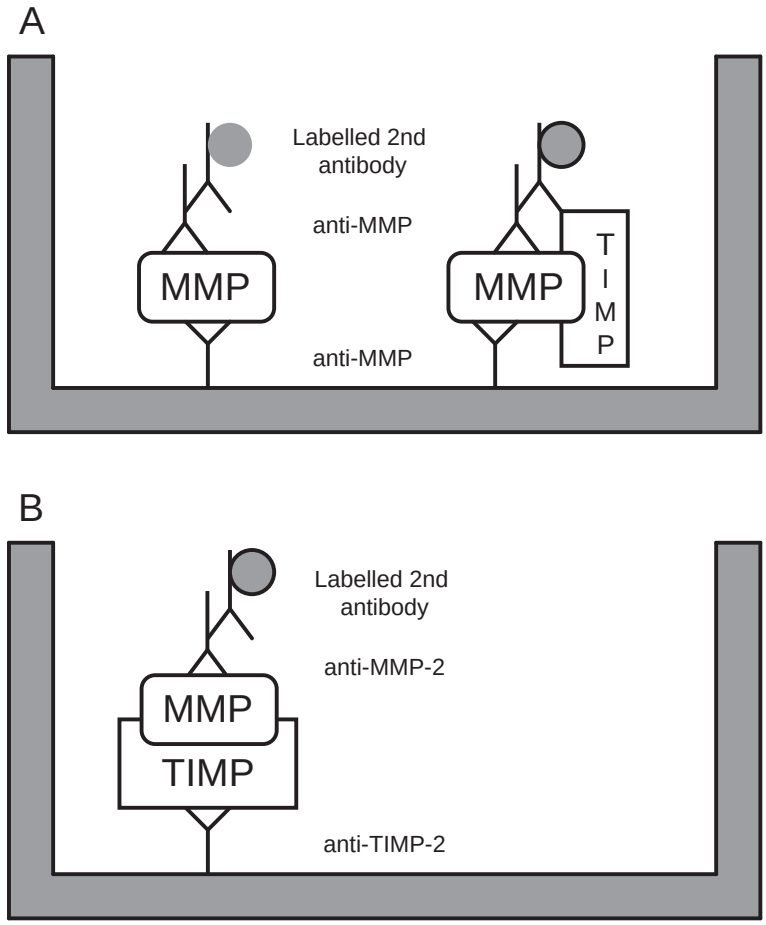

Fig. 3. Principles of ELISA for A) MMP-9, TIMP-1 and TIMP-2, and B) for proMMP-2/TIMP-2-complex assays.

4.4 Statistical analyses

All statistics were computed using SPSS software (Chicago, IL). Two-sided significance tests were used, and P-values less than 0.05 were considered significant. For categorical variables, the associations of clinicopathological parameters with the immunoreactive proteins studied were analysed using chi-squared test. For continuous variables, the tests were selected according to the normality of the distribution that was analysed using Kolmogorov-Smirnov's test.

For normally distributed continuous variables, T-test or one-way ANOVA was used in analysing the differences between groups. For skew distributions, Mann-Whitney U-test or Kruskall-Wallis tests were used. Correlations between two continuous variables were analysed using Pearson or Spearman correlation coefficients.

Lifetime analyses were conducted using Kaplan-Meier analysis and Log-rank or Breslow test for dichotomous variables. Receiving operating characteristics (ROC) curve was used to assess the cut-off points for continuous variables for Kaplan-Meier analyses. Cox regression analysis was used in univariate analysis to assess the hazard ratio for log-transformed continuous or dichotomous variables. Multivariate analysis was used to assess the independency of the prognostic variables. Multivariate analyses were done using Cox regression stepwise analysis. Relapse-free survival was determined as time elapsed from the time of diagnosis to the time of diagnosis of the relapse in months. Disease-specific survival was determined as the time (in months) elapsed from the primary diagnosis to the last clinical control visit, or to the time of death.

4.5 Ethical aspects

The study protocol was accepted by the Ethical committee of Oulu University Hospital. (EETTMK: 17:2002). For retrospective studies, the permission to use blood and tissue samples was obtained from the National Authority for Medicolegal Affairs (Ref.nr. 942/32/300/05). In the cases where the patients' permission to use their clinical data for research purposes was unobtainable due to death or discontinuation of treatment, the permission to use patient records was obtained from the Ministry of Health (STM/378/2005). In prospective series, all patients volunteered for the project, and received and signed an informed consent where they gave permission to use their blood samples and clinical data for research purposes.

The results of this research project have not affected patient care in any way. All data containing names and personal identification numbers have been stored separately from the data used in analyses. All tissue and blood samples have also been coded with numbers with no possibility to identify individual patients and their samples.

5 Results

5.1 Tumour tissue expression of TIMP-1 and TIMP-2 immunoreactive proteins in breast carcinoma (I, IV)

Pooled analysis of subpopulations 1 and 2 (n = 199) was conducted here in order to analyse the tissue TIMP-1 and -2 expression in primary breast carcinoma, and the results are presented in the following.

The expression of TIMP-1 and TIMP-2 proteins was found in 165 (82.8%) and 175 (88.8%) breast tumours, respectively. The expression of TIMP-1 was absent in 34 (17.1%), weak (>25% cells positive) in 44 (22.1%), moderate (25-50% cells positive) in 32 (16.1%) and strong (>50% cells positive) in 89 (44.7%) of the cases. The corresponding data for TIMP-2 were 22 (11.2%) cases with absent, 47 (23.9%) with weak, 27 (13.7%) with moderate, and 101 (51.3%) with strong immunoreaction. (Fig 4)

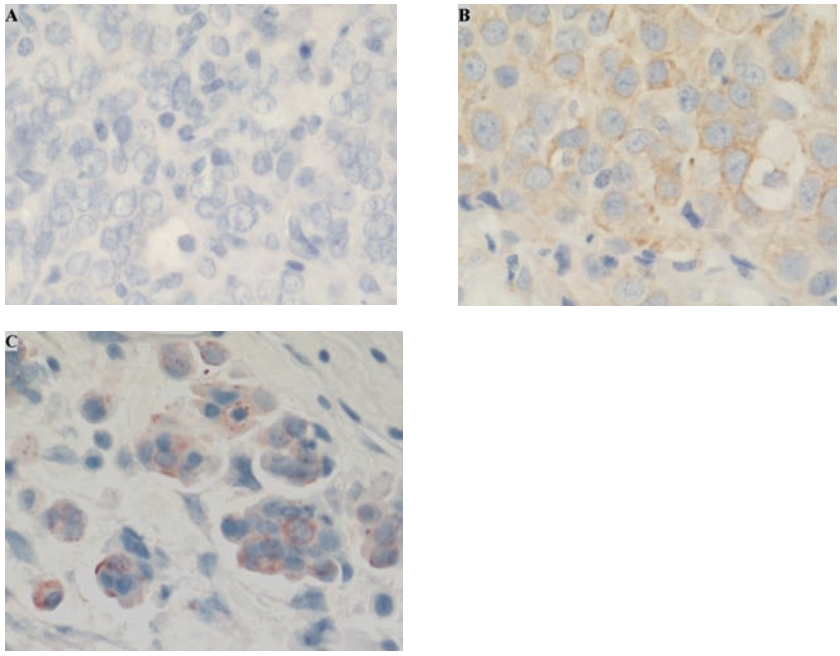

Fig. 4. The staining result for A) negative TIMP-1, B) positive TIMP-1, C) granular positive TIMP-2.

TIMP-1 immunoreactivity was found to associate with the disease-specific survival. When the entire patient material (n = 199) was analysed, the association failed to show any statistical significance, although the patient group with positive tumour TIMP-1 immunoreaction had worse survival (71.4%) than the TIMP-1 negative group (81.8%) (Fig. 5B).

In a subgroup of grade 2-3 tumours (n = 125), the patients with negative TIMP-1 immunoreactivity had an excellent survival of 90.0%, whereas patients with positive tumour TIMP-1 had a survival of only 64.8%. This difference was statistically significant (P = 0.03), giving HR 4.1 (95% CI 1.0 – 17.1) (Fig. 5A). No such associations were found for recurrence-free survival. For TIMP-2, no associations with survival were found.

