THE PROGNOSTIC ROLE OF MATRIX METALLOPROTEINASE-2 AND -9 AND THEIR TISSUE INHIBITOR-1 AND -2 IN ENDOMETRIAL CARCINOMA

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University of Oulu Graduate School; University of Oulu, Faculty of Medicine, Institute of Clinical Medicine, Department of Obstetrics and Gynecology; Oulu University Hospital; National Graduate School of Clinical Investigation

Abstract

Endometrial carcinoma is the most common gynecologic malignancy in developed countries. Due to early symptoms, including abnormal uterine bleeding, endometrial cancer is often diagnosed at an early stage and in that case usually has a good prognosis and high cure rates. However, the nature of the disease is heterogeneous.

During the last decades, the improvement in survival rates among endometrial cancer patients has not been significant, suggesting that the traditional clinicopathological factors may be inadequate to identify patients with high-risk disease. Furthermore, aggressive adjuvant treatments can be costly and very toxic. Therefore, better prognostic markers associated with biological aggressiveness of endometrial carcinoma are needed to identify the patients with high-risk disease, and to be able to select the treatment more individually.

Gelatinases (MMP-2 and MMP-9) and their tissue inhibitors (TIMP-1 and TIMP-2) have been found to play a role in tumor progression. In the present work, the expression and prognostic value of MMP-2, MMP-9, TIMP-1 and TIMP-2 were assessed in endometrial carcinoma. The patient material consisted of a total of 266 women diagnosed with primary endometrial carcinoma. The tissue expression of immunoreactive proteins was examined in paraffin-embedded tumor sections by immunohistochemical staining using specific antibodies, and the pretreatment serum levels of the proteins were quantitatively measured by ELISA.

Tissue MMP-2 expression associated with a worsened prognosis, whereas tissue TIMP-2 overexpression was an indicator of a favorable outcome. Furthermore, we observed a combination of strong MMP-2 and weak TIMP-2 tissue expression to identify a group of women at high risk of adverse outcome in endometrial carcinoma. Patients with negative MMP-2 immunostaining had the best prognosis, regardless of TIMP-2 staining result. In serum measurements, high preoperative TIMP-1 concentration was a prognostic indicator of unfavorable outcome.

These results indicate that tissue MMP-2 and TIMP-2 as well as circulating TIMP-1 may be prognostic markers in endometrial carcinoma. Of these, tissue MMP-2 seems to be the most potent prognostic marker. Studies with larger patient materials are needed to further explore the value of these enzymes in clinical practice in endometrial cancer.

Keywords: ELISA, endometrial neoplasms, enzyme-linked immunosorbent assay, immunohistochemistry, matrix metalloproteinase 2, matrix metalloproteinase 9, MMP-2, MMP-9, neoplasm invasiveness, prognosis, survival, TIMP-1, TIMP-2, tissue inhibitor of metalloproteinase-1, tissue inhibitor of metalloproteinase-2
Honkavuori-Toivola, Maria, Matriksin metalloproteinaasien-2 ja -9 sekä niiden kudosinhibiittoreiden-1 ja -2 ennustellinen merkitys kohdunrungon syövää.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteiden laitos, Synnytys ja naistenlaajentumis; Valtakunnallinen kliininen tutkijakoulu

**Tiivistelmä**

Kohdunrungon syöpä on yleisin gynekologinen maligniteetti kehittyneissä maissa. Vanhaisten oireiden, kuten poikkeavan verisen vuodon, vuoksi kohdunrungon syöpä havaitaan usein varhaisessa vaiheessa, jolloin sen ennuste on hyvä. Taudin käyttäytyminen voi kuitenkin olla moninaista.

Viime vuosikymmenten aikana kohdunrungon syöpään sairastuneiden ennuste ei ole merkitövästi parantunut. Vaikuttaisi siltä, että perinteiset ennustetekijät eivät ole riittävän tarkkoja ennustamaan syövän taudinkulua. Uusien biologisten ennustetekijöiden löytäminen olisi tärkeää, jotka aggressiivista syöpätyyppiä sairastavat potilaat pystyisivät ennustamaan entistä paremmin, ja hoito keytäisiäan räätälöimään yksilöllisemmän taudinkuvan vastaavasti.


Asiastat: ELISA, ennuste, immunohistokeemia, kasvainen invaasiivisuus, kohdunrungon syöpä, matriksimetalloproteinaasi 2, matriksimetalloproteinaasi 9, metalloproteinaasien kudosinhibiittori-1, metalloproteinaasien kudosinhibiittori-2, MMP-2, MMP-9, selviytyminen, TIMP-1, TIMP-2
To my family
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Oulu, March 2014

Maria Honkavuori-Toivola
### Abbreviations

<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>ADAM</td>
<td>a disintegrine and metalloproteinase</td>
</tr>
<tr>
<td>AP</td>
<td>activator protein</td>
</tr>
<tr>
<td>b-FGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>extracellular matrix metalloproteinase inducer</td>
</tr>
<tr>
<td>EPA</td>
<td>erythroid-potentiating activity</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>GPI</td>
<td>glycophosphatidylinositol</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>HER-2</td>
<td>human epidermal growth factor receptor 2</td>
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<tr>
<td>HNPCC</td>
<td>hereditary non-polyposis colorectal cancer</td>
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<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>ISH</td>
<td>in situ hybridization</td>
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<td>ISZ</td>
<td>in situ zymography</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>LVSI</td>
<td>lymphovascular space invasion</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSI</td>
<td>microsatellite instability</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane-type matrix metalloproteinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCPE</td>
<td>procollagen C-proteinase enhancer protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome ten</td>
</tr>
<tr>
<td>RASI-1</td>
<td>rheumatoid arthritis synovium inflamed-1</td>
</tr>
<tr>
<td>RECK</td>
<td>reversion-inducing cysteine-rich protein with Kazal motifs</td>
</tr>
<tr>
<td>ROC</td>
<td>receiving operating characteristics</td>
</tr>
<tr>
<td>TAH+BSO</td>
<td>total abdominal hysterectomy + bilateral salpingo-oophorectomy</td>
</tr>
<tr>
<td>TFPI-2</td>
<td>tissue factor pathway inhibitor 2</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TVU</td>
<td>transvaginal ultrasonography</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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</table>
List of original publications

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


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1 Introduction

Endometrial cancer is the most common gynecologic malignancy in industrialized countries. Annually, endometrial carcinoma is estimated to develop in 287,100 women worldwide (Jemal et al. 2011). In Finland, there were about 800 new cases in 2009, and the incidence, which is now 13.9 per 100,000 person-years, is expected to continue rising (Finnish Cancer Registry 2011). Endometrial carcinoma is primarily a disease of postmenopausal women. The median age at diagnosis is the sixth decade, although 20 to 25% of the cases are diagnosed premenopausally (Masciullo et al. 2010). The tumor often causes abnormal bleeding as a first symptom, and the majority of the cases are therefore diagnosed in the early stages. Although endometrial carcinoma is generally considered to have a good prognosis, approximately one fifth of these carcinomas recur, with limited effect of systemic therapies in metastatic disease (Salvesen et al. 2012).

Surgery is the primary therapy for endometrial cancer, and the use of adjuvant treatments is decided upon evaluation of traditional prognostic markers. Unfortunately, by this approach no significant improvement in survival has been observed over the last two decades (Susini et al. 2007). It seems that in some cases the classical pathologic parameters are unable to effectively identify patients with high-risk disease. On the other hand, some patients with less aggressive disease types are over-treated and suffer from the side effects of adjuvant therapies. Thus, new prognostic markers are needed for the decision process about the use of adjuvant therapies in endometrial carcinoma treatment.

The capability of tumors to invade into surrounding tissues and through tissue boundaries to form metastases at distant sites is characteristic of malignant cancer. Matrix metalloproteinases (MMPs) are a large family of enzymes that regulate the extracellular matrix turnover. Their subgroup of gelatinases, consisting of MMP-2 and MMP-9, are the main enzymes in degrading collagen IV, the major component of basement membranes. Enhanced expression of MMP-2 and MMP-9 is thus thought to play a critical role in the invasion through basement membranes. (Birkedal-Hansen et al. 1993) Accumulating data demonstrate that MMPs are also involved earlier in tumorigenesis, e.g., in malignant transformation and angiogenesis (Hua et al. 2011). Moreover, recent studies have suggested that MMPs play more complex roles that both promote and inhibit development of the tumor microenvironment, tumor progression and metastasis. The action is dependent on the MMP as well as the timing and location of its expression. (Stetler-Stevenson & Gavil 2014)
Tissue inhibitors of metalloproteinases (TIMPs) are the major endogenous inhibitors of the matrix metalloproteinases. The first suggestions on TIMP functions were solely based on their ability to inhibit MMP activities and thereby restrict tumor cell migration and invasion. However, today their multifunctional role in tumor progression is acknowledged. TIMPs have been shown to take part at least in the regulation of cell growth, angiogenesis and apoptosis. Paradoxically, TIMPs can also participate in the activation of MMPs. In addition to their MMP-dependent functions, TIMPs exhibit cellular activities independent of MMP inhibition. Their role in tumor progression is therefore controversial and far from clear. (Fassina et al. 2000, Lambert et al. 2004)

The expression of gelatinases and their tissue inhibitors has been associated with the clinical course in a wide variety of cancers (Turpeenniemi-Hujanen 2005). Data on the expression of gelatinases and their tissue inhibitors in endometrial carcinoma is very limited. Especially studies concerning their prognostic role in endometrial carcinoma are rare.

The aim of the present study was to evaluate the expression and clinical role of gelatinases and their tissue inhibitors in endometrial carcinoma. These markers were studied in both tumor tissue and serum samples from endometrial cancer patients.
2 Review of the literature

2.1 Endometrial carcinoma

Endometrial carcinoma is the most common invasive malignancy of the female reproductive tract in the Western world. The disease is often diagnosed at an early stage due to early clinical signs, including postmenopausal or prolonged menstrual bleeding. It is therefore a highly curable malignancy, with a 5-year overall survival rate of 82% (Siegel et al. 2012). However, the nature of the disease is heterogeneous and there is a group of patients with a high risk of cancer recurrence or metastatic spread.

2.1.1 Epidemiology

Worldwide, endometrial carcinoma is the seventh most common malignancy, but incidence varies among regions. Some of the highest incidence rates of endometrial carcinoma worldwide are found within European populations and in North America. In less developed countries, risk factors are less common and endometrial carcinoma is rare, but mortality is higher. (Amant et al. 2005, Bray et al. 2005, Parkin et al. 2005)

In Finland, there were 809 new cases in 2009, accounting for 5.9% of all newly appearing cancers in women. In 2009, the age-adjusted incidence rate and mortality rate per 100,000 person-years was 13.9 and 2.8, respectively. (Finnish Cancer Registry 2011)

Endometrial carcinoma mainly affects postmenopausal women. At diagnosis, only 4.7% of the patients in Finland are less than 50 years of age (Finnish Cancer Registry 2011). The annual incidence and the mortality rate of endometrial carcinoma are rising, despite the advances that have been made in the early detection and treatment in this disease (Linkov et al. 2008). In Finland in the 1970s, some 400 new endometrial carcinoma cases were diagnosed annually (Finnish Cancer Registry 2011). Thus, the incidence of endometrial carcinoma has doubled in forty years. The main factors contributing to the increase in incidence rates are increasing life-expectancy, obesity and tamoxifen, a widely used adjuvant treatment for breast cancer (Linkov et al. 2008).
2.1.2 Etiology

The human endometrium is a dynamic tissue that undergoes extensive cyclic degradation and renewal in response to fluctuations of estrogen and progesterone levels during the menstrual cycle. Excessive estrogen exposure without the differentiating effect of progestin is considered to be the primary etiological factor in the development of endometrial hyperplasia and adenocarcinoma. About 10% of endometrial cancers are high-grade tumors (poorly differentiated endometrioid or non-endometrioid histological type), which are not estrogen-driven and carry a high risk of relapse and metastatic disease. (Amant et al. 2005)

Factors associated with endometrial carcinoma risk

Obesity is a major risk factor for endometrial carcinoma development. The development of endometrial carcinoma in obese women is believed to be mediated by endogenous estrogen, through the conversion of androstenedione to estrone by aromatase in peripheral adipose tissue (Rose 1996). Obesity remains a risk factor for endometrial carcinoma even when circulating levels of estrogen are normal (Potischman et al. 1996). Morbid obesity also increases significantly the risk of mortality from endometrial cancer (Calle et al. 2003).

Other recognized risk factors are associated with a higher cumulative exposure to estrogen, such as early menarche, late menopause, nulliparity, a history of anovulatory cycles, and estrogen replacement therapy without concomitant use of progestin (Beral et al. 2005, Dossus et al. 2010, Hemminki et al. 2005). Unopposed estrogen replacement therapy has been found to increase the risk of endometrial cancer dose- and duration-dependently (Beral et al. 2005, Weiderpass et al. 1999). Progestins counteract the proliferative effect of estrogens on the endometrium, and the beneficial effect is greater the more days every month they are added to estrogen and the more obese the women are (Beral et al. 2005, Trabert et al. 2013). Sequential or continuous progestins have been added to the hormone replacement therapy in women who have not had a hysterectomy to minimize the increased risk of endometrial cancer. However, there appears to be growing evidence that certain regimens of use confer increased risk. Long duration of sequential combined estrogen-progestin regimens has been associated with increases in the risk of endometrial cancer – particularly for thin-to-normal weight women (Trabert et al. 2013, Weiderpass et al. 1999). Even continuous use
of progestin has been associated with increased risk of endometrial cancer compared to non-users of hormone replacement therapies (Lacey et al. 2005).

Women with breast cancer have an increased risk of primary endometrial cancer, partly due to common risk factors. Breast cancer can also in rare cases metastasize to the endometrium. (Harvey & Brinton 1985) An additional endometrial cancer risk has been related to long-term use of tamoxifen, a selective estrogen receptor modulator (SERM) used in the treatment of breast cancer. Tamoxifen is found to have a stimulating effect on the endometrium, but the underlying mechanism of tamoxifen-associated endometrial carcinoma development is not clear. (Swerdlow et al. 2005, Williams-Brown et al. 2011)

Approximately 5% of endometrial carcinomas are considered familial. Many of these are associated with hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, which is a dominant inherited syndrome with germline mutations in DNA mismatch repair genes, causing microsatellite instability of the genome. (Uharček 2008) Reported cancer risks for individuals with Lynch syndrome vary by population and by gene, but it seems that female carriers have a higher risk of endometrial cancer than of colorectal cancer (Aarnio et al. 1999). Women with Lynch syndrome have an estimated 40–60% lifetime risk of developing an endometrial cancer (Masuda et al. 2011). In Finnish population, the cumulative incidence rates at 70 years of age were 60% for female HNPCC carriers, as compared with only 1.3% in the Finnish population as a whole (Aarnio et al. 1999). Thus, the identification of women with HNPCC is desirable, because they can benefit from increased cancer surveillance.

