Gene signatures and prognostic factors in endometrial cancer

A study with special focus on vascular invasion

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CONTENTS

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Bergen, December 2010 Monica Mannelqvist

2. LIST OF PUBLICATIONS

The thesis in based on the following papers, which will be referred to by their Roman numerals:

I. **Mannelqvist M**, Stefansson I, Salvesen HB, Akslen LA: Importance of tumour cell invasion in blood and lymphatic vasculature among patients with endometrial carcinoma. *Histopathology 2009, 54:174-83.*

II. **Mannelqvist M,** Stefansson IM, Bredholt G, Bø TH, Øyan AM, Jonassen I, Kalland K-H, Salvesen HB, Akslen LA: Gene expression patterns related to vascular invasion and aggressive features in endometrial cancer. *Am J Pathol 2010 (in press).*

III. Engelsen IB, **Mannelqvist M**, Stefansson IM, Carter SL, Beroukhim R, Oyan AM, Otte AP, Kalland KH, Akslen LA, Salvesen HB: Low BMI-1 expression is associated with an activated BMI-1-driven signature, vascular invasion, and hormone receptor loss in endometrial carcinoma. *Br J Cancer 2008, 98:1662-9.*

IV. **Mannelqvist M**, Stefansson I, Salvesen HB, Akslen LA: Lipocalin 2 expression is associated with aggressive features of endometrial cancer. *Manuscript.*

3. ABBREVIATIONS

bFGF:	Basic fibroblast growth factor
BVI:	Blood vascular invasion
cRNA/cDNA:	Copy RNA/DNA
CSC:	Cancer stem cell
DNA:	Deoxyribonucleic acid
ECM:	Extracellular matrix
EEC:	Endometrioid endometrial carcinoma
EMT:	Epitelial mesenchymal transition
ER:	Estrogen receptor
FIGO:	International Federation of Gynecology and Obstetrics
GB:	Glomeruloid body
GMP:	Glomeruloid microvascular proliferation
H&E:	Hematoxylin and eosin
HNPCC:	hereditary non-polyposis colorectal cancer
HSC:	Haematopoietic stem cell
IHC:	Immunohistochemistry
LOH:	Loss of heterozygosity
LOOCV:	Leave one out cross validation
LVI:	Lymphatic vascular invasion
miRNA:	Micro RNA
MMP:	Matrix metalloproteinase
MMR:	Missmatch repair
mRNA:	Messenger RNA
MSI:	Microsatellite instability
MVD:	Microvessel density
NEEC:	Non-endometrioid endometrial carcinoma
PcG:	Polycomb group
PLI:	Perivascular lymphocytic infiltration
PR:	Progesterone receptor
qPCR	Quantitative polymerase chain reaction
RNA:	Ribonucleic acid
SAM:	Significance analysis of microarray
SE:	Standard error
SI:	Staining index
TAF:	Tumor angiogenic factor
TMA:	Tissue microarray
TIL:	Tumor infiltrating lymphocyte
TLDA:	Taqman low density array
VEGF:	Vascular endothelial growth factor
VI:	Vascular invasion
VIS:	Vascular invasion signature
WHO:	World Health Organisation

4. INTRODUCTION

Cancer affects people at all ages, but the risk increases with age, and malignant tumors accounted for 13% of all deaths worldwide in 2005.[1] Endometrial cancer is the most common pelvic gynecologic malignancy in industrialized countries, showing an increasing incidence rate.[2] Even though the majority of endometrial cancers are diagnosed at an early stage due to postmenopausal bleeding, 15-20% of the tumors recur and might then be unresponsive to systemic therapy.[3, 4] Markers to identify subgroups of aggressive endometrial cancers are needed to tailor treatment and follow-up.

4.1 ENDOMETRIUM

The uterus is specifically adapted for the reproductive process and is on a histological basis divided into the endometrium and myometrium. The endometrium is a mucosal layer composed by glandular epithelium and a highly cellular stroma which undergoes cyclic changes of growth, differentiation and shedding in response to ovarian sex steroids throughout a woman's reproductive life. The myometrium surrounds the endometrial lining of the uterine cavity and forms the major component of the uterine volume.[5]

4.2 EPIDEMIOLOGY

In developed countries, endometrial cancer is the most frequent malignant tumor in the female genital tract, and the fourth most common cancer after lung, breast and colorectal cancer among females.[6] Most patients are post-menopausal, and approximately 86% of the patients are over 50 years at diagnosis.[7] The incidence rate in the Norwegian population was 16.5 per 100 000 during 2004-2008 **(Figure 1)** and has increased since the beginning of the 1960's.[8] The incidence of endometrial cancer

increases especially in Eastern Asia and some Southern and Eastern European countries.[9] The mortality rate (per 100 000/year) for cancer in the corpus uteri in Norway was 1.7 in 2007 **(Figure 1)**.