Cox regression multivariate analysis was conducted to analyse the independency of the prognostic value of TIMP-1. In this material, in addition to TIMP-1 immunoreactivity, tumour size, nodal status and stage were prognostic in the univariate Cox regression analysis. When TIMP-1 was analysed together with these variables, it lost its significance in this material, and hence it was not an independent prognostic marker.

In the pooled analysis TIMP-1 immunoreactivity was found to correlate with histological grade of the tumour (P = 0.015, Table 6), and with TIMP-2 immunostaining (P = 0.008) No other correlations with the clinico-pathological parameters were found for TIMP-1 (Table 6) or TIMP-2 (data not shown).

Table 6. TIMP-1 immunoreactivity according to patient characteristics in the pooled analysis.

Clinical parameter	n	TIMP-1 positive/ (%)	Sig. (P-value)
All	199	165 (82.2)	
T-class			
1	83	66 (79.5)	
2	86	73 (84.9)	
3	17	16 (94.1)	
4	4	3 (75.0)	N.S.
missing	9		
N			
0	46	39 (84.8)	
1	139	115 (82.7)	
2	10	8 (80.0)	N.S.
missing	4		
Stage of the tumour			
I	29	25 (86.2)	
II	143	117 (81.8)	
III	21	18 (85.7)	N.S.
missing	6		
Nuclear grade			
1	9	5 (55.6)	
2	68	53 (77.9)	
3	57	52 (91.2)	**0.015**
missing	65		
ER status			
negative	36	30 (83.3)	
positive	105	83 (79.0)	N.S.
missing	58		
PR status			
negative	48	42 (87.5)	
positive	93	71 (76.3)	N.S.
missing	58		
Menopausal status			
premenopausal	93	76 (81.7)	
postmenopausal	96	82 (85.4)	N.S.
missing	10		

Tumour tissue expression of TIMP-1 and -2 was studied in original article I in a series of 132 node-positive patients (subpopulation 1). Positive intracytoplasmic staining for TIMP-1 immunoreactive protein in tumour cells was found in 107

(81.1%) of the primary tumours. TIMP-2 was found in 111 (84.1%) of the cases (I).

The staining pattern represented diffuse cytoplasmic staining in all TIMP-1 stainings, and in the majority of TIMP-2 stainings. In 4 cases TIMP-2 represented granular cytoplasmic staining (Fig. 4C)

In Kaplan-Meier survival analyses a statistically significant correlation was found between the absence of immunoreaction for TIMP-1 and a more favourable survival in high-grade (grade 2-3) tumours (P = 0.03) (I). A similar trend for a more favourable survival in TIMP-1 negative cases was observed when patients with grade 1 tumours were included, though it was no longer statistically significant (P = 0.07) (I).

Cox regression multivariate analysis was used to further test the prognostic value of tissue TIMP-1 in grade 2-3 tumours. TIMP-1, stage and tumour size were significant in Cox regression univariate analyses. In the multivariate analysis of TIMP-1, stage and tumour size, the relative risk of death for TIMP-1 positivity was 3.9 and the p-value for additional information chi-squared test was 0.021. However, TIMP-1 was not an independent prognostic variable (P = 0.06) (I).There was a positive correlation between high tumour grade (grade 2-3) and TIMP-1 immunoreaction (P = 0.047).

Tumour tissue TIMP-1 and -2 were also analysed in subpopulation 2, where both node-positive and node-negative patients were included. The results of TIMP-1 immunoreactivity were published in the original article IV. Tumour TIMP-1 positivity was observed in 55 cases (85.9%), and TIMP-2 positivity in 61 cases (98.4%). No associations with clinico-pathological parameters or survival were found in this material, although patients with moderate or high tumour TIMP-1 immunoreactivity demonstrated a trend towards worsened survival when compared with patients with negative or weak TIMP-1. The disease-specific survival was 84.0% after 10 years in the low TIMP-1 (weak/ negative immunoreaction) group and 76.3% in the high TIMP-1 (moderate/strong) positive group (data not shown) (IV)

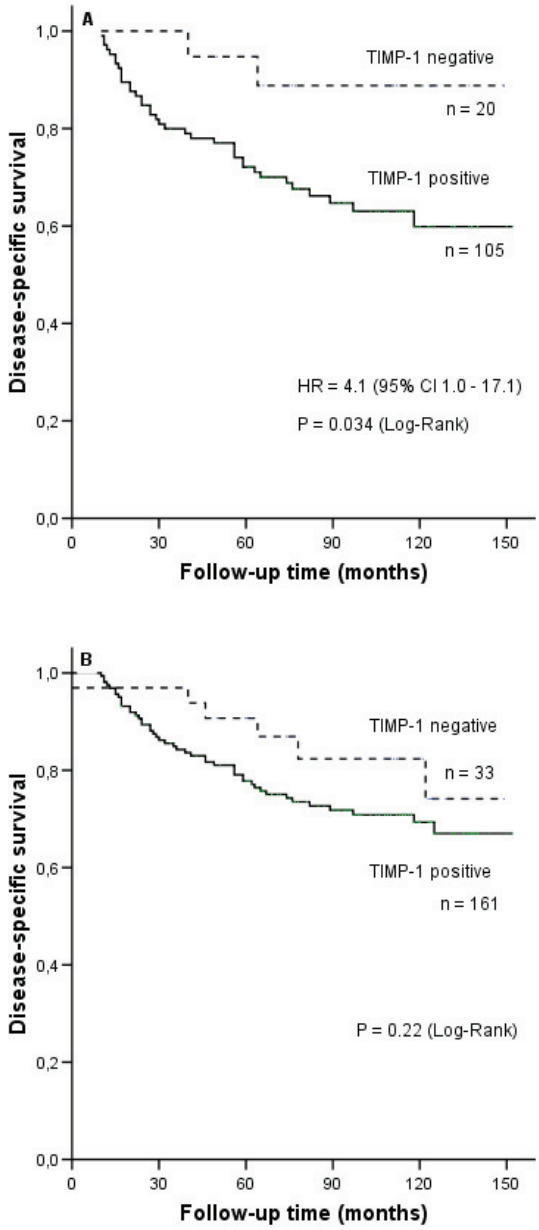

Fig. 5. Pooled survival analysis according to TIMP-1 immunoreactivity in A) grade 2-3 tumours (n = 125) and in B) all cases (n = 199).

5.2 Preoperative serum MMP-2 and TIMP-1 in breast carcinoma: correlations with clinico-pathological parameters and comparison with tumour tissue expression (II, IV)

Different forms of circulating preoperative MMP-2 and TIMP-1 levels were studied in subpopulation 2 and included in original articles II and IV.

The distributions of preoperative serum levels were found to be normal for total proMMP-2 and active MMP-2, and skew for proMMP2-TIMP2 complex, total TIMP-1 and uncomplexed TIMP-1. The means for total proMMP-2 and active MMP-2 were 1971.6 ng/ml and 41.2 ng/ml, respectively, and the medians for proMMP2-TIMP2 complex, total TIMP-1 and uncomplexed TIMP-1 were 520.6 ng/ml, 165.5 ng/ml and 232.3 ng/ml, respectively (Table 7). The levels of total proMMP-2 correlated with proMMP2-TIMP2 with Spearman's rho 0.6 ($p < 0.001$), but the active MMP-2 levels did not correlate with the total serum proMMP-2 levels (Pearson $R = -0.2$). The serum total TIMP-1 correlated with uncomplexed TIMP-1 with Spearman's rho 0.75 ($P < 0.001$).

The serum concentrations of different forms of circulating MMP-2 and TIMP-1 were analysed for correlation with clinico-pathological parameters. Total serum MMP-2 correlated inversely with stage of the disease, nodal status and histological grade of the tumour (Table 8). For active MMP-2 or proMMP2-TIMP2 complex no associations with clinico-pathological parameters were found (data not shown).