Pregnancy protects against endometrial carcinoma, possibly due to the continuous progesterone production throughout the pregnancy. In a study by Hinkula et al. (2002), multiple births, old age at first birth and a long birth period significantly reduced the risk of endometrial cancer in grand multiparous women. Anovulatory cycles are common before menopause, and the protective effect on endometrial cancer risk of old age at first birth and a long birth period were thought to be associated with a shortened delivery-free premenopausal period (Hinkula et al. 2002). Other protective factors are oral contraceptive use, physical activity and smoking (Cust 2011, Deligeoroglou et al. 2003, Zhou et al. 2008.). Smoking has been suggested to exert an antiestrogenic effect on the endometrium through weight reduction, earlier menopause, or alteration of hormone metabolism, and thus decrease the risk of endometrial carcinoma especially among postmenopausal women (Zhou et al. 2008). Table 1 lists the most significant factors associated with endometrial cancer risk.
Table 1. Risk factors for endometrial cancer. (Modified from Amant et al. 2005 & Fader et al. 2009).

<table>
<thead>
<tr>
<th>Factors increasing risk</th>
<th>Factors decreasing risk</th>
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<tbody>
<tr>
<td>Obesity</td>
<td>Grand multiparity</td>
</tr>
<tr>
<td>Nulliparity</td>
<td>Smoking</td>
</tr>
<tr>
<td>Years of menstruation</td>
<td>Oral contraceptive use</td>
</tr>
<tr>
<td>Exogenous unopposed estrogen</td>
<td>Physical activity</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td></td>
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<tr>
<td>Increasing age</td>
<td></td>
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<tr>
<td>Diabetes</td>
<td></td>
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<tr>
<td>Hypertension</td>
<td></td>
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<tr>
<td>History of breast cancer</td>
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<tr>
<td>HNPCC</td>
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<td>High energy intake</td>
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<td>Radiation therapy</td>
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</table>

**Molecular characteristics of endometrial carcinoma**

The molecular mechanisms of endometrial carcinogenesis are still largely unknown. PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a tumor-suppressor gene that is expressed most highly in an estrogen-rich environment. It has an important function in the regulation of the cell cycle and apoptosis, and through the phosphoinositide 3-kinase (PI3K)/Akt pathway it also regulates cell survival and proliferation. PTEN inactivation has been widely implicated in the development of endometrial cancer, especially well-differentiated endometrioid cancer. (Ellis & Ghaem-Maghami 2010, Terakawa et al. 2003) Other commonly observed defects in well-differentiated endometrioid cancers are microsatellite instability (MSI), β-Catenin, and K-ras oncogene mutations. By contrast, poorly differentiated endometrioid or non-endometrioid cancers are often associated with p53 mutations, loss of E-cadherin expression, p16 inactivation, and human epidermal growth factor receptor 2 (HER-2)/neu overexpression. (Bansal et al. 2009, Lax et al. 2000, Lax. 2004)

**2.1.3 Diagnosis and staging**

The most frequent symptom of endometrial carcinoma is abnormal uterine bleeding (Amant et al. 2007). The probability of endometrial cancer as the cause of postmenopausal bleeding increases with age and risk factors (Gredmark et al. 2007).
All postmenopausal women with bleeding or spotting should undergo further diagnostic assessment. Women with postmenopausal bleeding may be evaluated initially with either endometrial biopsy (Pipelle) or transvaginal ultrasonography (TVU). Endometrial thickness of 4 mm or less on TVU indicates a very low likelihood of cancer, and endometrial biopsy is not generally needed. (Doubilet 2011, Goldstein 2009) When the diagnosis is confirmed histopathologically, imaging is recommended to assess the stage radiologically in order to plan surgery (Kitchener 2006).

Surgical staging of endometrial cancer has been the cornerstone of management since 1988, when the International Federation of Gynecology and Obstetrics (FIGO) system was changed from clinical to surgical staging. In 2009, the revised FIGO staging system was published, replacing the 1988 FIGO staging system. In the revised FIGO staging system, FIGO 1988 stages IA (tumor limited to the endometrium) and IB (tumor limited to the inner one-half of the myometrium) were combined into a single substage, FIGO 2009 stage IA. In addition, stage II no longer has subsets A and B, since cervical glandular involvement was eliminated from the staging criteria and only women with cervical stromal invasion are classified as stage II. Third, peritoneal cytology was removed as a staging criterion. Finally, patients with nodal metastasis were stratified into stages IIIC1 (positive pelvic nodes) and IIIC2 (positive para-aortic nodes with or without positive pelvic nodes). The staging of endometrial carcinoma is presented in Table 2. (Lewin 2011, Creasman 2009)
Table 2. FIGO staging system (2009) for carcinoma of the endometrium. (Modified from Lewin 2011).

<table>
<thead>
<tr>
<th>FIGO Stages</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Tumor confined to the corpus uteri</td>
</tr>
<tr>
<td>IA</td>
<td>No or less than half myometrial invasion</td>
</tr>
<tr>
<td>IB</td>
<td>Invasion to or more than half of the myometrium</td>
</tr>
<tr>
<td>Stage II</td>
<td>Tumor invades cervical stroma, but does not extend beyond the uterus</td>
</tr>
<tr>
<td>Stage III</td>
<td>Local and/or regional spread of the tumor</td>
</tr>
<tr>
<td>IIIA</td>
<td>Tumor invades the serosa and/or adnexae</td>
</tr>
<tr>
<td>IIIB</td>
<td>Vaginal and/or parametrial involvement</td>
</tr>
<tr>
<td>IIIC</td>
<td>Metastases to the pelvic and/or para-aortic lymph nodes</td>
</tr>
<tr>
<td>IIIC1</td>
<td>Positive pelvic nodes</td>
</tr>
<tr>
<td>IIIC2</td>
<td>Positive para-aortic lymph nodes with or without positive pelvic lymph nodes</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Tumor invades bladder and/or bowel mucosa, and/or distant metastases</td>
</tr>
<tr>
<td>IVA</td>
<td>Tumor invasion of bladder and/or bowel mucosa</td>
</tr>
<tr>
<td>IVB</td>
<td>Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes</td>
</tr>
</tbody>
</table>

2.1.4 Prognostic factors and survival

Endometrial carcinoma has been described as consisting of two groups (Table 3). In 1983, Bokhman proposed the concept of type I and type II endometrial carcinomas based on clinical behavior and histopathology (Bokhman 1983). Type I tumors, consisting mainly of endometrioid adenocarcinomas and accounting for approximately 80% of all endometrial carcinomas, are believed to develop in an estrogen-related manner. These tumors are usually of low grade and well differentiated, thus carrying a better prognosis. Type II tumors, consisting mostly of serous and clear cell carcinomas, are unrelated to estrogen exposure and arise in a background of atrophic endometrium. These tumors are typically of high grade and poorly differentiated, thus characterized by a more aggressive clinical course and worse prognosis. (Bansal et al. 2009, Bokhman 1983, Dizon 2010)

Recently, the etiologic differences of type I and type II endometrial cancers were supported by Brinton et al. (2013). Furthermore, additional etiologic heterogeneity was observed within type II cancers based on tumor histology.
Table 3. Contrasting profiles of type I versus type II endometrial carcinoma. (Reproduced from Hamilton et al. 2008).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type I endometrial carcinoma</th>
<th>Type II endometrial carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>50–60</td>
<td>60–70</td>
</tr>
<tr>
<td>Low parity</td>
<td>Common</td>
<td>Less common</td>
</tr>
<tr>
<td>Obesity, hyperlipidemia, diabetes</td>
<td>Common</td>
<td>Less common</td>
</tr>
<tr>
<td>Estrogen related</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Background endometrium</td>
<td>Hyperplastic</td>
<td>Atrophic</td>
</tr>
<tr>
<td>Grade</td>
<td>Low</td>
<td>High (by definition)</td>
</tr>
<tr>
<td>Molecular alterations</td>
<td>PTEN, MSI</td>
<td>p53</td>
</tr>
<tr>
<td>Estrogen/progesterone receptor</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td>Clinical behavior</td>
<td>Indolent</td>
<td>Aggressive</td>
</tr>
</tbody>
</table>

Traditional prognostic factors for endometrial carcinoma include surgical FIGO stage, histological type, age, grade, depth of myometrial invasion, presence of lymph node metastases, and lymphovascular space invasion (LVSI) (Narayan et al. 2012). Serous and clear cell carcinomas carry a worse prognosis when compared with endometrioid and mucinous histology (Mendivil et al. 2009). In 2006 FIGO reported an all-stage 5-year survival rate of 83.2% for endometrioid carcinoma, compared with 62.5% for clear cell carcinoma and only 52.6% for serous histology (Creasman et al. 2006). In Finland, the corresponding figure with all the histological types included is 83% (Finnish Cancer Registry 2011). Survival rates according to stage and histological type are presented in Table 4.

Myometrial invasion has been found to be an independent predictor of outcome in several studies (Creasman et al. 1987, Creasman et al. 2006, Steiner et al. 2003). Histological grade is strongly associated with prognosis, and thus one of the prognostic factors applied in clinical decisions regarding treatment (Creasman et al. 2006, Uharček 2008). Younger patients tend to have a more favorable prognosis than older women, and usually have a history of estrogen- or hormone-related disorders, such as chronic anovulation, ovarian dysfunction, infertility, polycystic ovary syndrome, and obesity (Alektiar et al. 2003, Ota et al. 2005). Lymphovascular space invasion has been associated with the presence of lymph node metastasis. LVSI as well as the absolute number of positive nodes and lymph-node ratio are independent predictors of outcome. (Briët et al. 2005, Chan et al. 2007, Narayan et al. 2012)
Steroid hormones play an important role in the pathogenesis of endometrial cancer. The presence and quantity of steroid receptors (estrogen ER or progesterone receptor PR) have been correlated with stage, histological grade and survival. Several studies have reported that ER or PR status constitutes an independent prognostic factor (Kadar et al. 1993, Nyholm et al. 1995, Steiner et al. 2003). However, their role as independent prognostic factors for endometrial carcinoma has been questioned in some studies (Iversen et al. 1988, Jeon et al. 2006, Sivridis et al. 2001).

DNA aneuploidy has been associated with other traditional prognostic factors and recurrence in endometrial cancer (Larson et al. 1999, Susini et al. 1994). Some studies have suggested that DNA ploidy has an independent effect on survival (Santala & Talvensaari-Mattila. 2003, Song et al. 2011, Susini et al. 2007, Wik et al. 2009), but the results are equivocal (Larson et al. 1999, Pfisterer et al. 1995, Terada et al. 2004). CA 125 is the most widely used biochemical tumor marker. Elevations of serum CA 125 levels were first described in patients with recurrent and advanced endometrial cancer by Niloff et al. (1984). Since then, many subsequent studies have reported the association of elevated levels of preoperative CA 125 with advanced stage and the presence of extrauterine disease (Duk et al. 1986, Patsner et al. 1988), as well as with poor survival (Goksedef et al. 2011, Santala et al. 2003, Sood et al. 1997). However, the role of CA 125 in endometrial carcinoma has not been clearly established.
2.1.5 Treatment

Surgery is the primary treatment for endometrial cancer. The use of adjuvant treatment is decided based on clinicopathological criteria of tumor histological type, grade, myometrial invasion and the presence or absence of lymph node metastases (Faust et al. 2010). Endometrial carcinoma can be classified as low-risk, intermediate-risk and high-risk for lymph node metastases and/or early disease spread to the abdominal cavity and to distant sites. Low-risk endometrial cancers are FIGO (2009) stage IA, grade 1 or 2, of endometrioid type histology. High-risk cancers are FIGO stage IB of grade 3 or of non-endometrioid histology; or stage II or III endometrial carcinoma. All other stage I endometrial carcinomas are intermediate risk. This group can further be divided into low and high intermediate risk subgroups. (Creutzberg & Nout 2011)

Surgery

The majority of women with well- or moderately-differentiated endometrioid carcinoma can be cured by total hysterectomy with bilateral salpingo-oophorectomy. Pelvic and para-aortic lymphadenectomy is necessary for the surgical staging, but the role of lymphadenectomy or lymph node sampling has been widely debated (ASTEC study group et al. 2009, Benedetti Panici et al. 2008, Kehoe & Miller 2011, Todo et al. 2010). This has traditionally been performed by laparotomy, but the laparoscopic technique has become more frequent in recent years (Faust et al. 2010). No significant differences have been found in overall or relapse-free survival in patients operated either laparoscopically or with open surgery (Eltabbakh 2002, Malur et al. 2001, Tozzi et al. 2005b). The advantages of laparoscopic surgery are shorter hospitalization and recovery time and decrease in surgical morbidity (Malur et al. 2001, Mourits et al. 2010). Laparoscopic procedure is recommended especially for patients with increased surgical risks for complications (Tozzi et al. 2005a). A potential hazard associated with laparoscopy are port site tumors, but this is a rare event occurring in about 1% of cases according to literature (Abu-Rustum et al. 2004, Zivanovic et al. 2008).
**Adjuvant radiotherapy**

Only 20–30% of endometrial cancer patients are thought to require adjuvant therapy (Faust et al. 2010). Radiation can be delivered externally to the pelvis, as internal vaginal brachytherapy or as a combination of the two (Amant et al. 2005). Preoperative radiotherapy interferes with adequate surgical staging and has no demonstrated benefit over postoperative radiotherapy (Amant et al. 2007). Postoperative pelvic radiotherapy has been the traditional adjuvant therapy. It has been found to reduce local recurrences in early stage endometrial carcinomas, but the trials have shown no survival advantage for routine postoperative radiotherapy (ASTEC/EN.5 Study Group et al. 2009, Creutzberg et al. 2000, Keys et al. 2004). In low-risk patient group adjuvant pelvic radiotherapy seems to have no benefit at all and the use of external radiotherapy should therefore be limited to the group of patients with high-risk factors to warrant the risk of treatment-associated morbidity (Creutzberg & Nout 2011).

For intermediate-risk endometrial carcinoma, the vagina is the most frequent site of recurrence. The use of postoperative vaginal brachytherapy has been reported to be very effective in prevention of vaginal recurrence (Alektiar et al. 2005, Rittenberg et al. 2003, Rittenberg et al. 2005). A recent multicentre trial established vaginal brachytherapy to be as effective as pelvic external radiotherapy in obtaining local control with fewer side effects and better quality of life for patients with endometrial carcinoma of high-intermediate risk (Nout et al. 2010, Nout et al. 2011).

**Adjuvant chemotherapy**

The use of systemic chemotherapy has not been very common in endometrial cancer treatment. Cytotoxic chemotherapy is mostly used for advanced cases or recurrences, but accumulating data show that chemotherapy is increasingly integrated in the treatment of early stage diseases with high-risk features as well (Maggi et al. 2006, Susumu et al. 2008).