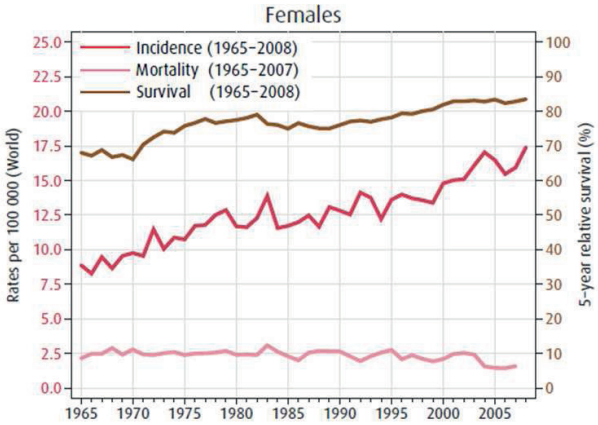

Figure 1.

Trends in incidence, mortality and five-year relative survival for women with cancer in corpus uteri in Norway. [8]

4.3 ETIOLOGY

Approximately 5-10% of endometrial cancer cases have a hereditary basis.[10] Hereditary non-polyposis colorectal cancer (HNPCC) is a dominantly inherited syndrome due to germline mutations in DNA-mismatch repair genes resulting in micro-satellite instability (MSI). Women with HNPCC have a ten-fold increased lifetime risk for developing endometrial carcinoma compared to that of the general population.[11, 12]

Several different risk factors are reported for sporadic endometrial cancer, many of them include lifestyle factors and unopposed estrogen stimulation. High body mass index and diabetes mellitus increase the risk,[13, 14] while physical activity reduces the

occurence.[15] The mechanism behind high body mass index and increased endometrial cancer risk is thought to be a disturbed balance between estrogen and progesterone. Estrogen produced in fat stores has a mitogenic effect on endometrial cells, and the estrogen is not balanced by progesterone in postmenopausal women. This mechanism seems to be irrelevant in younger premenopausal women.[14] Activity on the other hand reduces fat sources, leading to decreased estrogen levels.[15] Smoking and oral contraception seem to decrease the risk for getting endometrial cancer.[16, 17]

Endometrial cancers are divided into two clinico-pathological subtypes. Type I, including endometrioid endometrial cancers (EECs), is the most common type. It is well differentiated, associated with unopposed estrogen exposure or other hyperestrogenic risk factors and has a good prognosis.[2] Other risk factors for type I tumors are obesity, early menarche, late menopause and nulliparity.[18] Use of the breast cancer drug tamoxifen has been reported to be a risk factor for developing endometrial cancer for women older than 50 years.[19] Unopposed estrogen therapy and tamoxifen both exert proliferative effects on the endometrium.[18] Atypical endometrial hyperplasia is a known precursor lesion of endometrioid adenocarcinoma, and increases the risk for cancer development.[20] Type II endometrial carcinomas are of the non-endometrioid subtype (serous, clear cell), are poorly differentiated, not associated with estrogenic risk factors and have a poorer prognosis with a tendency to recur.[21] These women are more likely to have a history of additional primary tumors, normal weight, multiparity and older age at diagnosis compared with patients having type I endometrial cancer.[22]

4.4 HISTOPATHOLOGY

4.4.1 Histological type

Histologically, 85-90% of endometrial carcinomas are endometrioid adenocarcinomas,[4] while 10-15% represents the non-endometrioid cancers (NEECs)

comprised of serous carcinoma, clear-cell carcinoma and undifferentiated carcinomas. They usually arise from atrophic endometrium, have high histological grade and are poorly differentiated.[2] Carcinosarcomas are a subgroup of endometrial cancer composed of an admixture of malignant epithelial and mesenchymal components.[23]

4.4.2 Histological grade

Only the EECs are histologically graded in a routine setting, whereas serous and clear-cell carcinomas are considered high grade by definition. Histological grading is performed according to architecture and adjusted by severe nuclear atypia. Once the architectural grade has been established on the basis of the percentage of solid growth, notable nuclear atypia raises the grade of the tumor by one.[24]

4.4.3 Vascular invasion

Presence of tumor cells in vascular spaces is usually determined on standard H&E (hematoxylin and eosin) sections **(Figure 2)**. Vascular invasion is presently not integrated into any of the grading system for endometrial cancer, even though it is recommended.[24] Detection of vascular invasion on standard H&E sections may be challenging. Small vessels might be missed, and artificial tissue retraction can be mistaken for vascular invasion. Lately, the D2-40 antibody has been used as a specific marker for lymphatic vessels.[25] D2-40 in combination with CD31 (or CD34) are now important markers to distinguish between blood vessels and lymphatic vessels.

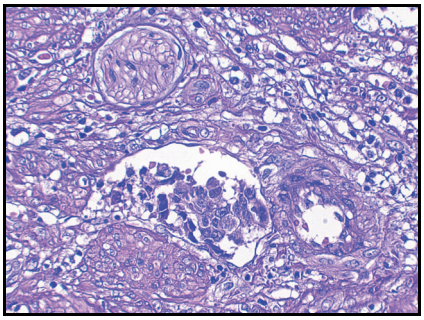

Figure 2.