Higher total serum TIMP-1 was found to correlate with oestrogen receptor positivity of the tumour ($P = 0.036$) (Table 8). Both total and uncomplexed TIMP-1 levels correlated with patient's age at diagnosis with Spearman's rho 0.358 ($P = 0.003$) and 0.434 ($P < 0.001$), respectively.

Table 7. Preoperative serum MMP-2 and TIMP-1 concentrations.

Measured protein	Nr of samples	Mean/ (ng/ml)*	Median/ (ng/ml)*	Range (ng/ml)
Total proMMP-2	69	1971.6		1060.8–3075.7
ProMMP2-TIMP2 complex	72		520.6	253.2–1126.8
Active MMP-2	69	41.2		14.8–75.0
Total TIMP-1	69		165.5	100.4–384.7
Uncomplexed TIMP-1	68		232.3	149.8–445.5

*According to normality of the distribution

Table 8. Circulating total MMP-2 and TIMP-1 concentrations according to patient characteristics.

Clinical parameter	Number of patients MMP-2/TIMP1	Mean total MMP-2 concentration (ng/ml)	Sig. (P – value)	Median total TIMP-1 concentration (ng/ml)	Sig. (P-value
T-class					
1	33/32	1964		159.6	
2	24/24	1912		170.2	
3	5/6	1766	0.585	157.3	0.12
missing	7/7				
N					
0	50/50	2044		162.8	
1	18/18	1793	**0.042**	173.2	0.749
missing	1/1				
Stage of the tumour					
1	32/31	2155		154.4	
2	32/33	1826		177.0	
3	4/4	1774	**0.008**	157.3	0.426
missing	1/1				
Nuclear grade					
1	1/1	2745			
2	7/7	1806		154.9	
3	9/9	1771	**0.047**	173.6	0.358
missing	52/52				
ER status					
Negative	15/16	1945		154.9	
Positive	34/34	1930	0.909	173.6	**0.036**
missing	20/19				
PR status					
Negative	20/21	1899		147.5	
Positive	29/29	1959	0.620	173.6	0.089
missing	20/19				
Menopausal status					
Premenopausal	20/19	1953		142.1	
Postmenopausal	42/44	2017	0.601	173.3	0.059
missing	7/6				

In survival analyses it was noticed that the patients with lower levels of circulating active MMP-2 had more often relapses than those with higher levels, but the difference was not statistically significant (P = 0.058). The best cut-off value found in ROC analysis for dividing the patients into groups of high or low levels (for Kaplan-Meier analyses) was found to be 40.7ng/ml. At 10 years of

follow-up only 50% of the patients with low levels of circulating active MMP-2 were relapse-free, compared with 74% in the patient group with higher serum levels (P = 0.058) (Fig. 6A). There was a similar trend in the disease-specific survival according to this division, though not statistically significant. In the patient group having low levels of active MMP-2 there were more deaths by the disease, the survival being 68% at 10 years of follow-up. In the group of high levels the survival was better, 83% at 10 years of follow-up (Fig. 6B). For total proMMP-2 or proMMP2-TIMP2 complex no associations with recurrence-free or disease-specific survival were found (data not shown). The survival analyses for circulating TIMP-1 are presented later in 5.4.2.

The circulating levels of different forms of MMP-2 and TIMP-1 were compared with their tissue expression in the primary tumour. The tissue expression of MMP-2 had an inverse correlation with high serum proMMP2-TIMP2 complex, the levels being significantly higher in the group of negative immunoreaction (P = 0.045) (Fig. 7A). For total proMMP-2 the trend was similar, though not significant (Fig. 7B). For active MMP-2 no differences were found according to the MMP-2 immunoreactivity (data not shown). For TIMP-1, there were no significant associations with serum TIMP-1 and tumour TIMP-1 immunoreactivity (data not shown).

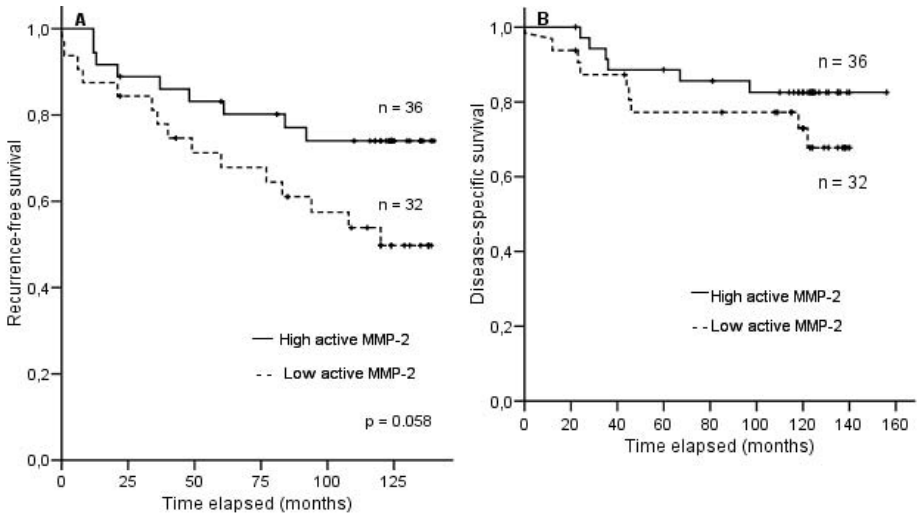

Fig. 6. A) Tumour recurrence and B) disease-specific survival according to high (>40.7ng/ml) or low (<40.7ng/ml) levels of preoperative serum active MMP-2 (II), Copyright from Elsevier

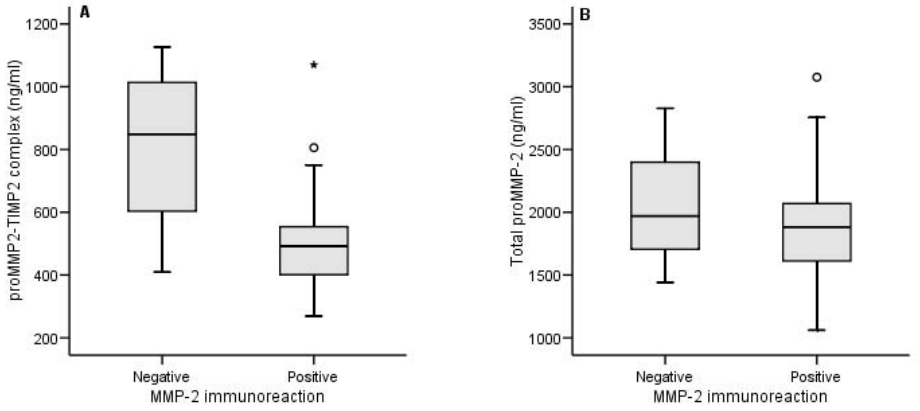

Fig. 7. The levels of A) proMMP2-TIMP2 complex and A) total proMMP-2 according to tissue MMP-2 immunoreactivity. Median for proMMP2-TIMP2 complex in the patient group of negative tumour MMP-2 immunoreactivity (n = 4) was 849.0 ng/ml and in the group of positive tumour MMP-2 (n = 36) 492.1 ng/ml (p = 0.045). Means for total proMMP-2 in the groups of negative and positive MMP-2 immunoreactivity were 2079.9 and 1880.4ng/ml, respectively (not significant) (II). Copyright from Elsevier.

5.3 Comparison of the levels of circulating gelatinases and their inhibitors in patients and controls (II, III)

The levels of circulating gelatinases and their inhibitors in patients and controls were studied in original articles II and III. In subpopulation 2, the levels of total proMMP-2, active MMP-2 and proMMP2-TIMP2 complex in serum were compared between patients and 27 healthy female controls. The patients had significantly higher serum total proMMP-2 ($P < 0.001$), but lower proMMP2-TIMP-2 complex levels than controls ($P < 0.001$). Hence, a higher proportion of circulating MMP-2 was in a TIMP-2-complexed form in healthy controls (Fig. 8).