In advanced or recurrent endometrial cancer phase II studies have shown response rates exceeding 20% mainly with anthracyclines, platinum compounds, and taxanes (Humber et al. 2007). Combination chemotherapy yields better response rates, but does not always improve survival. The combination of doxorubicin and cisplatin was for many years regarded as the standard treatment in endometrial cancer. Two large randomized phase III trials have examined the
combination of cisplatin and doxorubicin with doxorubicin alone (Aapro et al. 2003, Thigpen et al. 2004). In both studies, the combination therapy was found to give better response rates, but no significant differences in survival. The Gynecologic Oncology Group compared the cisplatin plus doxorubicin to three-drug regimen comprising doxorubicin, cisplatin, and paclitaxel (Fleming et al. 2004). Response rate, progression-free survival and overall survival were significantly better in the three-drug group. However, increasing efficacy also led to increasing toxicity and treatment-related mortality. Paclitaxel plus carboplatin is a commonly used and usually well-tolerated drug combination in another gynaecologic cancer types. Previous studies in endometrial cancer have demonstrated high response rates of 40–90%. Results from two ongoing trials with paclitaxel plus carboplatin are currently awaited. (Hogberg 2008, Hogberg 2011)

The combination of radiotherapy and chemotherapy seems promising in high-risk diseases and appears to be more effective than radiotherapy alone. Doxorubicin-cisplatin or carboplatin-paclitaxel seems to be the most appropriate regimen to combine with radiation therapy. (Hogberg et al. 2010, Homesley et al. 2009) The possible use of combined chemoradiotherapy is tested further in the PORTEC-3 trial (Dutch Cancer Society and UK National Cancer Research Network.).

Adjuvant endocrine therapy

The majority of endometrial cancers are hormonally sensitive tumors. The presence of estrogen (ER) and progesterone receptors (PR) has been documented in over 90% of endometrioid and about 30% of papillary-serous endometrial carcinomas. Clear-cell carcinomas typically do not express steroid receptors. (Mountzios et al. 2011) The percentage of ER/PR positive tumors decreases among patients with higher grade disease (Kounelis et al. 2000). Progestins have been the cornerstone of hormonal treatment of metastatic endometrial cancer. Response rates usually range from 15% to 20% (Amant et al. 2007, Sjoquist et al. 2011). However, the majority of the trials fail to demonstrate significant beneficial effect on survival with adjuvant progestagen therapy (Macdonald et al. 1988, Martin-Hirsch et al. 2011, von Minckwitz et al. 2002). Overall, hormonal therapy with progestins can be a valid option for patients with low-grade tumors with positive PRs and/or in women with a poor performance status due to the favorable toxicity profile (Mountzios et al. 2011, Temkin & Fleming 2009).
Other forms of adjuvant endocrine therapy are selective estrogen-receptor modulators (SERMs), aromatase inhibitors, and gonadotropin-releasing hormone agonists. Tamoxifen belongs to the SERM family and has been investigated in patients with advanced or recurrent endometrial carcinoma. (Mountzios et al. 2011) Only modest efficacies have been observed, response rates being 10% in phase II studies (Thigpen et al. 2001). The combination of tamoxifen with progestins has also been studied, since tamoxifen induces an increase in PRs, thus enhancing the effect of progestin therapy (Carlson et al. 1984). Although higher response rates have been reported than for progestins alone, no significant beneficial effects on progression-free intervals and overall survival rates have been demonstrated (Fiorica et al. 2004, Whitney et al. 2004). Other hormonal treatment options including aromatase inhibitors (anastrozole) and GnRH agonists (goserelin) have shown only minimal efficacy (Asbury et al. 2002, Rose et al. 2000). Studies combining hormonal therapy with chemotherapy have not shown added benefit compared with chemotherapy alone (Pectasides et al. 2007).

**Targeted therapy**

Advances in the understanding of molecular mechanisms of cancer have led to the development of targeted therapies. Several of these agents, including mammalian target of rapamycin (mTOR) inhibitors, antiangiogenics such as bevacizumab, HER-2 antibody trastuzumab and EGFR inhibitors, are currently under investigation in endometrial carcinoma. (Aghajanian et al. 2011, Fleming et al. 2010, Oza et al. 2008, Slomovitz et al. 2010) So far there is not enough data to assess their significance in the management of endometrial cancer. Investigations into molecular mechanisms and targeted therapies based on individual molecular profiles may be the next step in the treatment of advanced metastatic or recurrent endometrial cancer (Lee 2011).

### 2.2 Tumor invasion and metastasis

The ability to invade other tissues and spread to distant sites is characteristic of cancer cells. Since metastatic spread is the principal cause of death in cancer patients, a better understanding of the process of tumor invasion and metastasis is essential for the development and appropriate clinical use of new therapeutic targets (Chambers & Matrisian 1997, Zitka et al. 2010). Sequential steps necessary for metastasis are similar for all tumor types and include tumor cell
attachment, neovascularization for further growth of tumor, degradation of the basement membrane (BM) with subsequent invasion of malignant cells into the host stroma, intravasation into the blood or lymphatic circulation, extravasation at distant sites, and growth of cells to form metastatic foci in the new environment (Nelson et al. 2000).

During metastasis, tumor cells are involved in numerous interactions with the extracellular matrix (ECM) (Deryugina & Quigley 2006). The extracellular matrix is a dynamic meshwork of interacting components such as collagens, laminin, fibronectin and proteoglycans (Lambert et al. 2004). Proteolytic breakdown of components of the BM or ECM are essential steps in tumor invasion. There are four groups of proteases capable of degrading the BM and/or ECM - the aspartate and cysteine enzymes responsible for intracellular proteolysis, and the serine and metal-atom-dependent enzymes mainly involved in extracellular proteolysis. (Curran & Murray 1999, Zitka et al. 2010) Among these the matrix metalloproteinases are the most extensively studied.

2.3 Matrix metalloproteinases and their inhibitors

2.3.1 Matrix metalloproteinases

Matrix metalloproteinases, also known as matrixins, are a multigene family of zinc-dependent endopeptidases with a capacity to degrade virtually every component of the extracellular matrix (Nagase & Woessner 1999, Roy et al. 2009). In addition to the ECM breakdown, they are able to regulate a wide variety of cell functions, such as cell growth, differentiation, apoptosis, angiogenesis, immune responses, invasion and metastasis by cleaving and releasing bioactive molecules from the cell surface (Lynch & Matrisian 2002, Nagase et al. 2006). MMPs are involved in many physiological processes, including embryonic development, organ morphogenesis and wound healing. Abnormal activation of MMPs has been observed in numerous diseases and pathological processes, such as arthritis, athelosclerosis, neurological diseases, and cancer. (Hua et al. 2011, Nagase & Woessner 1999). To date more than 20 different types of MMPs have been identified and designated with numbers as MMP-1 to MMP-28 (Table 5) (Hua et al. 2011). The MMPs can be divided into two groups based on their cellular location (secreted versus membrane-bound) or into five main groups according to their structure and substrate specificity: collagenases, gelatinases,
stromelysins, matrilysins and membrane-type MMPs (MT-MMPs) (Bourboulia & Stetler-Stevenson 2010). However, the nomenclature is imprecise due to the high degree of overlap among MMP substrate specificities and the fact that they cleave a growing list of nonmatrix substrates as well (Stamenkovic 2003).

Table 5. Matrix metalloproteinase classification and main substrates. (Reproduced from Chakraborti et al. 2003, Murphy & Nagase 2008, Zitka et al. 2010)

<table>
<thead>
<tr>
<th>Enzyme subclass</th>
<th>Enzyme</th>
<th>Main substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>Collagenase 1,</td>
<td>collagens, gelatin, aggrecan, L-selectin, interleukin-1β, proteoglycans, entactin, ovostatin, MMP-2, -9</td>
</tr>
<tr>
<td></td>
<td>Interstitial collagenase</td>
<td></td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase 2,</td>
<td>collagens, gelatin, aggrecan, fibronectin</td>
</tr>
<tr>
<td></td>
<td>Neutrophil collagenase</td>
<td></td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase 3</td>
<td>collagens, gelatin, plasminogen, aggrecan, perlecan, fibronectin, osteonectin, MMP-9</td>
</tr>
<tr>
<td>MMP-18</td>
<td>Collagenase 4</td>
<td>collagens, gelatin, aggrecan</td>
</tr>
<tr>
<td>Gelatinases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>collagens, gelatin, elastin, fibronectin, aggrecan, osteonectin, laminin-1, MMP-1, -9, -13</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>collagens, gelatin, entactin, aggrecan, elastin, fibronectin, osteonectin, plasminogen</td>
</tr>
<tr>
<td>Stromelysins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin 1</td>
<td>collagens, gelatin, aggrecan, perlecan, decorin, laminin, elastin, casein, MMP-2/TIMP-2, MMP-7, -8, -9, -13</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin 2</td>
<td>collagens, gelatin, casein, aggrecan, elastin, MMP-1, -8</td>
</tr>
<tr>
<td>Matrilysins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin 1</td>
<td>collagens, gelatin, aggrecan, decorin, fibronectin, laminin, entactin, elastin, casein, MMP-9/TIMP-1, MMP-1, -2, -9</td>
</tr>
<tr>
<td>MMP-26</td>
<td>Matrilysin 2</td>
<td>unknown</td>
</tr>
<tr>
<td>Membrane-type MMPs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>collagens, gelatin, casein, fibronectin, laminin, vitronectin, entactin, proteoglycans, MMP-2, -13</td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>fibronectin, entactin, laminin, perlecan, MMP-2</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>collagen III, gelatin, casein, fibronectin, MMP-2</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td>unknown</td>
</tr>
<tr>
<td>MMP-24</td>
<td>MT5-MMP</td>
<td>unknown</td>
</tr>
<tr>
<td>MMP-25</td>
<td>MT6-MMP</td>
<td>pro-gelatinase A, fibrin, fibronectin, vitronectin</td>
</tr>
</tbody>
</table>
Enzyme subclass | Enzyme | Main substrates
--- | --- | ---
**MMP**
Other
MMP-12 | Macrophage elastase, Metalloelastase | collagen IV, gelatin, elastin, casein, fibronectin, vitronectin, laminin, entactin, fibrinogen
MMP-19 | RASI-1 (rheumatoid arthritis synovium inflamed-1) | gelatin, aggrecan, fibronectin
MMP-20 | Enamelysin | amelogenin, aggrecan
MMP-21 | XMMMP (Xenopus) | unknown
MMP-22 | CMMMP (Chicken) | unknown
MMP-23 | CA-MMP (Cysteine array) | unknown
MMP-27 | | unknown
MMP-28 | Epilysin | casein

**Structure and regulation of MMPs**

The members of the MMP family have major similarities in their structure. A typical MMP consists of an N-terminal signal sequence (predomain), a propeptide (prodomain), a catalytic metalloproteinase domain, a linker peptide, and a C-terminal hemopexin domain (Curran & Murray 1999, Nagase et al. 2006). The N-terminal predomain is required for the correct secretion of all MMPs and is removed after it directs their synthesis to the endoplasmic reticulum. The predomain is followed by a prodomain that maintains the latency of the enzymes, while the catalytic domain contains the zinc-binding region. Almost all MMPs have a hemopexin/vitronectin-like domain that is connected to the catalytic domain by a linker peptide or hinge region. The exceptions are MMP-7 and MMP-26, simply lacking the linker peptide and hemopexin domain, and MMP-23, which has a cysteine-rich and an immunoglobulin-like domain instead of the hemopexin domain. (Nagase et al. 2006, Sternlicht & Werb 2001) The hemopexin/vitronectin-like C-terminal domain functions as a recognition sequence for the substrates, influences TIMP binding and participates in the MMP activation process. The gelatinases (MMP-2 and MMP-9) further contain three fibronectin type II inserts in the catalytic domain that are required to bind gelatin and collagen. (Fig. 1) (Klein & Bischoff 2011, Sternlicht & Werb 2001)
MMP expression is generally low, which is essential for normal tissue homeostasis. The expression is induced when remodeling of the ECM is required. MMP function is regulated at many levels, including both transcriptional and post-transcriptional levels. The regulation also occurs at the protein level via their activators, inhibitors, and cell surface localization (Sternlicht & Werb 2001). The gene expression is enhanced by several cytokines, growth factors, physical stress, and chemical agents, such as phorbol esters. Suppressive factors, including transforming growth factor β (TGF-β), retinoid acids, and glucocorticoids, may downregulate the MMP expression. Besides the soluble factors, MMP expression can be regulated by cell-cell or cell-matrix interactions, such as extracellular matrix metalloproteinase inducers (EMMPRINs). (Nagase & Woessner 1999) The activator protein (AP) -1 site is known to play an important role in the activation of some MMP genes at transcriptional level. It is also involved in the repression of MMPs by TGF-β, retinoids and glucocorticoids. (Benbow & Brinkerhoff 1997) Post-transcriptionally the MMP gene expression can be regulated via the stabilization of messenger ribonucleic acid (mRNA) in the cytoplasm (Clark et al. 2008).

MMPs are synthesized as inactive proenzymes and need to be processed to become active proteases. The activation of MMPs can be obtained through three diverse mechanisms: by extracellular activation, by intracellular activation or by cell surface activation. Most MMPs are secreted as latent zymogens and activated in extracellular space by proteinases (serine proteases or other MMPs) or by chemical agents, such as thiol-modifying agents, oxidized glutathione, reactive
oxygen and low pH. In all cases the disruption of the Cys-Zn (cysteine switch) interaction is required for the activation. Some MMPs, for example MT-MMPs, contain a furin-like proprotein convertase recognition motif and are activated intracellularly and secreted or cell-surface-bound as active enzymes. (Nagase et al. 2006, Visse & Nagase 2003) The activation of proMMP-2 is an example of cell surface activation (Fig. 2). MT-MMPs, especially MT1-MMP, have an important role in this activation. First, the N-terminal of tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to MT1-MMP and the C-terminal acts as a receptor for proMMP-2, resulting in a trimolecular MT1-MMP*TIMP-2*proMMP-2 complex. Then another MT1-MMP molecule cleaves the propeptide domain of proMMP-2, and activated MMP-2 is released into the extracellular space. Although TIMP-2 is involved in the MMP-2 activation process, it has been found to inhibit MMP-2 in higher concentrations. (Strongin et al. 1995)

![Diagram of MMP activation](image)

**Fig. 2.** Cell surface activation of proMMP-2. Active MT1-MMP (MT-1) on the cell surface binds TIMP-2 (T-2) inhibiting its activity. ProMMP-2 (pM-2) binds to the C-terminal domain of TIMP-2 through its hemopexin domain. Then another active MT1-MMP cleaves the propeptide domain of proMMP-2. Activated MMP-2 (M-2) dissociates from the membrane. (Modified from Sternlicht & Werb 2001).
The activity of MMPs can be controlled by endogenous or exogenous inhibitors. There are two major types of endogenous inhibitors: α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs). α2-macroglobulin is a plasma glycoprotein that inhibits MMPs by entrapping the proteinase within the macroglobulin, and the complex is irreversibly cleared by scavenger receptors. (Nagase et al. 2006, Yang et al. 2001) However, the most thoroughly studied MMP inhibitors are TIMPs, which are discussed in more detail in 2.3.2. Several other proteins have also been reported to inhibit MMPs, including glycosylphosphatidylinositol (GPI)-anchored glycoprotein RECK (reversion-inducing cysteine-rich protein with Kazal motifs), the secreted form of β-amyloid precursor protein, procollagen C-proteinase enhancer protein (PCPE), thrombospondin-2, and tissue factor pathway inhibitor 2 (TFPI-2) (Baker et al. 2002, Higashi & Miyazaki 2003, Takagi et al. 2009). MMPs can be synthetically inhibited by novel drugs designed as MMP inhibitors, such as marimastat and batimastat, or by MMP transcription inhibitors including the epidermal growth factor receptor (EGFR) inhibitor gefitinib, chemically modified tetracyclines, and cyclooxygenase-2 (COX-2) inhibitors (Hua et al. 2011).