Presence of tumor cells in the vasculature by H&E stained section (magnification x 400).

Lymphatic vasculature

Lymphatic vessels begin as blind ends and are anatomically constructed to permit a continuous and rapid removal of transient interstitial fluids, plasma proteins, and cells from the interstitium.[26] Lymphatic vessels are often found in close contact with blood vessels and are present in almost all tissues.[27] The lymphatic capillaries consist of a single layer of lymphatic endothelial cells that lack tight junctions, basement membrane, pericytes and smooth muscle cells and are thought from its structure to be easier to penetrate than blood capillaries **(Figure 3)**.

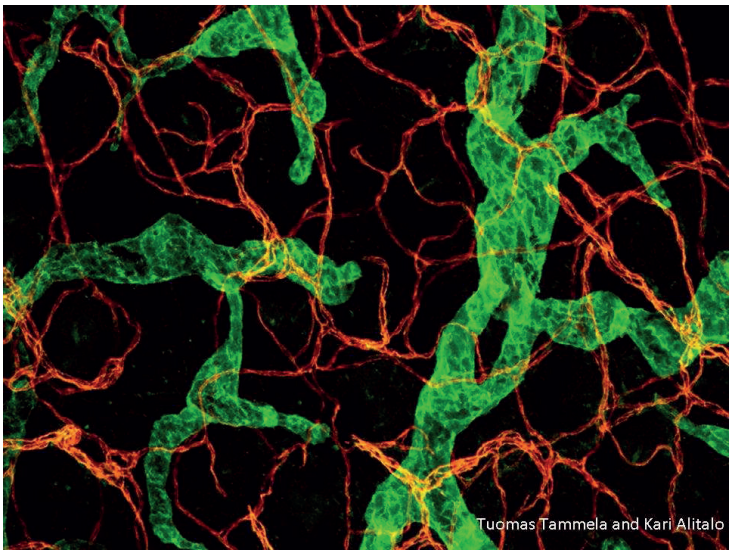

Figure 3.

Lymphatic vessels (*in green*) *start as blind ended and have a more open structure than blood vessels* (*in red*) (picture kindly provided by Professor Kari Alitalo).

Blood vessels

Blood vessels consist of a single layer of endothelial cells, covered by a vascular basement membrane followed by pericytes and smooth muscle cells. Adhesion between endothelial cells is mediated by various surface proteins, such as cadherins, integrins, immunoglobulins, and proteoglycans.[28]

4.4.4 Necrosis

Tumor necrosis is an indicator of fast growing tumors. Tumor cell necrosis was defined as areas of necrotic tumor cells bordering viable tumor cells.[29]

4.4.5 Other histopathological features

Other histopathological factors like solid tumor growth, high mitotic count, perivascular lymphocytic infiltration (PLI) and tumor infiltrating lymphocytes (TIL) have been reported to be markers of aggressive endometrial cancers.[29-32]

4.5 TUMOR BIOLOGY

Cancer is a group of diseases in which cells show uncontrolled growth, invasion and some times metastasis. Cancer development requires accumulation of heritable and sporadic changes in gene function. Those changes happen basically in tumor suppressor genes that inhibit cell growth and survival (*loss of function*) and oncogenes that promote cell growth and survival (*gain of function*). Oncogenes may become upregulated by gains of chromosomes, gene amplification, translocations and activating point mutations. Tumor suppressor genes may be inactivated by loss of whole chromosomes, gross deletions, intragenic deletions, point mutations and epigenetic silencing.[33] Malignant tumors are considered to have the following major hallmarks according to Hanahan and Weinberg: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and ability to invade tissues and metastasize.[34]

The tumor microenvironment is of critical importance for tumor development and metastatic spread, as was suggested by Stephen Paget in 1889 in the "*seed and soil*" hypothesis.[35] There is a complex crosstalk between malignant cells and their associated stroma in epithelial tumors. Stromal elements consist of extracellular matrix (ECM), fibroblasts, inflammatory cells, blood vessels, lymphatic vessels and nerves. Secreted factors from the stroma and the neoplastic cells are known to modify tumor cell proliferation, cell motility and alterations of the ECM.[36]

ECM is located outside the cell surface and regulates many aspects of cell behavior in addition to providing structural and functional integrity to connective tissues and organs. The main components of the ECM are structural proteins (e.g. collagen, laminins, fibronectin, vitronectin, elastin), specialized proteins (growth factors, small matricellular proteins, small integrin-binding glycoproteins) and proteoglycans. ECM is under constant remodeling, especially during tissue development, wound repair, in many disease states and in response to infectious agents. Structural changes can be induced in response to signals mediated by ECM receptors, by proteolytic cleavage (e.g. matrix metalloproteinases (MMPs), serine proteases, cysteine proteases) or by tensions (cellular or extracellular).[37]