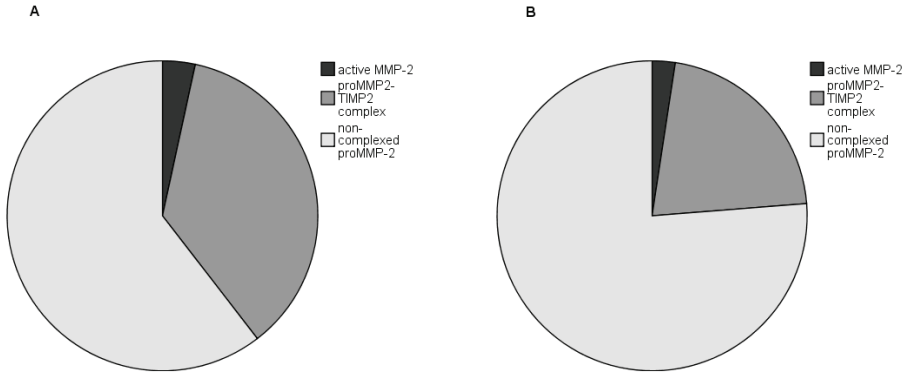

Fig. 8. Molar ratios of different forms of circulating MMP-2 in the serum (II) in A) healthy controls and B) patients. Copyright from Elsevier.

The circulating gelatinases and their inhibitors in patient and control groups were studied in original article III. This included 80 breast cancer patients and 26 healthy volunteers. MMP-9, proMMP2-TIMP2 complex, TIMP-1 and TIMP-2 were assayed and compared. Here, significant differences were found for preoperative plasma and serum MMP-9 and TIMP-2. ProMMP-9 concentrations in both plasma ($P = 0.002$) and serum ($P < 0.001$) were significantly lower in the blood of breast cancer patients than in control samples (Fig. 9A). The protein concentrations in both plasma ($P < 0.001$) and serum ($P < 0.001$) were also lower for TIMP-2 in patient samples compared with controls (Fig. 9B). The ranges for TIMP-1 concentrations were wider in both plasma and serum samples of breast cancer patients compared with healthy controls, although there were no significant differences between patients and controls for TIMP-1 in plasma or

serum (III). For the proMMP-2/TIMP-2 complex no significant differences were found between patients and controls in this material (data not shown).

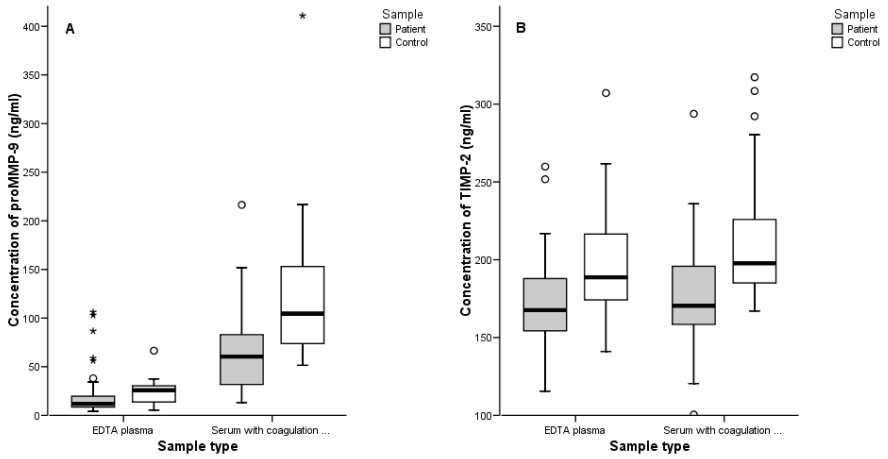

Fig. 9. Circulating plasma and serum levels of A) MMP-9 and B) TIMP-2 in patients and healthy controls.

5.4 Circulating TIMP-1 as a prognostic marker in primary breast carcinoma (IV, V)

5.4.1 Plasma TIMP-1 in pretreatment and follow-up samples (V)

Pre- and postoperative plasma TIMP-1 levels were studied in a population of 231 primary breast carcinoma patients (V). The preoperative and postoperative plasma TIMP-1 levels were not normally distributed. The median pre- and postoperative TIMP-1 levels were 239.2 ng/ml and 231.7 ng/ml, respectively (Table 9).

Preoperative plasma TIMP-1 was found prognostic for relapse. Preoperative TIMP-1 was analysed as log-transformed continuous variable with Cox regression analysis. In the univariate analysis, ln(TIMP-1) was prognostic, giving HR 8.1 (95% CI 1.8 – 37.6) with P-value 0.007. Of the traditional prognostic parameters analysed with univariate Cox regression analysis (stage, nodal status, ER/PR status, grade, HER-2, Ki67, patient's age) only nodal status was prognostic for relapse in this material. When ln(TIMP-1) was analysed together with nodal status in the multivariate analysis, ln(TIMP-1) was found to be an

independent prognostic variable superior to nodal status. The hazard ratio for high ln(TIMP-1) was 8.0 (95% CI 1.7 – 36.4, p = 0.007) and for positive nodal status 3.8 (95% CI 1.0 – 14.4, P = 0.049).

The TIMP-1 concentrations were divided into mathematical quartiles for Kaplan-Meier analyses. Patients belonging to the highest quartile were found to have significantly worse recurrence-free survival (79%) than patients in the lower quartiles (94%) at 40 months of follow-up (p = 0.016, Fig. 10). The receiving operating characteristics curve constructed showed a similar cut-off point 5.82 for ln(preoperative TIMP-1) as the 4th quartile limit 5.83. This cut-off point gave 54% sensitivity and 77% specificity (V) for relapse. In the subgroup analyses the difference was mostly demonstrated in the group of postmenopausal patients (data not shown).

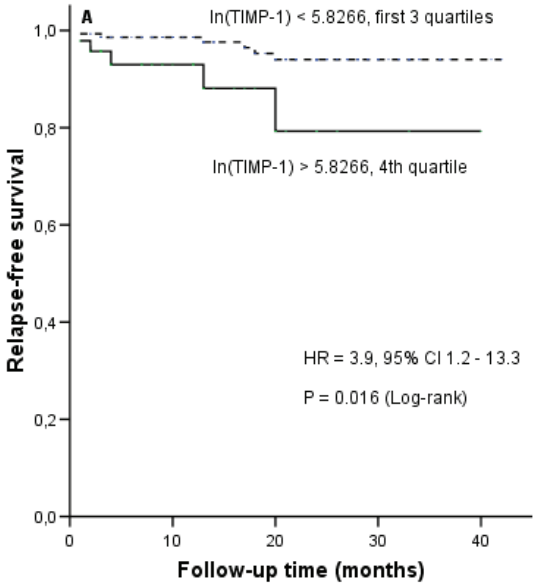

Fig. 10. Survival according to log-transformed preoperative plasma TIMP-1, 4th quartile versus lower quartiles.

Pre- and postoperative TIMP-1 levels had a strong correlation (Spearman's rho 0.747). High pre- and postoperative TIMP-1 levels were found to correlate with postmenopausal status (P < 0.001), and a correlation between patients' age at diagnosis and pre/postoperative TIMP-1 was also observed (Spearman's rho

0.359/0.374). Weak correlation was found between BMI and pre-/postoperative TIMP-1 (Spearman's rho 0.224/0.321). No other associations with clinico-pathological parameters were found for pre- and postoperative TIMP-1 (V).

The surgical operation did not significantly affect the TIMP-1 levels (Table 9). The TIMP-1 levels experienced a slight decrease in follow-up of at least one year, leaving TIMP-1 after follow-up at somewhat lower levels than preoperative TIMP-1 (Table 9, P = 0.04). These alterations (rise/decrease) in TIMP-1 plasma levels after surgery and in the follow-up were not associated with any clinico-pathological parameter studied (data not shown).

Table 9. The distribution of plasma TIMP-1.

Protein	n	median	range	One-sample T-test for normality
Preoperative TIMP-1	195	239.2	89.0–832.3	
Post-operative TIMP-1	180	231.7	136.0–980.9	
Difference after operation*	173	12.1	-249.3–563.2	N.S.
Difference after follow-up**	26	23.1	-96.8–223.4	p = 0.04***

*Computed as preoperative plasma TIMP-1 (ng/ml) – postoperative plasma TIMP-1 (ng/ml).