**Gelatinases**

Gelatinase A (MMP-2) and gelatinase B (MMP-9) form the subgroup of gelatinases, which are able to degrade several ECM and BM components, including the main component of the BM, type IV collagen. Thus, increased expression of gelatinases is thought to play a critical role in the invasion and metastasis of malignant cells. (Birkedal-Hansen et al. 1993) The development of new vascular system is also essential for expansive tumor growth and metastasis. Gelatinases are involved in angiogenesis by enabling proteolytic degradation of the vascular basement membrane, thus opening the way for endothelial cells to migrate and form new blood vessels (Klein & Bischoff 2011). They can also trigger angiogenic switch and promote angiogenesis by increasing the bioavailability of the pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and TGF-β (Bergers et al. 2000, Fang et al. 2000, Yu & Stamenkovic 2000). In MMP-2- or MMP-9-deficient mice reduced angiogenesis has been shown, suggesting a central role for gelatinases in tumor neovascularization (Huang et al. 2002, Itoh et al. 1998). However, gelatinases can have anti-angiogenic activities as well by releasing inhibitors of angiogenesis, such as angiostatin, in the proteolytic cleavage (Patterson & Sang 1997).
effect of gelatinases on angiogenesis may thus be pro- or anti-angiogenic, depending on which enzyme cascade is activated.

MMP-2 was first introduced as a type IV collagenase in metastatic murine tumors in the late 1970s by Liotta et al. (1979). The role of MMP-2 in BM degradation was soon confirmed by many studies (Liotta et al. 1981, Salo et al. 1983, Turpeenniemi-Hujanen et al. 1985). MMP-2 is expressed in many connective tissue cells, including fibroblasts, endothelial cells, keratinocytes and osteoblasts, as well as by many malignant cell lines (Birkedal-Hansen et al. 1993, Liotta et al. 1979, Salo et al. 1991). MMP-2 is secreted as a 72-kilodalton (kDa) inactive proenzyme and converted to a 62-kDa active enzyme by cleaving the prodomain (Strongin et al. 1995). MMP-2 is expressed constitutively and most pro-inflammatory stimuli fail to increase the expression, due to the lack of binding sites for pro-inflammatory transcription factors, such as AP-1 (Klein & Bischoff 2011). The main activation process occurs post-transcriptionally, unlike for the majority of MMPs that are mostly activated at the transcriptional level. The most important MMP-2 activation pathway is thought to be the MT1-MMP-mediated activation on the cell surface as described before. MMP-2 can also autoactivate itself, leading to a formation of multiple smaller activation products (Bergmann et al. 1995).

MMP-9 was first identified as a gelatin-binding protein secreted by human macrophages (Vartio et al. 1982). Unlike MMP-2, MMP-9 is expressed by only a few cell types including keratinocytes, trophoblasts, osteoclasts, macrophages, leucocytes and dendritic cells (Opdenakker et al. 2001b, Sternlicht & Werb 2001). MMP-9 expression is also highly inducible and regulated at the transcriptional level by growth factors, chemokines and other stimulatory signals in contrast to MMP-2. MMP-9 is secreted as a 92-kDa proenzyme and activated to an 83-kDa mature enzyme (Klein & Bischoff 2011). The basic structure is quite similar to that of MMP-2 with the fibronectin-like domain for gelatin and collagen binding, but it has additionally a unique collagen V-like insertion between the catalytic domain and the C-terminal domain, which probably provides the attachment sites of O-linked oligosaccharides (Opdenakker et al. 2001a).

### 2.3.2 Tissue inhibitors of metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) are considered some of the most important regulators of metalloproteinase activity. So far there are four TIMPs identified in humans, designated as TIMP-1, -2, -3 and -4. (Fassina et al. 2000)
TIMPs consist of 184–194 amino acids and have a molecular mass ranging from 21 to 34 kDa (Lambert et al. 2004, Nagase et al. 2006). They share substantial sequence homology and structural identity at the protein level (Stetler-Stevenson 2008). The basic structure of TIMPs consists of two domains, N- and C-terminal subdomains, each containing six conserved cysteine residues forming three disulfide bonds. The N-terminal domain is responsible for the MMP inhibitory activity, whereas the C-terminal of TIMPs is important for protein-protein interactions and binding to pro-MMPs. (Chirco et al. 2006)

Many external stimuli induce the expression of TIMP-1, whereas TIMP-2 expression is constitutive (Lambert et al. 2004). Growth factors, such as basic fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF), phorbol esters, oncostatin, retinoids, and cytokines, including interleukin (IL)-1, -6, and -1β, have all been reported to stimulate TIMP-1 expression in various cell types (Denhardt et al. 1993, Gomez et al. 1997). In addition, erythropoietin has been described to induce TIMP-1 secretion (Kadri et al. 2000). Tumor necrosis factor has been demonstrated to stimulate TIMP-1 production at low concentrations and suppress it in a dose-dependent manner at higher concentrations. Other agents suppressing TIMP-1 production are concanavalin A and dexamethasone. (Gomez et al. 1997)

TIMP-1 induction occurs primarily at the transcriptional level (Denhardt et al. 1993). Several DNA response elements in the 5’ region of the gene, such as a TPA-responsive element, AP-1, and IL-6/oncostatin M sites, control the transcription of TIMP-1. The AP-1 site can be activated by many of the same agents that activate collagenase, suggesting that it may have a critical role in the coordination of both MMP and TIMP genes induction. (Lambert et al. 2004) However, it seems that the expressions of MMPs and TIMPs can be regulated either in a coordinated or in a reciprocal manner (Overall 1994). Phorbol esters and interleukin have been found to upregulate both TIMP-1 and MMP expression (Overall 1994), whereas TGF-β and retinoids downregulate MMP and upregulate TIMP-1 expression (Clark et al. 1987, Overall et al. 1991). In addition, erythropoietin has also been demonstrated to regulate the expression of MMP-9 and TIMP-1 in a reciprocal manner (Kadri et al. 2000).

Hormonal regulation of TIMPs has been investigated in a few studies, but the results are conflicting. Progesterone and estradiol-17β have been found to stimulate the expression of TIMPs (Sakyo et al. 1986, Sato et al. 1991), but
opposite results have also been reported (van den Brule et al. 1992). Nevertheless, progesterone seems to be more effective than estrogen in the hormonal control of TIMPs. Luteinizing hormones (LH) and human chorionic gonadotropins (hCG) have also been demonstrated to upregulate the expression of TIMPs (Lind et al. 2006, Mann et al. 1993).

**MMP-dependent and -independent functions of TIMPs**

TIMPs inhibit MMPs proteolytic activity by forming 1:1 reversible noncovalent stoichiometric complexes. Although all TIMPs inhibit the proteolytic activity of MMPs, they diverge in many aspects including solubility, regulation of expression and interaction with the proenzymes (pro-MMPs). TIMP-1, -2 and -4 are present in soluble forms, whereas TIMP-3 is bound to the ECM. (Lambert et al. 2004) They are capable of inhibiting all MMPs, but TIMP-1 is a poor inhibitor of the MT-MMPs and MMP-19 (Baker et al. 2002). TIMP-1 prefers binding to proMMP-9, while TIMP-2 forms a preferential complex with proMMP-2, and TIMP-4 can also bind to the C-terminal domain of proMMP-2. TIMP-3 binds to both proMMP-2 and -9, but can also inhibit some members of the “disintegrin and metalloproteinase” (ADAM) family. (Lambert et al. 2004) TIMP-2 expression is widely expressed in the body, whereas TIMP-1, -3, and -4 expressions are often tissue-specific. TIMP-1 is expressed predominantly in reproductive organs, and TIMP-3 expression is found in the heart, kidney, and thymus. High TIMP-4 expression levels have been detected in the brain, heart, ovary, and skeletal muscle. (Crocker et al. 2004, Leco et al. 1997)

Originally, TIMPs were described solely as inhibitors of proteolysis. Numerous studies have reported a wide variety of other functions for TIMPs, including cell growth, angiogenesis, and apoptosis. Some of these functions are attributed to MMP inhibition, but TIMPs also exhibit cellular activities that seem to be independent of MMP inhibition. (Lambert et al. 2004) TIMP-1 and TIMP-2 were first characterized as proteins with erythroid-potentiating activity (EPA) (Hayakawa et al. 1992, Stetler-Stevenson et al. 1992). Since then, the growth-promoting effects of TIMP-1 and TIMP-2 have been extended to a wide range of cells, including keratinocytes, chondrocytes, fibroblasts, epithelial and endothelial cells, breast cancer cells, osteosarcoma cells, and fibrosarcoma cells (Lambert et al. 2004). These cell growth-promoting activities seem to be independent of MMP inhibition, and occur concentration-dependently. It has been shown that alkylated forms of TIMP-1 and -2 lacking the MMP inhibitory activity still maintain their
cell growth-promoting capacity. (Hayakawa et al. 1992, Hayakawa et al. 1994) Conflicting effects on growth have also been described, since TIMP-1 and -2 have also been demonstrated to inhibit the proliferation of endothelial and carcinoma cells (Lambert et al. 2004). These findings support the theory that TIMPs function in a contextual fashion so that the mechanism of action depends on the tissue microenvironment (Stetler-Stevenson 2008).

In addition to the growth-promoting activity, TIMPs can also regulate cell apoptosis. A traditional mechanism through which TIMPs modulate cell survival is by inhibiting MMP activity. However, a mounting literature has demonstrated TIMPs, especially TIMP-1, to regulate apoptosis independently of MMP inhibition. (Lambert et al. 2004) The anti-apoptotic activity of TIMP-1 has been demonstrated in human Burkitt’s lymphoma cell lines (Guedez et al. 1998). TIMP-1 expression has also been associated with resistance to apoptosis in normal tonsillar B cells and MCF10A malignant human breast epithelial cells (Li et al. 1999, Stetler-Stevenson et al. 1997). The anti-apoptotic activity is not restricted to TIMP-1, since TIMP-2 and TIMP-4 have been shown to suppress apoptosis (Jiang et al. 2001, Valente et al. 1998). TIMP-3, in contrast, may either promote (Ahonen et al. 1998) or inhibit (Fata et al. 2001) apoptosis.

TIMPs also participate in the regulation of angiogenesis. Involvement of TIMPs in angiogenesis was first demonstrated in chick embryo yolk-sac membranes where TIMP-1 and TIMP-2 inhibited polyamines-induced angiogenesis (Takigawa et al. 1990). Subsequently, at least TIMP-1, TIMP-2 and TIMP-3 have been shown inhibit angiogenesis. TIMP-1 has been demonstrated to inhibit endothelial cell migration both MMP-dependently and MMP-independently (Akahane et al. 2004). TIMP-2 seems to inhibit growth factor-stimulated endothelial cell proliferation independently of MMP inhibition and possibly mediated by cell surface receptor mechanism (Stetler-Stevenson & Seo 2005). TIMP-3 may inhibit angiogenesis by blocking the binding of VEGF to VEGF receptor-2 independently of its MMP-inhibitory activity (Qi et al. 2003). Relatively little is known about the role of TIMP-4 in neovascularization, and it has been suggested that it may not even modulate angiogenesis (Fernandez & Moses 2006). TIMPs have also been associated with steroidogenesis. Especially TIMP-1 has been shown to stimulate steroidogenesis, regulate steroid concentrations, and thus regulate germ cell development in both males and females (Boujrad et al. 1995). Additionally, TIMPs have also been shown to be involved in embryogenic activity (Behrendtansen & Werb 1997).
**Tissue inhibitor of metalloproteinase-1**

TIMP-1 is a soluble, glycosylated protein with a molecular mass ranging from 28.5 to 34 kDa, depending on the degree of glycosylation (Lambert et al. 2004). It was originally isolated from rabbit bone and characterized as a collagenase inhibitor (Sellers et al. 1979). Human TIMP-1 is located in the X chromosome and is encoded as a 0.9 kilobase (kb) mRNA. TIMP-1 is expressed by a variety of cultured cell types including fibroblasts, epithelial and endothelial cells, osteoblasts, chondrocytes, smooth muscle cells, and many tumor cells. (Lambert et al. 2004) Its expression is responsive to several external stimuli (Gomez et al. 1997). TIMP-1 inhibits MMP-9 by binding the hemopexin domain of proMMP-9, but it is not able to interact with MMP-2, because TIMP-1 lacks the critical C-terminal MMP-2-interacting residues that are present in TIMP-2 (Morgunova et al. 2002).

**Tissue inhibitor of metalloproteinase-2**

TIMP-2 is a soluble unglycosylated protein with a molecular mass of 21 kDa, sharing approximately 40% sequence identity with TIMP-1 (Fassina et al. 2000, Nagase et al. 2006). It was first described and sequenced in human melanoma cells in 1989 (Stetler-Stevenson et al. 1989). TIMP-2 is located in chromosome 17, and encoded as two transcripts of 3.5 and 1.0 kb. In contrast to TIMP-1, the expression of the TIMP-2 gene is typically constitutive. (Gomez et al. 1997). Activation of proMMP-2 has been indicated as the dominant function of TIMP-2 and in fact, TIMP-2 is required for efficient activation of proMMP-2 (Wang et al. 2000). Low concentrations of TIMP-2 have been associated with MMP-2 activation, and high concentrations with MMP-2 inhibition (Kinoshita et al. 1998, Kurschat et al. 1999). Unlike TIMP-1, TIMP-2 is also an effective inhibitor of the MT-MMPs (Albini et al. 1991). The molecular characteristics of TIMPs are summarized in Table 6.
Table 6. Molecular characteristics of TIMPs (modified from Lambert et al. 2004).

<table>
<thead>
<tr>
<th>Protein feature</th>
<th>TIMP-1</th>
<th>TIMP-2</th>
<th>TIMP-3</th>
<th>TIMP-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal location</td>
<td>Xp</td>
<td>17q</td>
<td>22q</td>
<td>3p</td>
</tr>
<tr>
<td>mRNA (kb)</td>
<td>0.9</td>
<td>3.5 – 1.0</td>
<td>5.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
<td>28.5</td>
<td>21</td>
<td>22/27</td>
<td>22</td>
</tr>
<tr>
<td>Protein localization</td>
<td>soluble</td>
<td>soluble/cell</td>
<td>ECM</td>
<td>soluble/cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td>surface</td>
<td></td>
<td>surface</td>
</tr>
<tr>
<td>Protein expression</td>
<td>inducible</td>
<td>constitutive</td>
<td>inducible</td>
<td>inducible</td>
</tr>
<tr>
<td>ProMMP association</td>
<td>proMMP-9</td>
<td>proMMP-2</td>
<td>proMMP-2/-9</td>
<td>proMMP-2</td>
</tr>
<tr>
<td>MMPs poorly inhibited</td>
<td>MT1-MMP</td>
<td>MT2-MMP</td>
<td>MT3-MMP</td>
<td>MT5-MMP</td>
</tr>
</tbody>
</table>

2.4 MMP-2, MMP-9 and their tissue inhibitors in cancer

An imbalance between MMP and TIMP activities results in an excessive ECM degradation that has been implicated in tumor invasion and metastasis. Accumulating evidence suggests a much more complex, even controversial, role for MMPs and TIMPs during tumor progression, in addition to their regulation of the ECM breakdown. Recent findings have emphasized the importance of the tumor microenvironment in determining the effects of MMPs and TIMPs on cell fate (Noël et al. 2008, Stetler-Stevenson 2008).