Endothelial cells are attached to ECM primarly through integrins on the endothelial cell surface. ECM acts like a scaffold supporting endothelial cell structure in addition to regulating many processes important for vessel formation. ECM signals regulate a molecular balance between vascular morphogenesis and vascular regression. For instance, collagens stimulate vascular formation while laminin appears to inhibit the formation process,[38, 39] and some MMPs control vascular morphogenesis whereas other MMPs control regression.[40] Degradation of ECM by MMPs creates a path for migrating endothelial cells which is an important factor for angiogenesis.[41]

4.5.1 Genetic factors

No single genetic alteration has been linked to endometrial cancer, but the genetic changes found differ between EECs and NEECs. EECs exhibit more often genetic changes as microsatellite instability (MSI), *PTEN* alternations, mutations in *PIK3CA*, *KRAS* and *CTNNB1* (β-catenin) than NEECs. In contrast, NEECs show higher rates of genetic alternations such as *TP53* mutations, *ERBB2* (encoding HER-2) amplifications, inactivation of p16 (*CDKN2A*) and absence E-cadherin (*CDH1)*,[42, 43] but overlap exists.

Microsatellite instability: Short tandem repeats, microsatellites, are susceptible for slippage during DNA replication. Defects in the DNA mismatch repair (MRR) system can induce microsatellite instability (MSI), resulting in a higher rate of mutations in both coding and non-coding regions.[44] The MSI phenotype is detected in approximately 20% of non-familiar endometrial cancers, and mainly in the endometrioid subtype.[45, 46]

Copy-number alterations: LOH and amplification of gene regions have been found on several chromosome arms, such as 1p, 3p, 17p, 8p and 10q in endometrial cancer.[47-50] This indicates regions containing putative tumor suppressor genes and oncogenes.

PTEN encodes a lipid phosphatase which maintains G1 arrest and regulates the PI3/AKT pathway.[51] An inactive *PTEN* gives a constantly active PI3K pathway. *PTEN* may be inactivated by combinations of mutations, deletions and loss of heterozygosity (LOH).[52] Mutations in the tumor suppressor gene *PTEN* have been detected in up to 34-55% of endometrial carcinomas and at a higher frequency among the endometrioid tumors.[52-54]

PIK3CA is the p110α catalytic subunit of PI3K. PI3K is involved in intracellular signaling networks regulating cell proliferation, cellular survival, apoptosis, adhesion and motility. *PIK3CA* somatic mutations are seen in 24-38% of endometrial cancers.[49, 55]

KRAS is a member of the small GTPase superfamily involved in signal transduction pathways between cell surface receptors and the nucleus.[42] Mutational activation of *KRAS* is observed in 10-30% of endometrial carcinomas.[56]

B-catenin: Mutations in *CTNNB1*, encoding β-catenin, have been described in 14-44% of EEC's.[57] β-catenin is an adherence junction protein that maintains cell polarity by interactions with E-cadherin, and it is also involved in the Wnt-pathway regulating gene transcription.[58]

TP53 is a tumor suppressor protein that accumulates during DNA damage and trigger DNA repair and promotes either cell cycle arrest or apoptosis. Mutation in the *TP53* gene gives a protein that accumulates in the nucleus, and increased TP53 expression is found in 31-66% of endometrial cancers.[59-61] Several studies of endometrial cancer show high TP53 expression to be associated with poor prognosis and an aggressive phenotype.[62-64]

ERBB2 encodes the HER-2 oncogenic growth factor. HER-2 is a transmembrane protein that undergoes hetero-dimerization with other HER family members. The intracellular tyrosine residues get phosphorylated, and thereby HER-2 induces several downstream processes.[65] Gene amplification of *ERBB2* has been found in about 20% of the NEECs but is infrequent in type I cancers.[66, 67]

P16 is a nuclear protein encoded by the tumor suppressor gene *CDKN2A*. Loss of p16 expression has been observed in 14-26% of endometrial cancers.[68-70] The underlying mechanism of p16 inactivation seems to be promoter hypermethylation, deletions and mutations. Promoter hypermethylation is reported in the wide range from 0.7-37%,[68, 69, 71, 72] In contrast, *CDKN2A* mutations and deletions are reported to be less than 5%,[70, 73, 74] although one study showed a deletion rate of 67%.[72]

E-cadherin: negative expression of the cell adhesion molecule epithelial cadherin was observed in 44-51% of the endometrial cancers and shows association with aggressive features.[75-77]

4.5.2 Cell cycle regulation

Genetic abnormalities in cell-cycle regulatory genes can result in uncontrolled neoplastic growth. Down-regulation of p27 and Rb2 as well as overexpression of CDK4, cyclin A, cyclin B1 and cyclin E are frequently observed in the more aggressive tumors. Cyclin D1 overexpression are typically found in endometrioid tumors.[78]