**Computed as preoperative plasma TIMP-1 (ng/ml) – plasma TIMP-1 after follow up of a minimum of 12 months.

***(95% CI 1.7–56.2)

Six patients with early (<24 months) occurring relapses and available follow-up samples were matched with patients with similar prognostic parameters of primary breast carcinoma inside the same patient material and paired for analysis of plasma TIMP-1 (V). Matching was done according to nodal status, stage, age, histological grade, ER/PR status, histological sybtype and primary treatment given (V). Four patients developed early systemic metastases, and they were observed to have higher preoperative TIMP-1 than did their matched pairs, and all had preoperative TIMP-1 higher than the 4th quartile limit. These patients developing early systemic metastases showed treatment failure and continuous progression (Fig. 11A-D).

Two patients developed isolated bone metastases, and their plasma TIMP-1 levels were both preoperatively and at follow-up at similar or lower levels that those of their matched pairs. These two patients had hormonal treatment for their bone metastases and their disease has shown no progression (Fig. 11E-F).

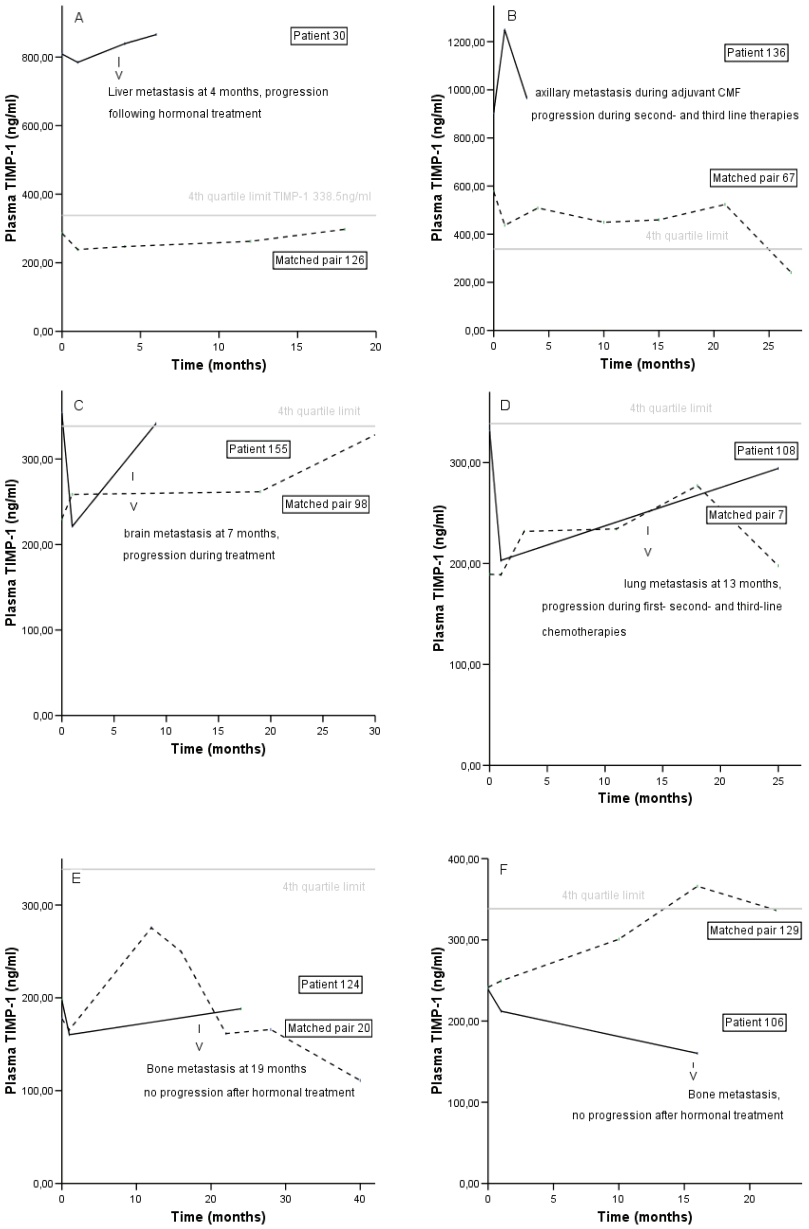

Fig. 11. Plasma TIMP-1 in matched patient pairs with similar prognostic parameters of primary tumour A-D) with had systemic distant metastasis and treatment failure or E-F) isolated bone metastasis and treatment response.

5.4.2 Validation of the prognostic value of serum TIMP-1 in breast carcinoma (IV)

The validity of preoperative serum TIMP-1 was assessed in original article IV. Previously in this material, high serum preoperative TIMP-1 was found prognostic (Talvensaari-Mattila and Turpeenniemi-Hujanen 2005a) for poor survival. In study IV, the preoperative serum TIMP-1 levels were analysed using independent ELISA in another laboratory. In addition, the free fraction of TIMP-1 in preoperative serum was determined.

TIMP-1 was found to maintain its prognostic value when analysed with a different ELISA. There was a correlation between these two ELISA analyses (Spearman's rho 0.39, P = 0.001). Serum preoperative TIMP-1 was analysed by dividing patients into mathematical quartiles according to serum TIMP-1 concentrations. The patients belonging to the highest quartile had shortened disease-specific survival (54%) compared to lower quartiles (86%) (Fig. 12, P = 0.005). When serum TIMP-1 was entered as a dichotomous variable into the Cox regression analysis, it was found to be an independent prognostic variable when analysed together with stage (HR = 3.5, 95% CI 1.3 – 9.8) or nodal status (HR = 3.9, 95% CI 1.4 – 10.8) and superior to nodal status as a prognostic variable. Also tumour TIMP-1 was inferior as a prognostic variable compared to preoperative serum TIMP-1 in this material.

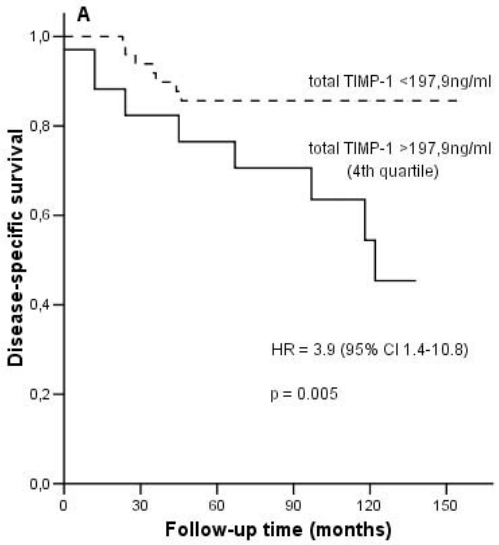

Fig. 12. Survival according to serum TIMP-1 (Copyright from IOSpress).

5.5 Comparison of different sample types in measuring gelatinases and their tissue inhibitors from circulation (III)

The effect of the sample type used in analysing gelatinases and their inhibitors was studied in original article III. The effect of 4 different sample types (2 serum samples and 2 plasma samples) was analysed in a group of twenty-six healthy volunteers. Sample type was found to affect both MMP and TIMP concentrations. Significant differences were observed in the assays for TIMP-1, proMMP2-TIMP2 complex, proMMP-9 and active MMP-2.

For TIMP-1, the plasma levels were significantly lower than the serum levels ($P < 0.001$). Coagulation activation in the serum samples and the type of anticoagulant used in the plasma samples did not affect the TIMP-1 levels (Fig. 13A).

ProMMP-9 serum levels were significantly affected by coagulation activation, giving the median 30.4ng/ml for native serum and the mean 124.8ng/ml for serum with coagulation activator ($P < 0.001$, Fig. 13B). ProMMP-9 levels were also significantly affected by anticoagulant type, with lower MMP-9 levels for LiHe plasma than for EDTA plasma ($P < 0.001$, Fig. 13C). The

proMMP-9 levels in the lithium-heparin plasma, and serum samples with coagulation activator (serum+) differed significantly from all other sample types, giving $P < 0.001$ in all subgroup analyses of 2 variables (Fig. 13B). When EDTA plasma and native serum were paired for comparison, no significant differences in the proMMP-9 levels were found in the U-test ($p = 0.06$, Fig. 13B).