MMPs have been implicated in all aspects of cancer progression and dissemination (Noël et al. 2008). Their expression has been found to be increased and to correlate with invasiveness and poor prognosis in virtually all cancer types. Especially MMP-2 and MMP-9 have been linked to aggressive clinical behavior in different tumor types. MMP-2 overexpression has been found to correlate with a more aggressive phenotype and/or poor prognosis in breast carcinoma (Sivula et al. 2005, Talvensaari-Mattila et al. 2003), ovarian carcinoma (Garzetti et al. 1995, Westerlund et al. 1999), cervical cancer (Rauvala et al. 2006), head and neck squamous cell carcinoma (HNSCC) (Ruokolainen et al. 2006), lung cancer (Leinonen et al. 2008), melanoma (Väisänen et al. 1998, Väisänen et al. 2008), colorectal (Hiilska et al. 2007) and bladder cancer (Vasala et al. 2003). In contrast, in hematological malignancies tissue MMP-2 seems to have favorable prognostic value (Kuittinen et al. 1999, Kuittinen et al. 2002).

The prognostic role of MMP-9 is somewhat more contradictory than the role of MMP-2. Tissue expression of MMP-9 has been associated with poor survival
in lung cancer (Cox et al. 2000, Sienel et al. 2003), HNSCC (Ruokolainen et al. 2004) and gastric carcinoma (Sier et al. 1996). In bladder cancer, in contrast, tissue MMP-9 overexpression has been found to be an independent marker of favorable prognosis (Vasala et al. 2008b). Especially in breast carcinoma the results concerning the prognostic role of tissue MMP-9 are conflicting. MMP-9 has been suggested to be an independent favorable prognostic factor in node-negative patients by Scorilas et al. (2001), while in a study by Rahko et al. (2004) MMP-9 had only modest prognostic value in breast carcinoma, since MMP-9 positivity was associated with early recurrence only in a patient subgroup with ER-negative tumors. The prognostic significance of MMP-9 may also vary according to its origin. Pellikainen et al. (2004) reported that positive stromal MMP-9 expression predicted shortened survival in hormone-responsive small tumors, whereas carcinoma cell MMP-9 positivity was associated with a favorable survival.

The effect of tissue inhibitors of metalloproteinases on the prognosis of different cancer types is somewhat complex as well. TIMP-1 tissue expression has been associated with shortened survival in breast carcinoma (Kuvaja et al. 2005), and similar results have also been reported in renal cell carcinoma, lung cancer, and HNSCC (Aljada et al. 2004, Kallakury et al. 2001, Ruokolainen et al. 2005b). A contradictory prognostic role for TIMP-1 has also been suggested at least in breast cancer, where TIMP-1 positivity in cancer cells was shown to be an independent indicator of favorable prognosis (Nakopoulou et al. 2003).

TIMP-2 studies have also given conflicting results concerning the relationship between TIMP-2 expression and prognosis. High TIMP-2 expression has been associated with poor survival in HNSCC, bladder cancer, and cervical carcinoma (Davidson et al. 1999, Grignon et al. 1996, Kanayama et al. 1998, Ruokolainen et al. 2006). On the other hand, correlation between tissue TIMP-2 positivity and patients’ favorable outcome was observed in gastric carcinoma (Grigioni et al. 1994) and pancreatic ductal adenocarcinoma (Giannopoulos et al. 2008). In breast carcinoma, TIMP-2 immunoreactive protein has been linked with favorable (Nakopoulou et al. 2002) as well as with adverse prognosis (Visscher et al. 1994) and distant metastases (Garcia et al. 2010, Vizoso et al. 2007).

Due to their relevant role in tumor invasion and metastasis, inhibition of MMP activity has been under investigation as a method of preventing or decreasing tumor spread. Unfortunately, clinical trials using synthetic metalloproteinase inhibitors in different cancer types have thus far failed to live up to expectations (Overall & Kleifeld 2006). There have been some promising
results (Batist et al. 2002), but in many cases, despite the decrease in biomarker levels, positive clinical outcomes were not necessarily observed (Roy et al. 2009). Part of the explanation for the failure of the MMP inhibitors in clinical trials may be the adverse effects that have limited the maximum-tolerated dose, thereby limiting drug efficacy. Secondly, the MMP inhibitors used in trials have not been selective, and therefore they may have affected a wide range of MMPs and physiological events in an unwelcome way. Finally, the patients recruited in the trials had advanced metastatic diseases, so it remains an open question to what extent tumors rely on MMP activity at such late stages. Therapy at earlier stages of cancer progression might enable the use of lower drug doses and thereby perhaps limit the toxicity. (Roy et al. 2009, Stamenkovic 2003)

2.4.1 MMP-2, MMP-9 and their tissue inhibitors in endometrial carcinoma

There are only a few studies concerning the role of MMP-2, MMP-9 and their tissue inhibitors in endometrial carcinoma. In immunohistochemical studies, epithelial tumor cells have been the main site of MMP-2 and MMP-9 proteins (Di Nezza et al. 2002, Inoue et al. 1997), whereas mRNA of MMP-2 and -9 is mainly found in stromal cells (Iurlaro et al. 1999, Park et al. 2001, Soini et al. 1997). TIMP-1 mRNA has been shown to be expressed by stromal cells, whereas TIMP-2 mRNA has also been present in carcinoma cells (Määttä et al. 2000). Epithelial tumor cells have been the main expression site of TIMP-1 and -2 in immunohistochemical studies (Amalinei et al. 2011). The expression of MMP-2 and -9 in endometrial tumors has been shown to be upregulated in endometrial carcinoma compared to benign endometrium (Iurlaro et al. 1999, Laird et al. 1999, Lopata et al. 2003) as well as to endometriosis (Weigel et al. 2012). Enhanced expression of TIMP-1 and -2 is also detected in endometrial carcinoma compared to benign endometrium, especially in less differentiated carcinomas (Määttä et al. 2000). In contrast, Amalinei et al. (2011) reported decreased TIMP-1 expression in neoplastic endometrium compared to non-neoplastic endometrium.

The role of MMP-2 and MMP-9 in endometrial carcinoma

The results on correlation between clinicopathological parameters and the expression of MMP-2 and -9 are not unanimous (Table 7). Upregulation of MMP-2 has been associated with increasing histologic grade (Aglund et al. 2004, Di
Nezza et al. 2002, Graesslin et al. 2006a, Graesslin et al. 2006b, Guo et al. 2002, Iurlaro et al. 1999). On the other hand, in some studies no correlation between MMP-2 and the grade of the tumor has been observed (Lopata et al. 2003, Talvensaari-Mattila et al. 2005, Yilmaz et al. 2011). Increasing MMP-9 protein production has been associated with advanced histologic grade in some studies (Aglund et al. 2004, Di Nezza et al. 2002, Guo et al. 2002, Iurlaro et al. 1999), whereas Lopata et al. (2003) reported no such correlation. MMP-9 correlated with stage in a study by Aglund et al. (2004). Association with the depth of myometrial invasion and MMP-9 (Di Nezza et al. 2002) or both gelatinases (Iurlaro et al. 1999, Zheng et al. 2002) has been found in several studies, but opposite results have also been reported (Aglund et al. 2004, Lopata et al. 2003, Shaco-Levy et al. 2008). Overexpression of MMP-2 and MMP-9 has been correlated to lymphatic and vascular invasion by Karahan et al. (2007), while in the work of Di Nezza et al. (2002) similar associations with vascular/lymphatic invasion were seen for MMP-9, but not for MMP-2. However, most studies show no correlation between gelatinase expression and lymphovascular space invasion (Graesslin et al. 2006b, Lopata et al. 2003, Yilmaz et al. 2011, Zheng et al. 2002). MMP-2 has been associated with lymph node metastasis (Graesslin et al. 2006b), whereas Lopata et al. (2003) found correlation between MMP-9, but not MMP-2, and nodal metastasis.

A few studies have looked for differences in the expression of gelatinases between the different histological tumor types of endometrial cancer with somewhat conflicting results. Increased MMP-2 expression has been associated with serous histology (Shaco-Levy et al. 2008). In contrast, Monaghan et al. (2007) noted significantly higher expression of MMP-2 and MMP-9 in endometrioid tumors compared to serous tumor types. On the other hand, Graesslin et al. (2006b) and Yilmaz et al. (2011) did not find any statistically significant differences in MMP-2 and/or MMP-9 expression according to the histologic subtype.

Survival analyses have rarely been conducted in previous studies. In a study by Talvensaari-Mattila et al. (2005), MMP-2 immunoreactive protein was associated with more aggressive disease with a somewhat increased risk. However, Moser et al. (1999) and Yilmaz et al. (2011) found no statistically significant correlation between MMP-2 and overall survival, and MMP-2 was considered not to be useful in predicting the prognosis of endometrial carcinoma patients. In a study by Aglund et al. (2004), an association between gelatinases and survival was seen, even though it was not statistically significant. They
further reported that in stage I disease, strong MMP-2 predicted four times poorer outcome than in MMP-2 negative cases (Aglund et al. 2004). For MMP-9, statistically significant associations with overall survival have not been observed (Aglund et al. 2004, Inoue et al. 1997).
Table 7. Summary of correlations between clinicopathological parameters and MMP-2 and MMP-9 expression in endometrial carcinoma studies.

<table>
<thead>
<tr>
<th>Author</th>
<th>n</th>
<th>Method</th>
<th>Stage</th>
<th>Grade</th>
<th>Myometrial invasion</th>
<th>LVSI</th>
<th>Lymph node metastasis</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MMP-2 MMP-9</td>
<td>MMP-2 MMP-9</td>
<td>MMP-2 MMP-9</td>
<td>MMP-2 MMP-9</td>
<td>MMP-2 MMP-9</td>
<td>MMP-2 MMP-9</td>
<td>MMP-2 MMP-9</td>
</tr>
<tr>
<td>Glund et al. 2004</td>
<td>88</td>
<td>IHC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Di Nezza et al. 2002</td>
<td>29</td>
<td>IHC, ISH, ISZ</td>
<td>↑</td>
<td>↑</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Graesslin et al. 2006a</td>
<td>38</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Graesslin et al. 2006b</td>
<td>50</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>Guo et al. 2002</td>
<td>37</td>
<td>IHC</td>
<td>↑</td>
<td>↑</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inoue et al. 1997</td>
<td>129</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Iurlo et al. 1999</td>
<td>64</td>
<td>IHC, ISH</td>
<td>↑</td>
<td>↑</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Karahan et al. 2007</td>
<td>42</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lopata et al. 2003</td>
<td>95</td>
<td>gelatine zymography</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Moser et al. 1999</td>
<td>103</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Shaco-Levy et al. 2008</td>
<td>29</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Talvensaari-Mattila et al. 2005</td>
<td>112</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Yilmaz et al. 2011</td>
<td>95</td>
<td>IHC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Zheng et al. 2002</td>
<td>31</td>
<td>ISZ, IHC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

IHC=immunohistochemistry, ISH=in situ hybridization, ISZ=in situ zymography, NS=not significant
The role of TIMPs in endometrial carcinoma

TIMPs are less studied molecules in endometrial cancer, and their prognostic role is far from clear. TIMP-2 downregulation has been related to high-grade endometrial cancers (Ferguson et al. 2004, Graesslin et al. 2006a, Graesslin et al. 2006b). For TIMP-1, Graesslin et al. (2006b) observed a tendency of declining expression as histological grade rose, but the difference did not reach statistical significance. In contrast, Guo et al. (2002) reported increasing TIMP-1 levels with the histologic grade of endometrial carcinoma. Low expression of TIMP-2 has been associated with the depth of myometrial invasion, lymphovascular space involvement and lymph node metastasis (Graesslin et al. 2006a, Graesslin et al. 2006b). For TIMP-1, no relations between its expression and FIGO stage, myometrial invasion or vascular/lymphatic invasion have been reported (Graesslin et al. 2006b, Obokata et al. 2007). However, increased TIMP-1 expression has been associated with endocervical invasion in one study (Shaco-Levy et al. 2008).

The expression of TIMPs has differed according to the histologic type. TIMP-2 expression has been found to be lower in serous and clear cell endometrial carcinomas than in endometrioid adenocarcinoma (Graesslin et al. 2006b). Nonendometrioid cancer types carry a worse prognosis, suggesting that low TIMP-2 could be linked to the aggressiveness of type II endometrial tumors. On the other hand, TIMP-1 has been shown to be higher in endometrial serous carcinomas as compared to endometrioid adenocarcinomas (Shaco-Levy et al. 2008), although in the study by Graesslin et al. (2006b) TIMP-1 did not differ according to the histologic type.

Data concerning the effect of TIMP-1 and TIMP-2 on survival are limited. TIMP-1 expression has not been correlated with recurrence or death from the disease, but proper survival analyses, i.e., Kaplan-Meier method or Cox regression analysis, were not performed (Graesslin et al. 2006a, Graesslin et al. 2006b, Obokata et al. 2007, Shaco-Levy et al. 2008). The same applies to TIMP-2, for which no correlations between its expression and survival were seen (Graesslin et al. 2006a, Graesslin et al. 2006b). The only study that conducted survival analyses was by Moser et al. (1999), in which TIMP-2 was not associated with overall survival in endometrial carcinoma.
2.5 MMP-2, MMP-9 and their tissue inhibitors as circulating markers

The potential of circulating MMP-2, MMP-9 and their tissue inhibitors as prognostic markers in solid malignant tumors has been under evaluation in recent years. Several studies have reported higher levels of circulating gelatinases and their tissue inhibitors in cancer patients compared with healthy controls (Holten-Andersen et al. 1999, La Rocca et al. 2004, Oberg et al. 2000).

Circulating gelatinases and their tissue inhibitors can be measured from plasma or serum, preoperatively or postoperatively. In preoperative serum samples, high MMP-2 levels were reported to correlate with advanced stage and lymph node status in breast cancer (Sheen-Chen et al. 2001). In addition, high serum postoperative MMP-2 level in breast cancer patients has been observed to predict worse overall and disease-free survival (Leppä et al. 2004). In a study by Kuvaja et al. (2006), an association between low circulating levels of active MMP-2 in the preoperative serum of breast cancer and shorter relapse-free survival was seen, while low levels of proMMP-2 correlated with high grade and stage. In colorectal carcinoma, high preoperative serum MMP-2 was associated with shorter survival (Oberg et al. 2000). Also in urothelial carcinomas a correlation between elevated proMMP-2 levels and tumor recurrence has been found (Gohji et al. 1996). In contrast, low serum levels of pro-MMP-2 and MMP-2/TIMP-2 complex were found to correlate with adverse prognosis in bladder cancer (Vasala et al. 2008a, Vasala & Turpeenniemi-Hujanen 2007).

Low preoperative serum levels of MMP-9 have been associated with a three-fold risk of relapse in primary breast carcinoma (Talvensaari-Mattila & Turpeenniemi-Hujanen 2005b). On the contrary, high levels of preoperative MMP-9 have been reported to correlate with shortened relapse-free and cause-specific survival in HNSCC (Ruokolainen et al. 2005a). High serum levels of MMP-9 have also been associated with poor survival in lung cancer (Ylisirniö et al. 2000).