4.5.3 Apoptosis

Apoptosis, programmed cell death, is an active process to eliminate unwanted or damaged cells. Inhibition of apoptosis gives a longer life-time for the cells, which increases the possibility for accumulation of genetic changes and malignant transformation. The apoptosis inhibiting protein Bcl-2 has been shown to be positively correlated with hormone receptor status in hormone responsive tissue like prostate, breast and endometrium.[79-81] High Bcl-2 expression shows an association with favorable features of endometrial cancer. Apoptosis appears to be decreased in endometrial cancer compared with normal endometrium,[82, 83] while another study showed apoptosis to increase in endometrial cancers compared with normal and hyperplastic endometrium.[84]

4.5.4 Angiogenesis

Lewis suggested already in 1927 that the tumor environment had an impact on tumor growth.[85] In 1971, Judah Folkman stated that angiogenesis drives aggressive tumor growth and that inhibition of angiogenesis could be a way to block tumor expansion.[86] This seminal paper initiated the era of modern angiogenesis research.

The term angiogenesis is generally applied to the process of new blood-vessel growth from preexisting microvasculature, a process that is coordinated by a range of angiogenic factors and inhibitors.[87] The process in which a dormant, microscopic and non-angiogenic tumor of ~1 mm^3 or less turn into a growing angiogenic tumor is a process called the *angiogenic switch.*[88, 89] The earliest angiogenic factors, called tumor angiogenic factors (TAFs), were isolated from animal tumors and shown to be mitogenic for endothelial cells and responsible for formation of new capillaries.[90] The tumor vasculature is highly heterogeneous and does not have the same morphology as normal vasculature.[91] Abnormalities involve all components of the vessel wall: endothelial cells, basement membrane and the pericytes. Tumor vessels often have irregular diameters, abnormal branching patterns, and a defective wall structure. They may also have an incomplete vascular basement membrane and an abnormal pericyte coat.[92] The angiogenic vessels are more accessible to tumor cells than mature vessels due to their physical properties.[93]

One of the earliest endothelial cell growth factors to be isolated was the basic fibroblast growth factor (bFGF).[94] A factor secreted from tumor cells called vascular permeable factor was isolated by Dvorak's team and shown to increase the permeability of vessels.[95] The same factor was later isolated by other groups and named vascular endothelial growth factor (VEGF).[96] Several potential regulators of angiogenesis have later been identified like angiopoietins, aFGF, TGF-α, TGF-β and TNF-α.[97, 98]

4.5.5 Invasion and Metastasis

Metastasis, the spread of malignant tumors from its primary origin to a new distant organ, is the major cause of death for patients with solid malignant tumors. As mentioned, Stephen Paget proposed his "seed and soil" theory in 1889.[35] He observed that metastases did not occur in random organs, since the tumor cells (*seed*) and the

microenvironment of the distant organ (*soil*) had to be compatible. The metastatic process contains several critical steps. First of all, tumor cells must infiltrate the surrounding tissue, a process called invasion. Then tumor cells might invade blood or lymphatic vessels and must survive attacks from the immune system and forces in the vessels. Eventually, cells extravasate and colonize in a secondary organ. However, typically less than 0.01% of the tumor cells that reaches the vessels form metastasis.[99, 100] Which kind of vessel a tumor cell manage to invade might be restricted by the physical nature of the vessels.[99] The invasion process consists of changes in the adherence between tumor cells and ECM and other cells. Carcinomas are epithelial cells tightly connected to each other by E-cadherin-based cell–cell junctions and are initially separated from the stroma by the basement membranes.[101] Epithelial–mesenchymal transition (EMT) is a process where epithelial tumor cells loose their junctions to the neighboring cells which allows them to migrate through the basement membrane and into the matrix.[102] E-cadherin is down-regulated while N-cadherin, facilitating the binding between tumor cells and the stroma, is up-regulated.[103] Tumor cell adherence to the extracellular matrix is mediated by integrin cell surface receptors.[104] The basement membrane is composed of Type IV collagen, laminin, heparan sulfate proteoglycan, entactin, and fibronectin,[105] and collagen α (IV) chains seems to be lost in the early stages of invasive cancers.[106, 107] The matrix degrading proteases are upregulated in the ECM and creates a path for the moving tumor cells.[108] Many cancers express chemokines and chemokine receptors, which all have many roles in tumor progression. These cytokines are probably helping the tumor cells during invasion rather than being involved in host anti-tumor response.[109]

4.5.6 Cancer stem cells

Cancer stem cells (CSC) have the ability to self-renew and to undergo differentiation into cells that comprise the bulk of a tumor. It may not be the CSCs that initiate tumorigenesis, but over time they might represent the cell population that maintains the tumor.[110] Stem cells are long-lived cells in many tissues, and early transforming

mutations may accumulate in them. Only a minority (11-35% in breast cancer) of the cells that comprise a tumor have stem cell-like or tumor forming properties.[111] Identification of endometrial stem cells have been difficult due to lack of specific markers.[112] Still, cells showing properties for epithelial stem cells, progenitor cells and CSCs have been found in the endometrium and endometrial cancer.[113-115] EZH2, a member of the polycomb repressive complex 2, has been considered to play an essential role in maintaining self-renewal capacity of hepatic stem/progenitor cells.[116] High EZH2 expression in endometrial cancer and other tumors shows an association to aggressive features of the cancer and reduced survival.[117]

BMI-1.