For the proMMP2-TIMP2 complex, the protein concentrations in the EDTA plasma were lower than in other sample types ($p < 0.001$, Fig.13C). For active MMP-2, LiHe plasma levels were significantly lower than the corresponding serum levels (Fig.13D). For TIMP-2 and proMMP-2, no significant differences were found between the sample types (III).

Similar effects caused by sample type were discovered when blood samples of 80 breast cancer patients were analysed. For these samples, the levels of TIMP-1, proMMP-9, proMMP2-TIMP2 complex and TIMP-2 were compared in serum with coagulation activator (serum +) and in EDTA plasma. TIMP-1 levels were significantly lower in plasma (median 237.8ng/ml) than in serum (median 408.4ng/ml) ($P < 0.001$). Although the levels were different, the ranges were overlapping and there was a strong linear correlation between plasma and serum concentrations with Pearson $r = 0.79$ and R-squared 0.61 (III). For the proMMP2-TIMP2 complex, the levels were significantly lower in EDTA plasma (median 960.9ng/ml) than in serum (mean 1296.9ng/ml) samples ($P < 0.001$). Despite the differences in the mean/median levels, there was a strong linear correlation between plasma and serum values with Pearson $r = 0.89$ and R-squared 0.79 (III). ProMMP-9 concentrations were found to be significantly lower in plasma samples than in serum + samples ($P < 0.001$). There was a weak linear correlation between plasma and serum concentrations, but the linear regression model of plasma proMMP-9 explained only 13% of the total variation of serum proMMP-9 values (III) For TIMP-2 no differences were found between plasma and serum samples.

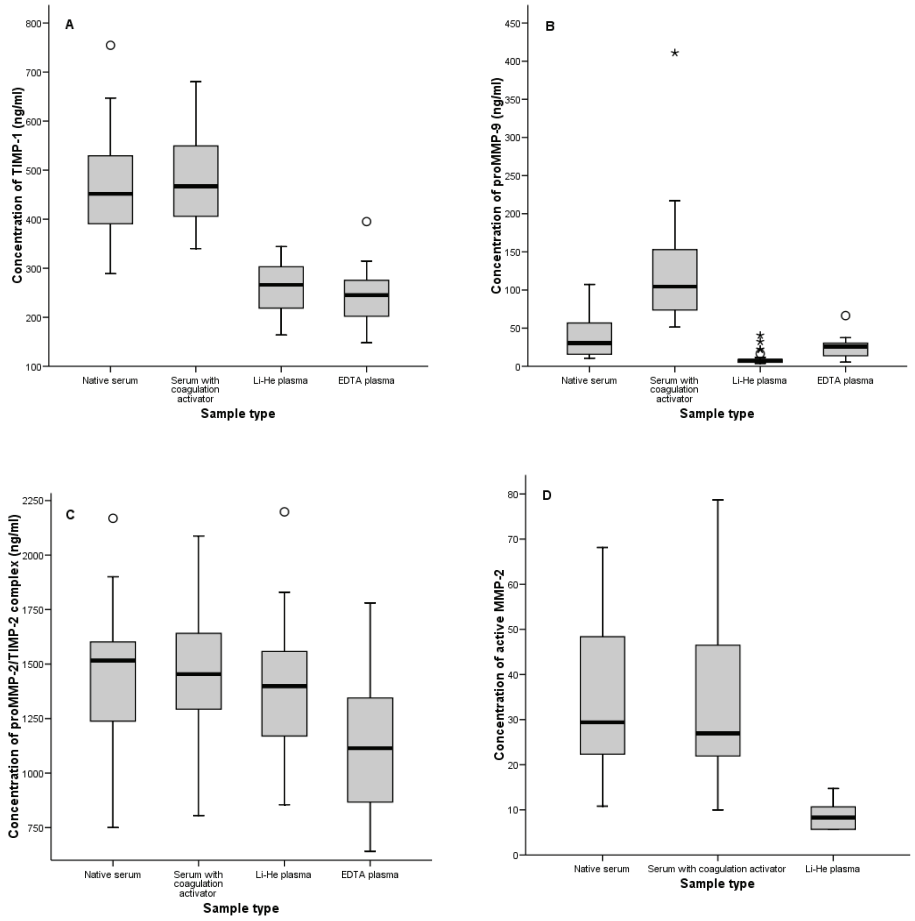

Fig. 13. Sample type effect in measuring A)TIMP-1 B) MMP-9, C) proMMP2-TIMP2 complex, D) active MMP-2.

6 Discussion

6.1 Evaluation of the clinical relevance of TIMP-1 as prognostic marker in primary breast carcinoma

Today, breast carcinoma prognosis is determined through evaluation of traditional prognostic markers, such as tumour size, nodal status, histological type and grade. Biological markers that give information on some specific trait of behaviour of the carcinoma tissue, e.g. hormone-dependency for growth and hormone-resistance, are needed. An optimal biological marker would also be a predictive marker for treatment, as in the case of ER or HER2 expression of the tumour. When novel treatment modalities are developed, biological markers are often used in order to find new suitable drug target molecules. However, a prognostic marker does not automatically become predictive if a new treatment is developed. Therefore, new prognostic markers need to be introduced and studied to find those that are the most suitable and have the greatest potential to be used in clinical practise.

In breast carcinoma, there are several very effective treatment modalities available. Adjuvant systemic therapy is recommended when the probability of recurrence exceeds 10% in 10-years follow-up time (Goldhirsch *et al.* 2005). Applying all of them in the primary treatment may lead to overtreatment of patients who already have a good prognosis, such as the majority of cases with T1 and N0 tumours. Only a minority of patients in this group benefit from adjuvant treatment, but today, there are no means of identifying those who will. There is thus a need for prognostic markers that identify the patients with a very good prognosis. On the other hand, it would be beneficial to immediately identify chemotherapy-resistant forms of the disease, in order to arrange more effective treatment, e.g. combine novel biological therapies, or spare patients from ineffective treatment.

Studying circulating cancer biomarkers is intriguing because it is much easier to measure tumour markers from the blood than to obtain tumour tissue for marker assays, as the majority of newly diagnosed breast carcinomas today are small tumours, and obtaining proper, representative samples might be difficult. In addition, if a marker is to be used in the follow-up, easy access to sample material becomes a crucial issue. TIMP-1 has recently been proposed as a novel

prognostic marker in breast carcinoma, in both tissue extracts and preoperative serum.

In this thesis work, the high immunohistochemical expression of TIMP-1 in the primary tumour and high preoperative plasma TIMP-1 were for the first time found to be prognostic for shortened disease-specific survival and relapse, respectively. In addition, high preoperative serum TIMP-1 was shown to maintain its prognostic value when assessed independently.

High TIMP-1 was a marker of worsened prognosis in all subpopulations studied, although tumour tissue expression of TIMP-1 was significantly prognostic only in a subpopulation of patients with grade 2-3 tumours. In this material, the majority of the cases included in grade 2-3 groups represented node-positive disease. The survival of nearly 90% at 10 years in the TIMP-1 negative subgroup was, therefore, exceptional.

In clinical studies using tumour tissue, the expression of TIMP-1 has been shown to correlate with poor prognosis in breast carcinoma using different methodologies (Hansen Ree *et al.* 1997; McCarthy *et al.* 1999; Nakopoulou *et al.* 2002b, Schrohl *et al.* 2004). In our tissue material, the TIMP-1 protein was evaluated in cancer cells by immunohistochemistry. High TIMP-1 positively associated with the high histological grade of the tumour, and in this subgroup it was quite well able to distinguish patients into groups of excellent and worsened prognosis. However, some bias may be caused by the fact that only 134 cases out of 199 had the information on nuclear grade available in this tissue material.

High circulating preoperative TIMP-1 was found to be an independent prognostic variable both in the serum and the plasma. In the population of 195 prospectively collected preoperative plasma samples, ln(TIMP-1) in the univariate analysis gave high HR 8.1 (95% CI 1.8 – 37.6) for relapse when there were only eleven relapses present in the material.