High preoperative circulating levels of TIMP-1 have been found to indicate a more aggressive clinical course and poor survival at least in breast, ovarian, lung, colorectal and head and neck carcinomas (Holten-Andersen et al. 2000, Rauvala et al. 2005, Ruokolainen et al. 2005b, Talvensaari-Mattila & Turpeenniemi-Hujanen 2005a, Ylisirniö et al. 2000). Furthermore, preoperative plasma TIMP-1 was found to be an independent prognostic factor for early systemic relapse in
primary breast carcinoma, whereas the postoperative levels of TIMP-1 were not prognostic for relapse (Kuvaja et al. 2008).

The data on the prognostic value of circulating TIMP-2 are more limited. In breast cancer, high levels of circulating TIMP-2 have been found to correlate with adverse prognosis (Remacle et al. 2000). In contrast, in bladder cancer high levels of circulating TIMP-2 were associated with a favorable prognosis (Vasala & Turpeenniemi-Hujanen 2007).

There is only one study so far concerning the role of circulating gelatinases and their tissue inhibitors in endometrial carcinoma. Adamiak et al. (2000) found serum MMP-2 levels to be statistically higher in clinically advanced stages of endometrial carcinoma in a small Polish patient series in a study of which only the abstract is available in English.
3 Aims of the present study

The expression of gelatinases and their tissue inhibitors has been associated with the clinical course in a wide variety of solid cancers. Only few studies have attempted to explore the role of gelatinases and their tissue inhibitors as prognostic factors in patients with endometrial carcinoma with somewhat conflicting results.

The specific aims of this study were:

1. To evaluate the significance of the tissue expression of MMP-2 and MMP-9 as prognostic markers in endometrial carcinoma and their associations with conventional prognostic markers.
2. To examine the prognostic and clinical implications of the tissue expression of TIMP-1 and TIMP-2 in endometrial carcinoma.
3. To evaluate the effect of MMP-2 and TIMP-2 immunoreactive protein combinations on the prognosis of endometrial carcinoma. In addition, to find out whether MMP-2 or TIMP-2 is a more powerful prognostic factor in endometrial cancer.
4. To evaluate the circulating levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex, and to study their connections with the clinical behavior of endometrial cancer.
4 Materials and methods

4.1 Patients

The patient material consisted of a total of 266 women diagnosed with primary endometrial carcinoma in Oulu University Hospital between the years 1992 and 2000. The histological material of endometrial carcinoma lesions was obtained during routine diagnostic procedures. Formalin-fixed, paraffin-embedded tumor samples were used for the study. Venous blood samples were obtained from 93 out of the 266 patients before the operation or adjuvant treatments.

The median age of the patients was 65 years (range 37–98). All the cases were staged according to the FIGO (1988) criteria. Description of the patients’ demographic data is presented in Table 8.

The treatment strategies for endometrial carcinoma patients were carried out according to the local protocol for treatment. In most cases, the primary treatments were extrafascial hysterectomy, bilateral salpingo-oophorectomy and pelvic lymphadenectomy. Preoperative cisplatin-based chemotherapy was given to five patients, while 57 patients received adjuvant chemotherapy postoperatively. Thirty-five patients had postoperative vaginal cuff brachytherapy and 140 patients underwent postoperative external whole pelvic irradiation.

The patients were followed up for a minimum of five years, the median follow-up time being 79 months (range 0–136). Immunostaining of the cancer tissue samples was performed for cases where a representative amount of tissue was available: in 266 cases for MMP-2 (I, III), in 261 cases for MMP-9 (I, III), in 230 cases for TIMP-1 (II, III) and in 241 cases for TIMP-2 (II, III). For MMPP-2, TIMP-2 and MMP-2/TIMP-2 complex ELISA analyses pretreatment serum samples from 93 cases were available, whereas the number of available serum samples for MMP-9 analysis and TIMP-1 analysis was 90 and 90, respectively (IV).
Table 8. Clinical data of the endometrial carcinoma patients (n=266).

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>179</td>
<td>67</td>
</tr>
<tr>
<td>IA</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>38</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>42</td>
<td>16</td>
</tr>
<tr>
<td>IV</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>135</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>92</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma</td>
<td>249</td>
<td>94</td>
</tr>
<tr>
<td>Adenoacanthoma</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Serous papillary</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Clear cell</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td>266</td>
<td>100</td>
</tr>
<tr>
<td>TAH+BSO without lymphadenectomy</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>TAH+BSO+pelvic lymphadenectomy</td>
<td>260</td>
<td>98</td>
</tr>
<tr>
<td>TAH+BSO+pelvic&amp;para-aortic lymphadenectomy</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>175</td>
<td>66</td>
</tr>
<tr>
<td>Chemotherapy</td>
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<td>23</td>
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<tr>
<td>Recurrences</td>
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<td>15</td>
</tr>
<tr>
<td><strong>Status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>196</td>
<td>74</td>
</tr>
<tr>
<td>Death from disease</td>
<td>42</td>
<td>16</td>
</tr>
<tr>
<td>Death from another cause</td>
<td>28</td>
<td>11</td>
</tr>
</tbody>
</table>

TAH+BSO=total abdominal hysterectomy + bilateral salpingo-oophorectomy

4.2 Immunohistochemistry

4.2.1 Staining protocol

The endometrium tissue samples from the primary operation were fixed in formalin and embedded in paraffin. Paraffin-embedded tissues were cut into 4µm
slices and incubated at 37°C for at least 4 hours, usually overnight, deparaffinized in a histological clearing agent, Histo-Clear (National Diagnostics, Atlanta, GA, USA) and hydrated in descending alcohol series. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide/absolute methanol in MMP-2 and MMP-9 stainings, and in 3% hydrogen peroxide/distilled water in TIMP-1 and TIMP-2 stainings. Non-specific binding was blocked with goat serum in MMP-2 and MMP-9 stainings, while Antibody Diluent (DakoCytomaton, Inc., Glostrup, Denmark) was used when diluting the primary TIMP-1 and TIMP-2 antibody.

The primary antibodies, antibody dilutions and the incubation times used in this study are listed in Table 9. The specimens were incubated in a humidity chamber at room temperature for 20h in MMP-2 and MMP-9 stainings and for one hour in TIMP-1 and TIMP-2 stainings. The immunohistochemical staining was continued using the Histostain bulk kit (Zymed Laboratory, San Francisco, CA, USA) in MMP-2 and MMP-9 stainings and LSAB2 System-HRP kit (Dako, DakoCytomation, Inc., Carpinteria, CA, USA) in TIMP-1 and TIMP-2 stainings according to the manufacturer’s instructions. Biotinylated antihomous immunoglobulin IgG was used as a second antibody, and the peroxidase was introduced using a streptavidin conjugate. The slides were washed thoroughly with phosphate-buffered saline (PBS) after each stage of the procedure. The antibody reaction was visualized by using a fresh substrate solution containing aminoethyl carbazol substrate kit (AEC Sigma) in MMP-2 and MMP-9 stainings and 3-3’ diaminobenzidine (DAB) in TIMP-1 and TIMP-2 stainings. The sections were counterstained with hematoxylin, dehydrated and mounted in Immu-Mount (Shanon, Pittsburgh, PA, USA) in MMP-2 and MMP-9 stainings and in Histomount (National Diagnostics, New Jersey, USA) in TIMP-1 and TIMP-2 stainings. For negative controls, the primary antibody was replaced by PBS. Each set of staining also included a control sample of endometrial carcinoma tissue previously known as positive for MMP-2, MMP-9, TIMP-1 or TIMP-2.
Table 9. Antigens and their respective antibodies used in immunohistochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Concentration</th>
<th>Incubation time</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>mouse monoclonal</td>
<td>5 µg/ml</td>
<td>20h</td>
<td>Diabor Ltd, Oulu, Finland</td>
</tr>
<tr>
<td></td>
<td>(CA-4001)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>mouse monoclonal</td>
<td>10 µg/ml</td>
<td>20h</td>
<td>Diabor Ltd, Oulu, Finland</td>
</tr>
<tr>
<td></td>
<td>(GE-231)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>mouse monoclonal</td>
<td>1:75</td>
<td>1h</td>
<td>Novocastra, Newcastle upon Tyne, UK</td>
</tr>
<tr>
<td></td>
<td>(NCL-TIMP-1-485)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>mouse monoclonal</td>
<td>15 µg/ml</td>
<td>1h</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
</tr>
<tr>
<td></td>
<td>(MAB-971)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.2.2 Evaluation of samples

The samples were evaluated with a microscope using 10x, 20x and 40x objectives by two independent observers blinded from the clinical data. The results were verified by a pathologist. The samples were considered as negative or positive according to the absence or presence of immunoreaction for MMP-2, MMP-9, TIMP-1 or TIMP-2 protein in the cytoplasm of the tumor cells. Depending on the extent of staining, the cases were scored as 0–3 (II), as 0–2 (I) or simply as positive or negative (III). The case was considered positive when >1% of the neoplastic cells showed a positive staining (Talvensaari-Mattila et al. 2005), with the exception of study III, where the case was considered positive for TIMP-2 when >25% of the tumor cells showed a positive staining. In study II, weak positivity was scored as 1 (1% <tumor cells with positive reaction ≤25%) and moderate positivity was scored as 2 (25% <tumor cells with positive immunoreaction ≤50%). In study I, 1% <tumor cells with positive reaction ≤50% were scored as 1. The staining was considered intensive (score 2 in study I, score 3 in study II) when more than 50% of the neoplastic cells showed a positive reaction for MMP-2, MMP-9, TIMP-1 or TIMP-2.

4.3 Enzyme-linked immunoassay (ELISA)

Venous blood samples were collected prior to surgery. Sera were obtained by centrifugation without using any artificial coagulation activator and stored frozen at -20°C. The enzyme-linked immunosorbent assay (ELISA) was used to detect the circulating MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex.
levels from serum samples. ELISA assays were performed on 8-well EIA/RIA microtiter plates (Corning Inc., Corning, NY, USA) using the standard protocols. Standard samples were included in every plate, and the standard curves were required to be similar in each lot. All measurements were performed in duplicate to minimize intra-assay variation.

The wells were coated overnight at 4°C with specific monoclonal antibodies for MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex provided by SBA Sciences, Oulu, Finland. Following coating, diluted serum samples and standards for TIMP-1, TIMP-2 and MMP-2/TIMP-2 were incubated for 60 minutes, and overnight in the case of MMP-9. Non-specific binding was blocked with phosphate-buffered saline containing 1% bovine serum albumin (BSA-PBS). The wells were thoroughly washed before each stage of the procedure, in the first phase with PBS and at the later stages with PBST (0.05% Tween 20 in PBS). The bound proteins were detected with polyclonal antibodies against each of the analytes (SBA Sciences, Oulu, Finland). A peroxidase-conjugated anti-chicken antibody (Chemicon International, CA, USA) was used to detect the bound polyclonal antibody, and an OPD solution (o-phenylenediamine dihydrochloride) (P-1526, Sigma, Steinheim, Germany) was used to visualize the peroxidase conjugate. The reaction was stopped with 1.8 M H2SO4. Color formation was measured on 492 nm with a microplate reader (Anthos Reader 2001, Anthos Labtec Instruments, Walls, Austria) using the Windows-based control and evaluation software for Rosys Anthos microplate readers (Anthos Labtec Instruments, Walls, Austria). The sensitivity of the assays was 2 ng/mL for MMP-9, 1 ng/mL for TIMP-1, 2 ng/mL for TIMP-2 and 2 ng/mL for MMP-2/TIMP-2 complex.

Serum MMP-2 concentration was determined by using human MMP-2 ELISA (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer’s instructions. This assay recognizes the precursor of MMP-2 (proMMP-2), i.e. free proMMP-2 and that complexed with TIMP-2, but not the active form of MMP-2. The sensitivity of the assay for MMP-2 was 0.37ng/mL. The antibodies used in ELISA analyses are listed in detail in Table 10.
Table 10. Coating antibodies and secondary antibodies used in ELISA analyses.

<table>
<thead>
<tr>
<th>Detected protein</th>
<th>Coating antibody (monoclonal)</th>
<th>Secondary antibody (polyclonal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>anti-MMP-2 (RPN2617, Amersham Biosciences, Buckinghamshire, UK)</td>
<td>peroxidase labeled Fab 1 antibody to MMP-2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>anti-MMP-9 (GE-231, SBA Sciences, Oulu, Finland)</td>
<td>anti-MMP-9 (DB-209, SBA Sciences, Oulu, Finland)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>anti-TIMP-1 (DB-102, SBA Sciences, Oulu, Finland)</td>
<td>anti-TIMP-1 (SBA Sciences, Oulu, Finland)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>anti-TIMP-2 (T2-101, SBA Sciences, Oulu, Finland)</td>
<td>anti-TIMP-2 (DB-205, SBA Sciences, Oulu, Finland)</td>
</tr>
<tr>
<td>MMP-2/TIMP-2 complex</td>
<td>anti-TIMP-2 (T2-101, SBA Sciences, Oulu, Finland)</td>
<td>anti-MMP-2 (DB-202, SBA Sciences, Oulu, Finland)</td>
</tr>
</tbody>
</table>

4.4 Statistical analysis

All statistical analyses were carried out by using the SPSS software (Chicago, IL, USA). P-values less than 0.05 were considered statistically significant. The relationships between the clinicopathological categorical variables and the expression of tissue and serum immunoreactive proteins were assessed with Fisher’s exact test. For continuous variables, the Mann-Whitney U test was used. The cancer-specific and recurrence-free survival rates were assessed by the Kaplan-Meier method. The differences in survival between the subgroups were compared by means of a log-rank test. Cancer-specific survival was defined as the time from the date of the diagnosis to the date of the last follow-up visit or death from endometrial cancer. Recurrence-free survival was calculated from the date of the diagnosis to the date of the last follow-up visit or the first relapse. Receiving operating characteristics (ROC) curve was used to assess the cut-off points for continuous variables for the Kaplan-Meier analyses. Cox regression model was used in multivariate analysis to assess the independence of the prognostic variables.

4.5 Ethical aspects

The results of this research project have not affected the treatment or the follow-up of the patients in any way. All information associated with patients’ identities has been stored separately from the data used in analyses. All tissue and serum
samples have also been coded with no possibility to link individual patients to their samples.

This study is part of the metalloproteinase study protocol that was accepted by the Ethical Committee of Oulu University Hospital (EETTMK: 17/2002). Permission to use histological samples retrospectively was given by the National Supervisory Authority for Welfare and Health.
5 Results

5.1 Expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in endometrial carcinoma tissue (I, II, III)

Positive immunoreaction was observed as diffuse staining localized in the cytoplasm of the carcinoma cells (Fig. 3). There were no cell types in stroma that would have stained systemically. Expression of MMP-2 immunoreactive protein was found in 88% of cases, whereas 70% showed positive staining for MMP-9. TIMP-1 immunoreactive protein expression was found in 88% of the cases. For TIMP-2, 86% of primary tumors showed positive staining. The levels of positivity for different immunoreactive proteins are listed in Table 11.