Conserved heritable cellular memory of chromatin modifications can be maintained by the transcriptional activator genes in the trithorax group and the transcriptional repressor genes in the polycomb group. Both groups form multiprotein complexes that control chromatin accessibility. BMI-1 is a component of the polycomb repressive complex 1 which controls gene activity by epigenetic changes like acetylation, methylation and mono-ubiquitination of histones, and chromatin methylation.[118, 119] BMI-1 seems to be essential for self-renewal of normal and leukaemic haematopoietic stem cells, with $p16^{Ink4a}$ and $p19^{Arf}$ as critical downstream effectors.[120-122] A BMI-1 driven signature consisting of 11 genes has been proposed to be a strong prognostic factor in many cancers.[123] BMI-1 has been associated with a stem cell phenotype and aggressive features of some malignant tumors,[124-126] and it was therefore of interest to see whether this protein was involved in tumor-vascular interactions in endometrial cancers.

4.5.7 Cancer and inflammation

It has been well documented that several types of inflammation can promote cancer development and progression, and up to 20% of all cancers are linked to a chronic

infection.[127] Examples are persistent Helicobacter Pylori infection and its association with gastric cancer and MALT lymphomas, Hepatitis B and C virus infection with hepatocellular carcinoma,[128] colitis and colon cancer,[129] and HPV infection with cervical carcinogenesis.[130] Also, tumors that epidemiologically are not linked to inflammation might have inflammatory components in their microenvironment, and inflammation has been suggested to be the seventh hallmark of cancer.[131] Typical characteristics of cancer initiated inflammation is infiltration of white blood cells, mainly tumor associated macrophages, tissue remodeling and angiogenesis.[131, 132] Tumor infiltrating immune cells secrete several cytokines that recruit more inflammatory cells which might act on all stages of tumorigenesis from initiation of mutations with enhanced proliferation to metastatic spread.[133] Oncogenic transcription factors NF-κB and STAT3 are activated by inflammatory cytokines and are found in over 50% of all cancers.[134]

4.6 GENE EXPRESSION IN ENDOMETRIAL CANCER

Transcription of DNA into mRNA followed by protein translation is considered the central dogma of molecular biology.[135] Epigenetic factors that structurally regulate the accessibility to DNA segments represent a critical aspect of transcriptional regulation. The process from pre-mRNA to a functional mRNA involves many highly regulated steps determined by several RNA binding proteins.[136] The mRNA is regulated by small RNAs in the cytoplasm. MicroRNA (miRNA) was identified 1993 as small non-coding RNA molecules that bind to their target mRNA with complementary sequence.[137] MiRNAs repress protein expression, either by inhibiting the translation process or by mRNA degradation.

Several gene signatures have been presented for endometrial cancer. In 2009, Salvesen and collaborators discovered a gene signature distinguishing between two major tumor clusters with strikingly different phenotypes,[49] and then found the PI3K

pathway to be important for aggressive endometrial cancer. Another gene expression study of endometrial cancer revealed three distinct clusters showing differences in grade and stage, which appear to group tumors with specific clinical behavior.[138] Comparison of type I endometrial cancer with normal tissue showed 621 different expressed genes that could contribute to the understanding of the biological mechanisms.[139] Also, a prognosis signature for type I endometrial cancers has been presented.[140] Even though there are several gene expression signatures correlated with endometrial cancer phenotypes, there are none yet applied in the clinical routine for handling this patient group.

MiRNA often shows an altered expression pattern in cancer. Many tumor-suppressors and oncogenes seem to be regulated by certain miRNAs. A disturbed expression of miRNAs can cause higher expression of tumor oncogenes and lower expression of tumor-suppressor genes.[141] Also, several miRNA gene expression analyses on endometrial cancer have identified miRNA signatures that differ between normal endometrium and the cancer, and also among different cancer subtypes.[142, 143]

4.7 TREATMENT

Endometrial carcinoma has since 1988 been surgically staged according to the International Federation of Gynecology and Obstetretics (FIGO) staging systems, revised in 2009.[144] Stage I tumors are limited to the corpus, stage II tumors involves the cervical stroma, in stage III there is local and/or regional spread of the tumor, and stage IV tumors invade the bladder and/or bowel mucosa or carry distant metastasis.[144] Correct staging is critical for the choice of treatment.