In the population of 68 patients, high preoperative serum TIMP-1 was prognostic for shortened disease-specific survival, giving HR 3.9 (95% CI 1.4 – 10.8) in Cox regression univariate analysis as a dichotomous variable. In both studies on circulating TIMP-1, it was found to be superior to nodal status as a prognostic variable. This is highly significant, since nodal status is today viewed as the most important traditional prognostic factor in breast carcinoma. The results on the prognostic value of TIMP-1 obtained in this thesis work are summarized in Table 10.

Table 10. Results on the prognostic value of high TIMP-1 in primary breast carcinoma.

Ref	Material	n	Prognostic for	Independent prognostic variable	Superior to	HR, 95% CI (univariate analysis)	Sig
I,II	Primary tumour tissue	199	DSS in grade 2-3	no		4.1 (1.0 – 17.1)	0.034
IV	Preoperative serum	68	DSS	yes, as a dichotomous variable	nodal status	3.9 (1.4 – 10.8)	0.005
V	Preoperative plasma	195	RFS	yes, as a continuous variable	nodal status	8.1 (1.8 – 37.6)	0.007

Circulating serum TIMP-1 was originally found to be prognostic for breast carcinoma in a study by Talvensaari-Mattila and Turpeenniemi-Hujanen (2005a). This result was validated here in another laboratory using a different ELISA. It was shown that the serum TIMP-1 maintained its prognostic value and the results on the two independent ELISAs correlated, although the measurements were done at different times and the serum tubes were stored in -20ºC. This indicated that TIMP-1 might indeed be used in clinical work, as it seems not to be very sensitive for storage conditions or storage time.

Inconsistent data exist on circulating TIMP-1 of cancer patients and healthy controls. It has been suggested that cancer patients have generally more elevated levels of TIMP-1 than healthy controls (Holten-Andersen *et al.* 1999). This was not observed here when the levels of plasma and serum TIMP-1 of 26 healthy controls and 80 breast cancer patients were compared (III).

The potential of TIMP-1 as a prognostic factor has been studied in several cancer forms during the last few years. Large studies, especially on colorectal and breast cancer, have found high TIMP-1 to be prognostic for worsened survival. The recent findings on the prognostic significance of TIMP-1 are summarized in Table 11.

Table 11. Recently published significant findings of prognostic value of high TIMP-1.

Malignancy	n	Material used	HR (95% CI) for DSS, Sig	Ref
breast ca	2984	cytosolic extracts of primary tumours	1.32 (1.17-1.49) P < 0.001	Schrohl *et al.* 2004
breast ca	71	preoperative serum	3.4 (1.2- 9.8) P = 0.02	Talvensaari-Mattila & Turpeenniemi-Hujanen 2005a
breast ca	341	cytosolic extracts of primary tumours, TIMP-1 as a combined score of total and uncomplexed TIMP-1	3.3 (1.8–6.3) P < 0.001	Wurtz *et al.* 2005a
colon ca	588	preoperative plasma	3.3 (2.6 – 4.2) P < 0.001	Holten-Andersen *et al.* 2000
colon ca	280	postoperative plasma	2.0 (1.3 – 2.9) P < 0.001	Holten-Andersen *et al.* 2006

In some studies using breast carcinoma tissue material the expression of TIMP-1 has been found to correlate with known clinico-pathological parameters. In two studies correlation with ER expression of the tumour was found (McCarthy *et al.* 1999; Nakopoulou *et al.* 2002b), but in the former the correlation of TIMP-1 and ER expression was inverse, whereas in the latter it was not. In one study a positive correlation between TIMP-1 and c-erbB2 expression was found (Nakopoulou *et al.* 2002b), and in two studies a correlation between positive lymph nodes and high TIMP-1 (Hansen Ree *et al.* 1997; Nakopoulou *et al.* 2002b), whereas in a study by McCarthy *et al.* (1997) a positive correlation between TIMP-1 and T-class of the tumour, but not nodal status, was reported (McCarthy *et al.* 1997). In one study a positive correlation between TIMP-1 and histological grade was previously found (Brummer *et al.* 1999). However, these correlations have not been a consistent finding, and in one study where multivariate analysis was done, high TIMP-1 was an independent prognostic marker for worsened survival when mRNA analysis was done on tumour tissue and surrounding stroma (Nakopoulou *et al.* 2002b).

Inconclusive data exist on the origin of tissue TIMP-1. In many studies TIMP-1 expression has been reported to be mainly found in the surrounding stromal tissue (Brummer *et al.* 1999, Nakopoulou *et al.* 2002b), but as in our own experiments, TIMP-1 expression in tumour cells has also been observed. It is possible that TIMP-1 is produced mainly as a stromal reaction, but cancer cells might be able to trap and use it. The results on the prognostic value of high TIMP-1 have shown considerable similarity, irrespective of the expression site.

The origin of circulating TIMP-1 is also unclear. It has been shown that neutrophils and platelets might produce some TIMP-1 when serum is assayed (Holten-Andersen *et al.* 2002; Sheu *et al.* 2004), and we have observed generally higher levels of TIMP-1 in serum compared with plasma (III). In study IV, we found no correlation between tumour and circulating TIMP-1, and it is possible that some of the circulating protein came from other sources, such as inflammatory cells. We did not observe a correlation between circulating and tumour cell TIMP-1 immunoreactivity, and it is probable that circulating TIMP-1 partly is of a stromal origin, and may partly originate in other components, such as inflammatory cells.

When tumour tissue extracts are used as a sample source, there is also other tissue material (stroma, inflammatory cells) present, and these extracts do not directly reflect tumour TIMP-1 content. It has previously been suggested that free fraction of TIMP-1 is prognostic when measured from tumour tissue extracts (Würtz *et al.* 2005a). In study IV, free fraction of TIMP was studied in serum and was not found to be prognostic, as it was for tumour extracts (Würtz *et al.* 2005a). In some cases, free TIMP-1 displayed higher serum concentrations than total TIMP-1. This was probably due to higher antibody affinities when measuring free TIMP-1, since it was noticed before when the same antibodies were used in ELISA assays (Würtz *et al.* 2005a).

Taken together, high TIMP-1 was found to be a powerful prognostic variable for relapse, and superior to nodal status in preoperative plasma. This observation was new. The material of 195 patients with only eleven relapses, with median survival of only 18 months, gave the highly significant HR of 8.1 for relapse in univariate analysis (Table 10). In previous reports on the prognostic value of TIMP-1 in breast carcinoma where tumour tissue extracts have been used as a sample source, the observed hazard ratios have not exceeded 3.3 (Table 11). In the study where serum TIMP-1 was measured the HR was 3.3, being similar to that observed in the validation experiment (IV), although in this material the minimum follow-up time was 120 months.

Other proteolytic enzymes, such as cathepsin D (Tandon *et al.* 1990; Rodriguez *et al.* 2005), uPa and PAI-1 have been implicated in breast carcinoma as novel prognostic markers, of which the latter two have been validated in a series of more than 8,000 patients where tumour tissue extracts were used as samples (Look *et al.* 2002). In this study, the combination of uPa/PAI-1 was quite well able to distinguish patients in the T1N0 group into high- or low-risk subgroups, with HR 2.58 (95% CI 2.24 – 2.98). A multicentre chemotherapy trial

of node-negative patients showed that by stratifying patients into high- or low-risk groups by uPa/PAI-1 analysis, about half of the patients could be spared from adjuvant chemotherapy (Jänicke *et al.* 2001). One recent study has suggested that measuring tumour TIMP-1 in addition to PAI-1 adds to the prognostic accuracy (Schrohl *et al.* 2003). Today, no prognostic factor that relates to proteolysis is in standard use, and as several strong candidates have recently been suggested (reviewed in Würtz *et al.* 2005b), it is reasonable to expect some of these to be included in clinical decision-making after careful validation processes.