The immunostaining patterns of MMP-2 and MMP-9 or TIMP-1 and TIMP-2 did not correlate with each other. In TIMP-2 stainings, it was observed that moderate and intensive positivity for TIMP-2 was only seen in endometrioid adenocarcinomas, whereas in other histologic subtypes of endometrial carcinoma, the staining remained negative or weakly positive.
Fig. 3. The staining result for A) negative MMP-2, B) positive MMP-2, C) negative MMP-9, D) positive MMP-9, E) negative TIMP-1, F) positive TIMP-1, G) negative TIMP-2 and H) positive TIMP-2.
Table 11. MMP-2, MMP-9, TIMP-1 and TIMP-2 immunostaining of endometrial carcinoma patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MMP-2*</th>
<th>MMP-9*</th>
<th>TIMP-1**</th>
<th>TIMP-2**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>266</td>
<td>261</td>
<td>230</td>
<td>241</td>
</tr>
<tr>
<td>Positive</td>
<td>233 (88%)</td>
<td>183 (70%)</td>
<td>202 (88%)</td>
<td>207 (86%)</td>
</tr>
<tr>
<td>+</td>
<td>125 (47%)</td>
<td>99 (38%)</td>
<td>94 (41%)</td>
<td>108 (45%)</td>
</tr>
<tr>
<td>++</td>
<td>108 (41%)</td>
<td>84 (32%)</td>
<td>55 (24%)</td>
<td>45 (19%)</td>
</tr>
<tr>
<td>+++</td>
<td></td>
<td></td>
<td>53 (23%)</td>
<td>54 (22%)</td>
</tr>
<tr>
<td>Negative</td>
<td>33 (12%)</td>
<td>78 (30%)</td>
<td>28 (12%)</td>
<td>34 (14%)</td>
</tr>
</tbody>
</table>

* the scale of immunoreaction 0–2
** the scale of immunoreaction 0–3

In the third study, subgroups with different MMP-2 and TIMP-2 staining results were formed. Out of the total of 237 patients, 224 (95%) were endometrioid adenocarcinomas while 13 (5%) cases presented with another histologic subtype of endometrial carcinoma. It was notable that ten out of 13 (77%) cases with histology other than endometrioid adenocarcinoma showed a positive immunostaining for MMP-2 and were negative for TIMP-2. Of the endometrioid adenocarcinomas, 93 (42%) were positive for MMP-2 and TIMP-2, whereas in 18 (8%) cases both the immunoreactive proteins showed negative immunostaining. In 107 (48%) cases, MMP-2 was positive and TIMP-2 negative, and 6 (3%) cases were negative for MMP-2 and positive for TIMP-2. The combinations and frequencies of different subgroups of MMP-2 and TIMP-2 according to the histologic subtype are presented in Table 12.
Table 12. MMP-2 and TIMP-2 immunostaining combinations according to the histologic subtype of endometrial carcinoma (n=237).

<table>
<thead>
<tr>
<th>Immunostaining</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrioid adenocarcinoma</td>
<td>224</td>
<td></td>
</tr>
<tr>
<td>MMP-2 + and TIMP-2 +</td>
<td>93</td>
<td>42</td>
</tr>
<tr>
<td>MMP-2 + and TIMP-2 -</td>
<td>107</td>
<td>48</td>
</tr>
<tr>
<td>MMP-2 - and TIMP-2 -</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>MMP-2 - and TIMP-2 +</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Serous papillary</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MMP-2 + and TIMP-2 +</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MMP-2 + and TIMP-2 -</td>
<td>5</td>
<td>83</td>
</tr>
<tr>
<td>MMP-2 - and TIMP-2 -</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>MMP-2 - and TIMP-2 +</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clear cell</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MMP-2 + and TIMP-2 +</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MMP-2 + and TIMP-2 -</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>MMP-2 - and TIMP-2 -</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MMP-2 - and TIMP-2 +</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenoacanthoma</td>
<td>3</td>
<td></td>
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5.2 Pretreatment serum levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex (IV)

The study population was divided into low-risk and high-risk patient groups based on conventional prognostic parameters. The low-risk group included patients with stage Ia-Ib and histological grade 1–2 disease (n=47). The high-risk group included patients with disease of higher stage and histological grade 3 or showing either vascular or lymphatic invasion (n=46).

We observed elevated serum levels of TIMP-1 in the high-risk patient group. The median concentration of TIMP-1 was 441 ng/mL in the low-risk patient group compared to 517 ng/mL in the high-risk patient group (p=0.018). The
MMP-2/TIMP-2 complex serum levels were also elevated in the high-risk patient group compared to the low-risk patient group, but the difference did not quite reach statistical significance ($p=0.051$). No statistically significant differences were found between the groups in the serum concentration levels of MMP-2, MMP-9 or TIMP-2.

5.3 Correlation of MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex with conventional clinicopathological parameters

We could not find any correlation between the tissue overexpression of MMP-2, MMP-9, TIMP-1 or TIMP-2 immunoreactive proteins and the stage of the disease or the histological grade of the tumor. Neither was there any correlation between patients’ age, depth of invasion, peritoneal cytology, chemotherapy or radiation therapy and the positive immunoreaction for MMP-2, MMP-9, TIMP-1 or TIMP-2 in the primary tumor.

Preoperative serum levels of CA 125 were available for 253 patients. CA 125 levels were found to be higher in patients with positive immunostaining for MMP-2 than in patients with negative immunoreaction. The median CA 125 concentration for the patients presenting with a positive immunoreaction was 13 U/mL (range 5–1215), whereas the corresponding figure for the patients with a negative immunoreaction was 8 U/mL (range 5–144) ($p=0.03$). Furthermore, the preoperative serum levels of CA 125 were highest in the patients with intensive immunoreaction for MMP-2 (Fig. 4).
Fig. 4. Box plot and whiskers of preoperative serum CA 125 concentration. The boxes include 50% of the values, and the horizontal line indicates the median. The whiskers are extended to the 10\textsuperscript{th} and 90\textsuperscript{th} percentiles, and the dots and asterisks represent the outliers and extreme values, respectively. The \( p \) value for the difference in the serum CA 125 levels between negative (\( n=29 \)) and positive (\( n=224 \)) MMP-2 immunostaining was 0.03. Reprinted with permission from Elsevier.

None of the serum levels of MMP-2, MMP-9, TIMP-1, TIMP-2 or MMP-2/TIMP-2 correlated with the clinical stage of the disease or with the histological grade of the tumor. Nor was there any significant correlation between the serum levels of gelatinases or tissue inhibitors of metalloproteinases and age.

5.4 Prognostic value of MMP-2, MMP-9 and their tissue inhibitors in endometrial carcinoma

5.4.1 Tissue MMP-2 and MMP-9 as prognostic markers (I)

In this study, tissue MMP-2 positivity was found to be an indicator of adverse prognosis in cases of both recurrence-free and cancer-specific survival in endometrial carcinoma. In turn, no correlations between MMP-9 expression and the outcome of endometrial carcinoma could be detected.

MMP-2 negativity was associated with favorable prognosis. Only one of the 33 (3.0\%) patients presenting with negative MMP-2 immunostaining developed recurrent disease and died during the follow-up, whereas the corresponding figure
for the patients presenting with positive MMP-2 immunostaining was 41 out of 233 (17.6%). The Kaplan-Meier analysis showed that the 5-year cancer-specific survival rate of the patients with positive MMP-2 immunostaining was 81%, whereas 96% of the patients with negative MMP-2 immunostaining were alive at that time (p=0.05; Fig. 5A). The survival analysis also showed a statistically significant correlation between tumor immunoreactive protein expression of MMP-2 and recurrence-free survival (p=0.04; Fig. 5B). In Cox regression multivariate analysis clinical stage, histological grade and preoperative CA 125 were significant predictors of cancer-specific survival.

![Fig. 5. Kaplan-Meier A) cancer-specific and B) recurrence-free survival according to MMP-2. Reprinted with permission from Elsevier.](image)

### 5.4.2 Tissue TIMP-1 and TIMP-2 as prognostic markers (II)

Positive TIMP-2 immunostaining of endometrial cancer cells appeared to have an association with favorable prognosis. This was most apparent when the superficial (stage Ia) carcinomas were excluded, suggesting that TIMP-2 may have a role in inhibition of tumor cell invasion.

In survival analyses, the staining results of TIMP-1 and TIMP-2 were categorized into two groups representing negative and weak positivity (negative group) tumors versus moderate and intensive positivity (positive group) tumors.
During the follow-up, 39 out of 241 (16%) patients died of endometrial cancer. Ten out of 99 patients (10%) presenting with TIMP-2 positive immunostaining died of the disease, whereas the corresponding figure for the patients presenting with TIMP-2 negative staining was 29 out of 142 (20%). In Kaplan-Meier analysis, it was shown that the cumulative cancer-specific survival of patients with TIMP-2 positive immunostaining was 89% and that of the TIMP-2 negative patients 78%. The difference was statistically significant ($p=0.041$; Fig. 6A). A statistically significant correlation was also found between overall survival and TIMP-2 immunoreaction ($p=0.036$). Including only endometrioid adenocarcinoma patients ($n=228$), the overexpression of TIMP-2 seemed to predict a more favorable survival although statistical significance was not reached ($p=0.069$; Fig. 6B). However, when stage Ia carcinomas were excluded ($n=198$), a clear difference in survival was observed between the patient groups with statistical significance ($p=0.034$; Fig. 6C).
The difference in cancer-specific survival between TIMP-2 negative and positive patients was seen after two years of follow-up. The survival curves show that the difference increased until approximately five years and remained the same until the end of the follow-up time.

For TIMP-1, there was no statistically significant association with the overall or cancer-specific survival.

Using the Cox regression multivariate analysis (cutoff \( p<0.10 \)), stage \( (p<0.001) \), grade \( (p<0.001) \) and TIMP-2 \( (p=0.054) \) were predictors of survival. In endometrial adenocarcinoma patients, stage IA carcinomas excluded \( (n=198) \), were stage \( (p=0.02) \), grade \( (p=0.006) \) and TIMP-2 \( (p=0.057) \) prognostic indicators in the Cox multivariate analysis.
5.4.3 Tissue MMP-2 and TIMP-2 combinations as prognostic markers

Only endometrioid adenocarcinomas were included in survival analyses (n=224). During the follow-up, 35 (16%) patients died of endometrial carcinoma. Interestingly, 24 (69%) of the deaths occurred in the subgroup of positive MMP-2 and negative TIMP-2 immunoreaction, meaning that 22% of the patients in this group died of endometrial carcinoma, whereas no deaths of the disease were seen in the subgroup of MMP-2 being negative and TIMP-2 positive. Ten out of 93 (11%) patients died of the disease in the subgroup of both MMP-2 and TIMP-2 being positive, while only one death (6%) was observed in the subgroup of both immunoreactive proteins being negative. A trend for a more favorable survival was observed in Kaplan-Meier analysis among the patients presenting with MMP-2 negative immunostaining (p=0.085). The 5-year cancer-specific survival rate of the patients with MMP-2 negative and TIMP-2 positive staining was 100%. In contrast, only 78% of the patients with positive MMP-2 and negative TIMP-2 immunoreaction were alive at that time. The corresponding figures for subgroups with MMP2-/TIMP2- and MMP2+/TIMP2+ immunostaining were 93% and 90%, respectively.

Due to the small number of patients in the two subgroups with negative MMP-2 immunostaining, the MMP-2 negative subgroups with either negative or positive TIMP-2 staining were combined for further survival analyses. In Kaplan-Meier analysis the 5-year cancer-specific survival rate for the patients with negative MMP-2 immunoreaction was 95%. The difference in survival between the groups was statistically significant (p=0.039; Fig. 7). When only the MMP-2 positive patients were included in Kaplan-Meier analysis, a difference in survival between these subgroups with either negative or positive TIMP-2 immunostaining was also found (p=0.048). In Cox regression multivariate analysis, stage (p<0.001) and grade (p=0.003) were the most significant prognostic factors. However, MMP-2 & TIMP-2 may also have prognostic value in this analysis, since patients with positive MMP-2 and negative TIMP-2 immunoreaction had a 4.7-fold relative risk of death compared to patients with negative MMP-2 and positive TIMP-2 immunostaining, although it did not quite reach statistical significance (p=0.073).
5.4.4 Circulating MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex as prognostic markers (IV)

Different cut-off values were defined by utilizing the ROC curve when analyzing the possible correlations of serum immunoreactive protein levels with survival. These values were more powerful in differentiating the cases according to their prognosis than the median or mean serum levels would have been. For TIMP-1, a cut-off value of 536 ng/mL was used to divide the serum values into two groups. Preoperative serum TIMP-1 level was found to associate with cancer-specific survival ($p=0.029$; Fig. 8A). The Kaplan-Meier analysis showed that the 5-year cancer-specific survival rate of the patients with high TIMP-1 values was 68%. The corresponding figure for the patients with low TIMP-1 values was 87%. A statistically significant correlation was also found between relapse-free survival and serum TIMP-1 levels ($p=0.036$; Fig. 8B). No differences in survival were detected among endometrial carcinoma patients when analyzed by groups according to the different cut-off values for serum MMP-2, MMP-9, TIMP-2 or

![Graph showing the effect of MMP-2 and TIMP-2 immunoreactive protein combinations on cancer-specific survival.](image-url)
MMP-2/TIMP-2 complex. Neither were there any associations with the serum levels of these immunoreactive proteins and the time of relapse.

Fig. 8. A) Cancer-specific and B) relapse-free survival according to preoperative serum TIMP-1 (s-TIMP-1 ≤ 536 ng/ml, n=63; s-TIMP-1 > 536 ng/ml, n=27). Reprinted with permission from IIAR.
6 Discussion

6.1 MMP-2 expression associates with an adverse prognosis

Little is still known about gelatinases in endometrial carcinoma, even though MMPs have long been associated with malignancy. The present study is the largest gelatinase immunohistochemistry study of endometrial carcinoma published so far.

Here, we evaluated MMP-2 and MMP-9 immunoreactive proteins from primary tumors of endometrial carcinoma in 266 specimens. Positive immunoreactions for MMP-9 and especially for MMP-2 were commonly seen in the cytoplasm of the carcinoma cells. We observed a shortened recurrence-free and cancer-specific survival in patients with positive MMP-2 immunostaining. Survival analyses have rarely been done in previous studies. This is the first immunohistochemical study to show an association between recurrence-free and cancer-specific survival and MMP-2 immunoreaction in endometrial carcinoma. Associations between MMP-2 overexpression and unfavorable prognosis in endometrial cancer have previously been reported, but statistical significance has not been reached, probably due to a small number of events (Aglund et al. 2004, Talvensaari-Mattila et al. 2005). In this study, MMP-9 expression did not correlate with survival parameters. This is in agreement with the results from previous studies, in which MMP-9 has not been useful in predicting the prognosis of endometrial cancer patients (Aglund et al. 2004, Inoue et al. 1997).