4.7.1 Primary surgery

Early stage I endometrial cancers are treated with hysterectomy with bilateral salpingoophorectomy and in some cases removal of lymph nodes. Depending on the lymph node status, radiotherapy or chemotherapy is added postoperatively.[145] Stage II cancers with infiltration of the cervical stroma are treated by radical hysterectomy. For more advances stages, the therapy is individualized, depending on tumor burden and patient performance status, aiming for removal of the uterus and tumor debulking surgery when possible.[146] The value of para-aortic lymph node removal is controversial, but advocated for high risk endometrial carcinomas with endometrioid high grade and non endometrioid carcinomas.[147]

4.7.2 Adjuvant therapy

FIGO stage I cancers are categorized from low to high risk. Patients within FIGO stage IA and IB (FIGO 1988 criteria) with grade 1and 2 are considered as low risk cancers, and those with grade 3 as intermediate cancers. FIGO stage IC with grade 1 and 2 are considered to be an intermediate subgroup and grade 3 as high risk cancers. All FIGO stage I cancers that are papillary serous/clear cell are high risk. Low risk, early-stage cancers are effectively treated surgically, commonly without adjuvant therapy, and have good prognosis regarding survival.[148] A pooled trial containing 905 women from seven countries with early stage cancers, intermediate or high risk, were randomized into groups with surgery alone or with surgery and additional external beam radiotherapy.[149] After a median follow-up time of 58 months, there was no difference in overall survival between the women with or without external beam radiotherapy.

The treatment of high-risk and advanced disease is more complex. Management and adjuvant treatment after surgery depends upon patients risk factors for recurrence. Options include vaginal brachytherapy, pelvic external-beam radiation therapy and/or

chemotherapy. For patients with an intermediate risk for recurrence, there is no advantage of adjuvant radiotherapy in randomized trials.[150-152] Women with advanced stage-disease have a poor survival and high risk for recurrence. There are no prospective randomized trials that show adjuvant radiation to improve survival in this group.[153] Some studies indicate that radiation combined with chemotherapy might improve overall survival in patients with endometrial cancers,[154, 155] while another study did not show chemotherapy to improve overall survival or decrease the recurrence rate.[156] Randomized trials show no survival benefit from adjuvant hormonal treatment.[157, 158]

Patients with recurrent and metastatic disease may be treated with radiotherapy, surgery, endocrine therapy and chemotherapy. Patients with localized pelvic recurrences should be evaluated for surgery at relapse,[159] or can be treated with pelvic radiotherapy if they have not previously received pelvic irradiation.[160] Systemic treatment is palliative, and response to treatment is generally partial and last for an average of 3-6 months. Response to hormonal treatment is best for receptor positive tumors.[161] Chemotherapy has a limited place in the management of advanced or recurrent endometrial cancer. Recent chemotherapy trials in advanced endometrial cancer have focused on a combination of agents that have shown effects as single agents.[161, 162]

4.7.3 Clinical trials

There are ongoing clinical trials, based on molecular mechanisms, to identify novel targeted therapy.[163] These studies are designed mainly for advanced or recurrent endometrial cancers. Against angiogenesis, humanized mAbs that binds and inhibit VEGF have been designed (*e.g.* bevacizumab and VEGF-TRAP), or small molecule inhibitors targeting VEGF receptors (e.g. sorafenib and sunitinib) may be an option. Loss of *PTEN* results in activation of AKT followed by upregulation of mTOR activity. Therefore, tumors with loss of PTEN might be candidates for mTOR

inhibitors temsirolimus, everolimus and deforolimus. There are several drugs targeting the EGFR family, *e.g.* lapatinib, targeting booth EGFR and HER-2, and gefitinib.[164] Hormonal receptor PR is an important target and also aromatase inhibitors against estrogen synthesis, *e.g.* letrozole.[165] TP53, PIK3CA, new ER antagonists and transmembrane tight junction proteins claudins have been proposed to be potential targets.[165, 166]

4.8 PROGNOSIS

The EUROCARE database, based on cancer registries from 17 European countries, shows a 5-year survival of 75% for endometrial cancer patients.[167] Decrease of incidence and mortality of endometrial cancer is unlikely in the next few years, as early detection and treatment modalities have not been proven to have a major impact on mortality.[145]

4.8.1 Clinical factors

Age

Younger women with endometrial cancer generally have a better prognosis than older women **(Figure 4)**.[8] Histological grade and in particular depth of myometrial invasion appear to increase with age. The observed poorer prognosis at higher age may to some degree relate to a lack of surgical staging in these individuals and also less aggressive therapy postoperatively.[168]

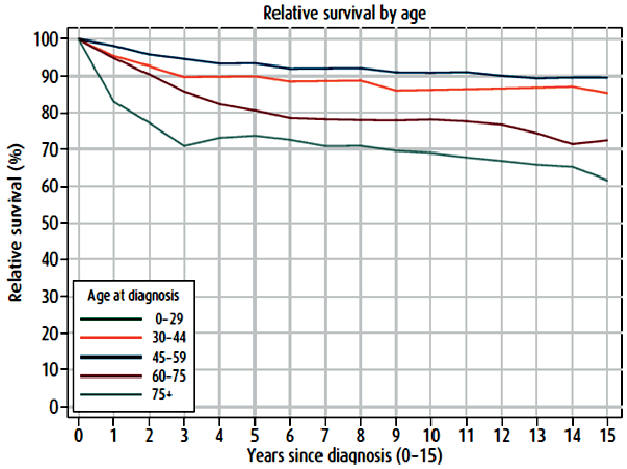

Figure 4.