6.2 The prognostic role of circulating MMP-2 in breast carcinoma

The circulating forms of MMP-2 were studied in the original article II. Surprisingly, it was discovered that lower levels of total serum proMMP-2 correlated with more advanced disease stage, positive nodal status and high histological grade. However, no associations with survival were found for total proMMP-2. For active MMP-2, an association of borderline significance was found for lower serum active MMP-2 and shorter recurrence-free survival. The tissue expression of proMMP-2 correlated inversely with circulating proMMP2-TIMP2 complex levels. In addition, healthy controls had lower total proMMP-2, but higher proMMP2-TIMP2 complex levels than patients.

These results indicate a very different perspective from those previously reported for the role of MMP-2 in breast carcinoma, since the tumour expression of MMP-2 has in many studies been shown to correlate with poor prognosis (Talvensaari-Mattila *et al.* 1998; 2005; Sivula *et al.* 2005). In addition, in one study where postoperative levels of circulating serum MMP-2 were studied higher MMP-2 was associated with a poor prognosis.

It was discovered here that high serum content of total proMMP-2 generally associated with cancer, but within patient material, low levels associated with advanced disease. Since the expression of MMP-2 is constitutive and not transcriptionally altered as that of for example MMP-9 (Sternlicht and Werb 2001), it might indicate higher usage of the existing circulating MMP-2 in the tumour. This was partly supported by the inverse correlation of tumour tissue MMP-2 expression and circulating proMMP2-TIMP2 complex levels, since a similar trend, although not significant, was also present for total serum proMMP-2. There has been a hypothesis that tumour tissue can enhance the activation of MMPs by trapping them onto the cell surface, via EMMPRINs, integrins or MT-

MMP molecules (Brooks *et al.* 1996; Sier *et al.* 2006). The present results support this hypothesis at least for MMP-2.

In study III it was discovered that in measuring proMMP-2 sample type used did not produce any significant variations in the MMP-2 levels detected. However, for active MMP-2, plasma levels were significantly lower than the serum levels detected.

MMP-2 is a multifunctional molecule that can act as a regulator of angiogenesis and apoptosis. MMP-2 can suppress angiogenesis by cleavage of plasminogen which can produce angiostatin, a potent inhibitor of angiogenesis (O'Reilly *et al.* 1999). The association of high serum levels with less advanced disease could be due to anti-angiogenic properties of MMP-2. It has also been suggested that high levels of MMP-2 and enhanced proteolysis might result in loss of contact with basement membranes, leading to increased apoptotic activities (Pereira *et al.* 2005; Simian *et al.* 2006)

Since MMP-2 is a multifunctional molecule, its net effect on the tumour might be pro- or antitumorigenic, and by measuring circulating MMP-2 it is very difficult to determine which previously described mechanisms might be active.

6.3 Physiological and preanalytical variants in determining gelatinases and their inhibitors from circulation

It has been suggested that preanalytical aspects play a very crucial role in measuring circulating gelatinases and their inhibitors. Especially for MMP-9 and TIMP-1 the use of serum as sample material has raised strong criticism since inflammatory cells and platelets contain them both and can cause much higher levels in serum samples (Jung *et al.* 1998, 2001, Jung 2005).

In our experiment it became evident that assaying proMMP-9 is very sensitive for several prenanalytical aspects, such as sample type selection and storage conditions (Rouy *et al.* 2005). It has been shown that MMP-9 levels are decreased, event though the samples are stored in -80ºC (Rouy *et al.* 2005). Some studies have suggested that the use of citrate plasma might be the safest option to determine proMMP-9 (Mannello 2003a, Mannello *et al.* 2003b), but when valid tumour markers are searched, the most sensitive ones, such as proMMP-9, might not be practical since the standard sample types used vary between laboratories and keeping the samples under precisely the same conditions might be very difficult. However, for proMMP-2 or TIMP-2 no significant effects of sample types were detected, and for TIMP-1 and the proMMP2-TIMP-2 complex there

was a good correlation between serum and plasma levels of the measured proteins.

As for TIMP-1, our results indicate a strong correlation between serum and plasma levels of TIMP-1, although serum levels are generally higher. The validation experiment in IV using serum samples resulted in good replicability of the results in terms of the prognostic value, even though the storage time varied and storage temperature was only -20ºC. Others have also reported that TIMP-1 is not particularly sensitive to storage conditions (Holten-Andersen *et al.* 2003), whereas proMMP-9 might still degrade heavily over time, even though the samples are kept in -80ºC (Rouy *et al.* 2005).

Some physiological variations might cause differences in circulating gelatinase and inhibitor levels. The one consistently registered in this study was age: higher circulating TIMP-1 levels correlated with patient's age at diagnosis and menopausal status. This has been reported previously (Schrohl *et al.* 2003; Würtz *et al.* 2005a). Also some other physiological aspects such as heavy exercise or alcohol consumption and liver fibrosis might alter the measured circulating TIMP-1 levels (Li *et al.* 1994; Koskinen *et al.* 2001). The identification of these physiological variants and careful validation of these biomarkers is therefore crucial before they can be adopted into clinical decision-making.

7 Conclusions

In this study, expression of tumour tissue TIMP-1 and -2 and their prognostic role was studied in breast carcinoma. The potential of circulating TIMP-1 and MMP-2 as prognostic markers was also explored. Additionally, the effect of different sample types on the levels of circulating gelatinases and their inhibitors was evaluated.

It is shown here that circulating TIMP-1 may have a role as a powerful new prognostic marker in breast carcinoma.

The specific conclusions of this study were:

1. Tumour tissue TIMP-1 expression associated with high grade and more aggressive disease course in breast carcinoma, and was a prognostic variable in a high-grade subpopulation.
2. Preoperative plasma TIMP-1 may be an independent prognostic marker for relapse, and seemed to be superior to nodal status as a prognostic indicator.
3. Preoperative serum TIMP-1 may be prognostic for survival in breast carcinoma: serum is a valid sample type, since the results were reproducible when two independent assays were utilized.
4. Low circulating preoperative serum proMMP-2 associated with aggressive features in breast carcinoma. The levels of circulating proMMP2-TIMP2 complex had an inverse correlation with tumour MMP-2 protein expression. In healthy controls, a larger portion of proMMP-2 was in a proMMP-2-TIMP2 complex form than in breast cancer patients.
5. Sample type is a critical preanalytical factor when circulating gelatinases and their inhibitors are measured. ProMMP-9 is very sensitive for preanalytical conditions. Serum measurement of TIMP-1 generally produces higher levels than plasma measurement since platelets contain TIMP-1, but there is a strong correlation between plasma and serum levels. Plasma might be a better sample type when assaying TIMP-1. The use of EDTA plasma as sample material might lower the measured proMMP2-TIMP2 complex levels.

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Original articles

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

I Kuvaja P, Talvensaari-Mattila A, Pääkkö P & Turpeenniemi-Hujanen T (2005) The absence of immunoreactivity for Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), but not for TIMP-2, protein is associated with a favorable prognosis in aggressive breast carcinoma. Oncology 68: 196-203

II Kuvaja P, Talvensaari-Mattila A, Pääkkö P & Turpeenniemi-Hujanen T (2006) Low serum level of pro-matrix metalloproteinase 2 correlates with aggressive behavior in breast carcinoma. Hum Pathol 37: 1316-1323

III Kuvaja P, Talvensaari-Mattila A &Turpeenniemi-Hujanen T (2007) The sample type used affects the levels of gelatinases (MMP-2 and -9) and their inhibitors (TIMP-1 and -2) in circulating blood of healthy controls and breast cancer patients. Biomarker Insights 2: 117-127

IV Kuvaja P*, Würtz SØ*, Talvensaari-Mattila A, Brünner N, Pääkkö P & Turpeenniemi-Hujanen T (2007) High serum TIMP-1 correlates with poor prognosis in breast carcinoma – a validation study. Cancer Biomark. In press.

V Kuvaja P, Talvensaari-Mattila A & Turpeenniemi-Hujanen T (2007) High preoperative plasma TIMP-1 is prognostic for early relapse in primary breast carcinoma. Manuscript.

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Original publications are not included in the electronic version of the dissertation.

933. Kangasniemi, Mari (2007) Monoliittisesta trilogiseen tasa-arvoon. Tasa-arvo hoitotyön etiikan tutkimuksessa

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