CA 125 is the most widely used biochemical tumor marker in gynecological malignancies. In endometrial carcinoma its role has not been clearly established, although some studies have reported associations of elevated CA 125 levels with advanced stage and the presence of extraterine disease (Duk et al. 1986, Niloff et al. 1984, Patsner et al. 1988, Sood et al. 1997). Interestingly, preoperative serum levels of CA 125 were higher in the patients presenting with MMP-2 positive tumors than in those with negative MMP-2 immunostaining. We suggest that MMP-2 may degrade the tumor basement membrane, thus enabling and increasing the transition of CA 125 from tumor tissue into the circulation. Since elevated serum levels of CA 125 predict a poor outcome (Santala & Talvensaari-Mattila 2003, Santala et al. 2003) and CA 125 levels were elevated in MMP-2 positive patients, we suggest that MMP-2 may also be linked with the biologically
more aggressive nature of endometrial carcinoma. In Cox multivariate analysis stage, grade and preoperative CA 125 were significant predictors of survival.

Taken together, MMP-2 seems to be more often identified as a prognostic factor than MMP-9 in endometrial carcinoma. Our data suggest that MMP-2 is a prognostic variable for recurrence and survival, but the traditional prognostic markers seem to be superior to MMP-2 in assessing the clinical course of endometrial carcinoma.

6.2 TIMP-2 expression is associated with a favorable prognosis in endometrial carcinoma

The results of our study indicate for the first time that positive TIMP-2 immunoreactive protein expression associates with favorable outcome in endometrial carcinoma. Positive immunoreaction for TIMP-2 correlated with favorable cancer-specific and overall survival. Tissue TIMP-1, in turn, did not yield any useful clinical data.

TIMP-2 has a dual role in the regulation of MMP-2 expression: low concentrations of TIMP-2 have been associated with MMP-2 activation and high concentrations with MMP-2 inhibition (Kinoshita et al. 1998, Kurschat et al. 1999). Hence, our results are in line with the main function of TIMP-2 and with the results derived from our previous study.

The difference in survival was seen only after two years of follow-up, which highlights the significance of long follow-up in this cancer type. Survival curves demonstrate that the difference in survival increased until approximately five years and remained the same until the end of the follow-up time. The majority of adjuvant therapies are given during the first two years after diagnosis, which could at least partly explain why almost the entire study group survived the first years in a similar manner. The results of our study suggest that TIMP-2 might be useful in identifying the patients with more aggressive disease at the time of the diagnosis. Patients presenting with negative TIMP-2 immunoreaction may benefit from close follow-up, especially during the first years after surgery. Our results also imply that TIMP-2 could add some value as a prognostic marker in adjuvant treatment decision-making.

Balance between active metalloproteinase and its inhibitor has been suggested to play a critical role in tumor cell invasiveness. In this study, the overexpression of TIMP-2 seemed to predict a more favorable survival among endometrioid adenocarcinoma patients. Interestingly, the difference in survival
between TIMP-2 negative and positive patients became more apparent as well as statistically significant when further superficial stage Ia tumors were excluded. Therefore, we suggest that TIMP-2 inhibits tumor cell invasion in endometrial carcinoma. Our results indirectly support the primary theory that TIMP-2 inhibits MMP-2 in infiltrating endometrial cancer.

### 6.3 MMP-2 and TIMP-2 as co-factors in malignant growth

The significance of the MMPs and TIMPs as regulators of the ECM degradation is undeniable. However, activation of multiple different genes and their products is required for malignant growth. Therefore, measuring one factor reveals only a fraction of the underlying malignant process in cancer progression, and good responses are probably not achieved by affecting malignant growth through single factors.

In this study, we evaluated the combined effects of MMP-2 and TIMP-2 protein immunoreactivities on the prognosis in endometrial carcinoma. We observed a combination of high MMP-2 and low TIMP-2 expression to identify a group of women at high risk of aggressive endometrial carcinoma. Patients with negative MMP-2 immunostaining had the best prognosis, regardless of TIMP-2 staining result. Our results confirm our previous findings concerning the prognostic role of separately assessed MMP-2 and TIMP-2 expressions in endometrial carcinoma, in which high tissue MMP-2 expression was found to correlate with poor prognosis and high TIMP-2 immunoexpression was associated with favorable survival in endometrial carcinoma. Furthermore, our results increase the knowledge about their mutual value in determining the prognosis in this cancer type. In line with our results are also Graesslin et al. (2006a & 2006b), who suggested high MMP-2 and low TIMP-2 immunoreactive protein expressions to be the most potent markers of endometrial malignancies with a high risk of local and distant spread.

The molar ratio of MMPs to TIMPs has been found to be higher in carcinoma tissues compared to non-neoplastic control tissues, which suggests the possibility of imbalance in favor of the proteinases (Ueno et al. 1999). However, it has not been evident which of the gelatinases or their tissue inhibitors has a more important role in cancer progression and determining the prognosis. The value of MMPs as well as TIMPs as prognostic indicators seems to vary in different types of malignomas (Turpeenniemi-Hujanen 2005). In the present study, negative MMP-2 immunoreaction correlated with favorable survival and was superior to
TIMP-2 in determining the prognosis of endometrial cancer. Previous studies suggest that MMP-2 is the main metalloproteinase involved in the malignant behavior of endometrial cancer (Park et al. 2001, Tamakoshi et al. 1995), which was verified in this thesis as well.

The majority of other histologies than endometrioid adenocarcinoma showed positive immunoreaction for MMP-2 and negative for TIMP-2. Type II tumors, consisting mainly of serous and clear cell carcinomas, are known to carry a poorer prognosis than type I tumors (mostly endometrioid adenocarcinomas). We suggest that high MMP-2 and low TIMP-2 expression levels may be linked to the aggressiveness of endometrial cancers. Increased MMP-2 expression has previously been associated with serous histology (Shaco-Levy et al. 2008). Graesslin et al. (2006b) observed lower TIMP-2 expression levels in serous and clear cell carcinomas than in endometrioid adenocarcinomas, which is in line with the results obtained in this study. In contrast, Monaghan et al. (2007) reported significantly stronger expression of MMP-2 and MMP-9 in endometrioid tumors compared to serous tumor types.

Our results suggest that TIMP-2, and especially MMP-2, may be useful for differentiating patients at low risk from those at high risk of relapse or death from the disease. Strong MMP-2 and weak TIMP-2 expression profiles define a subgroup of endometrial cancers with a more aggressive clinical course, suggesting they may have prognostic potential. Moreover, MMP-2 seems to be superior to TIMP-2 in determining the prognosis in endometrial cancer, if used separately.

6.4 High serum TIMP-1 level is an indicator of poor survival in endometrial carcinoma

Measuring circulating cancer biomarkers from blood samples is a fascinating possibility. Obtaining serum or plasma samples from cancer patients is much easier and less invasive than to get tumor tissue specimens for marker assays. Therefore, they could be very useful in the patient follow-up due to the easy access to sample material.

A statistically significant difference was found between the median values of circulating TIMP-1 in high-risk and low-risk patient groups. In this study, high preoperative serum TIMP-1 was for the first time found to be prognostic for poor cancer-specific and relapse-free survival. The range of serum TIMP-1 values was wide, but the correlation between high preoperative serum TIMP-1 and poor
survival seems evident. Previously, elevated levels of circulating TIMP-1 have been associated with poor prognosis in ovarian, breast, head and neck, lung and colorectal carcinoma (Holten-Andersen et al. 2000, Manenti et al. 2003, Rauvala et al. 2005, Ruokolainen et al. 2005b, Ruokolainen et al. 2006, Talvensaari-Mattila & Turpeenniemi-Hujanen 2005a, Ylisirniö et al. 2000). As regards endometrial carcinoma, there are no studies concerning the prognostic role of circulating TIMP-1 in this cancer type.

The MMP-2/TIMP-2 complex was also elevated in the high-risk patient group compared to the low-risk group, although the difference did not quite reach statistical significance. It is possible that the difference is clinically relevant but the number of patients in this study too small to show a clear statistically significant correlation.

In the present work, no correlation was observed between the MMP-2, MMP-9, TIMP-1, TIMP-2 or MMP-2/TIMP-2 complex serum levels and the conventional prognostic factors of endometrial cancer. Only one previous study has investigated circulating matrix metalloproteinases in endometrial carcinoma (Adamiak et al. 2000). They reported serum MMP-2 levels to be statistically higher in clinically advanced stages of endometrial carcinoma. Unfortunately, the patient series was small \( n=30 \) and no survival analyses were conducted.

Taken together, preoperative serum TIMP-1 measurement might be beneficial in deciding about the use of primary adjuvant treatment in endometrial carcinoma as well as in the follow-up of patients treated for endometrial cancer. On the other hand, serum MMP-2, MMP-9, TIMP-2 and possibly MMP-2/TIMP-2 complex measurements do not seem to add value to clinical decision-making.

6.5 Critical evaluation of the study material, methods and clinical implications

Endometrial carcinoma is generally considered to have a good prognosis; especially endometrioid adenocarcinoma is known to carry high survival rates. The majority of the patients in our study presented with type I tumors, and in some analyses we only included endometrioid adenocarcinomas to make the study group more homogeneous. Therefore, death was relatively infrequent in our study material, although the size of the study group was quite large. This means that when comparisons are made between different prognostic factors, the number of events in each group is small. This may affect the results, or at least make it hard to establish differences between the subgroups. Especially in the serum study
the study population was quite small, which may have caused the low power of significance in the analysis of MMP-2/TIMP-2 complex levels.

An advantage of our study is that all the patients were operated upon and treated in the same gynecological oncological unit in a university hospital. Furthermore, all the patients were carefully staged according to the FIGO (1988) criteria. Histological specimens were evaluated by experienced doctors specialized in gynecological pathology. After the operation, the follow-up was systematic and long enough to show the relapses and deaths from the disease. For the study, the stained tumor sections were analyzed by two independent observers. However, evaluating immunohistochemical staining is always to some extent subjective.

We included all the endometrial carcinoma patients with clinical data available treated in the Oulu University Hospital between 1992 and 2000. We also did analyses where only endometrioid adenocarcinomas were included, but the results were essentially the same. The number of other histologies than endometrioid adenocarcinoma was too small to be further analyzed separately.

The use of serum as a sample material has raised strong criticism. Several studies have reported higher MMP-9 and TIMP-1 concentrations in serum samples than in plasma samples (Jung et al. 2001, Jung 2005, Mannello 2003). Inflammatory cells and platelets contain high concentrations of MMP-9 and TIMP-1, which can be released during clotting, causing artificially elevated levels of MMP-9 and TIMP-1 in the serum. This can be further accelerated by using coagulation activators in serum tubes. (Jung et al. 2001, Jung 2005) Therefore, the preanalytical aspects have to be taken account when measuring the concentrations of gelatinases and their tissue inhibitors in different blood sample types. Sample type was found to have an effect on the concentrations of gelatinases and their tissue inhibitors in circulating blood in a study by Kuvaja et al. (2007). However, a strong correlation was found between serum and plasma levels of TIMP-1, justifying the use of serum in TIMP-1 tumor marker studies as long as the generally higher levels in serum are taken into account. In addition, assaying proMMP-9 was reported to be very sensitive for preanalytical aspects, such as the presence of blood coagulation activators. In our study, however, no coagulation activators were used.

Storage conditions and repeated freezing and thawing have also been reported to alter the serum levels of gelatinases and their tissue inhibitors; especially MMP-9 has been found to be sensitive to these preanalytical conditions (Holten-Andersen et al. 2003, Rouy et al. 2005). Rouy et al. (2005) evaluated the effect of
storage time on TIMP-1 and found no significant decrease in two years, when the samples were kept at -80°C. In this study, the storage time of serum samples before ELISA analyses was longer and the samples were kept at -20°C. No freezing and thawing was performed, since all the analyses were conducted at the same time and only once. Kuvaja et al. (2007) observed good replicability of the results in terms of prognostic value of TIMP-1, although the storage time varied and the storage temperature was only -20°C. This validates our findings concerning the prognostic value of pretreatment serum TIMP-1 levels.

Serum CA 125 levels were found to be higher in patients with positive MMP-2 immunoreaction than in patients presenting with negative MMP-2 immunostaining. The correlation was evident, even though the range of the serum values of CA 125 in patients with positive MMP-2 was wide and the difference in median values between the MMP-2 positive and negative patients was not great. In carcinoma patients, however, even smaller differences can be biologically significant. Santala et al. (2003) reported that even slightly elevated preoperative serum CA 125 levels might be helpful in identifying the patients at high risk. It has also been suggested that an elevated serum CA 125 level may predict advanced disease even in patients with apparently favorable histology and clinical stage (Rose et al. 1993).

Further studies with larger patient materials are needed to confirm the preliminary results obtained in this study. However, we succeeded in revealing new insights into the clinical relevance of gelatinases and their tissue inhibitors in endometrial carcinoma. Tissue MMP-2 was found to be the most potent prognostic marker in endometrial cancer, whereas in serum measurements, TIMP-1 seems most promising. In the future, pharmacological targeting of cancer by the development of a new generation of effective and highly selective MMP inhibitors is a promising area of research. Given our findings, especially inhibition of MMP-2 activity may prove to be of use in endometrial carcinoma treatment.
7 Conclusions

In the present study, expression of tumor tissue MMP-2, MMP-9, TIMP-1 and TIMP-2 and their prognostic role in endometrial carcinoma was studied. The potential of circulating MMP-2, MMP-9, TIMP-1, TIMP-2 and MMP-2/TIMP-2 complex as prognostic markers was also evaluated.

It is shown here that tissue MMP-2 and TIMP-2 as well as circulating TIMP-1 are prognostic factors in patients with endometrial carcinoma.

The specific conclusions of this study are as follows:

1. Tissue MMP-2 expression was found to be an indicator of adverse prognosis in cases of both recurrence-free and cancer-specific survival in endometrial carcinoma. MMP-2 may be associated with the biologically more aggressive nature of this cancer type.

2. Tissue expression of TIMP-2 immunoreactive protein associated with favorable outcome in endometrial carcinoma.

3. Strong MMP-2 and weak TIMP-2 immunoexpression were the most potent markers of endometrial cancer aggressiveness, suggesting they may have prognostic potential. Moreover, MMP-2 seems to be the main metalloproteinase determining the prognosis in endometrial carcinoma.

4. High preoperative serum TIMP-1 concentration was a prognostic indicator of unfavorable survival.

In conclusion, measuring tissue MMP-2, TIMP-2 and circulating TIMP-1 may be beneficial in the clinical practice of patients with endometrial carcinoma. However, the traditional prognostic markers are superior in predicting the clinical course of this disease, but tissue MMP-2, TIMP-2 and circulating TIMP-1 can add some value when deciding about treatment modalities. If immunohistochemical detection or blood component measurements of MMPs or TIMPs are planned to be used as a prognostic device, the methods, optimal sample source and cut-off values for positivity should be further studied and confirmed in larger studies.
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THE PROGNOSTIC ROLE OF MATRIX METALLOPROTEINASE-2 AND -9 AND THEIR TISSUE INHIBITOR-1 AND -2 IN ENDOMETRIAL CARCINOMA

UNIVERSITY OF OULU GRADUATE SCHOOL; UNIVERSITY OF OULU, FACULTY OF MEDICINE, INSTITUTE OF CLINICAL MEDICINE, DEPARTMENT OF OBSTETRICS AND GYNECOLOGY, OULU UNIVERSITY HOSPITAL; NATIONAL GRADUATE SCHOOL OF CLINICAL INVESTIGATION