Relative survival according to age at diagnosis among women with endometrial cancer. [8]

FIGO stage

The survival of patients with cancer in the corpus uteri decreases dramatically from stage IA with a five year survival between 91-100% to stage IV with a five year survival of 0-22% (FIGO 1988 criteria).[30, 168-170]

4.8.2 Histopathological factors

Histological type

The endometrioid endometrial carcinomas have a 75-83% 5-year survival while the non-endometrioid carcinomas only have 35-45% 5-year survival.[2, 3, 168, 171]

Histological grade

Grade and depth of myometrial infiltration is related to the risk of metastatic spread.[168] The five year survival in grade 1 is between 96-98% and decreases to 58-

76% in grade 3.[30, 170, 172] In all stages of endometrial cancer, grade 2 gives a hazard ratio between 1.3-1.6 and grade 3 a hazard ratio between 2.1-2.6.[168] Univariate survival analysis also show histological grade to be significantly associated with survival.[30, 173]

Myometrial infiltration

The depth of myometrial invasion in patients with endometrial cancer correlates strongly with the prevalence of lymph node metastasis and with patient survival.[30, 173, 174] Patients with more than 50% myometrial invasion are at marked risk for extrauterine metastases, including pelvic and para-aortic lymph node metastases [175]. Tumors with myometrial invasion below 50% have less than 5% prevalence of nodal spread.[176]

Vascular invasion

Vascular invasion has shown to be a marker of unfavorable prognosis in endometrial cancer and associated with several aggressive clinico-pathological features.[30, 177, 178] The antibody D2-40 is reported to be a good lymphatic vessel marker and has allowed studies on specific vascular invasion in many cancers like breast, colorectal, oral squamous cell and renal cell carcinoma, and shows in those cancers associations with aggressive features.[179-182] High lymphatic vessel density shows associations with several aggressive characteristics including vascular invasion in endometrial cancer.[183]

Necrosis

Several types of cancers with presence of necrosis show a correlation with increased angiogenesis in tumors and poor prognosis.[184-187] Presence of necrosis in endometrial cancer is of prognostic importance.[30, 172]

4.8.3 Other histopathological features

In endometrial cancer, the growth patterns show conflicting results regarding implications for prognosis.[30, 172, 188] Increased solid growth is of strong prognostic importance, both by univariate and multivariate analysis.[30, 172, 188] High mitotic count is also an indicator of poor prognosis of endometrial cancer.[30, 188] Presence of $CD8^+$ and $CD45R0^+$ TIL shows associations to favorable prognostic markers and disease specific survival in endometrial cancer,[189] and also in other types of cancers.[190] In endometrial cancer, PLI shows an association to vascular invasion.[191]

4.8.4 Biological markers

Steroid hormone receptors

Endometrial cell proliferation is under control of both estrogen and progesterone. Expression of estrogen- and progesterone receptors (ER, PR) is shown to have a favorable prognosis in patients with endometrial cancer.[192, 193]

DNA ploidy

A normal cell is diploid and contains one set of chromosomes from each parent, while an aneuploid cell is having an abnormal number of chromosomes. Flow cytometric analysis of DNA ploidy shows that aneuploidy is associated with poor prognosis in endometrial cancer.[194, 195]

Oncogenes and tumor suppressor genes

Mutations in the *PTEN* tumor suppressor gene leading to gene inactivation is found in 20-80% of endometrial carcinomas, most of them in the endometrioid subtype. The presence of mutations in hyperplasia indicates that this is an early event in endometrial carcinogenesis,[196-198] and mutations and loss of PTEN show associations

with a good prognosis.[54, 199-201] In contrast, others show that loss of PTEN is associated with poor prognosis.[202, 203] *KRAS* mutations have been reported in 10-30% of endometrial cancers.[57] Most studies do not find any association between *KRAS* mutations and clinico-pathological factors,[204-206] although one study reports mutations in *KRAS* to be associated with favorable prognosis.[207] HER-2 status has been shown to be an independent prognostic marker in endometrial cancer, and of particular importance in high-risk tumors in several studies,[66, 208, 209] while other studies do not find HER-2 to be an independent prognostic marker.[210, 211] TP53, regulating cell cycle progression by transcriptional activation of different genes, is described to be an independent prognostic factor in endometrial cancer,[63, 212] and to be associated with unfavorable clinico-pathological features.[203, 213, 214] Inactivation of p16, caused by LOH, deletions, point mutations or promoter hypermethylation, is thought to be involved in tumor progression and poor prognosis,[68, 69] and to be associated with aggressive phenotypes in endometrial cancer.[212] Whether MSI is a prognostic factor is not clear. Some studies show better survival for MSI positive endometrial carcinomas as well as an association with the endometrioid subtype,[215] while another study indicated poorer prognosis for MSI positive tumors,[216] and others did not show any relation between MSI and survival.[217-219]

Cell cycle related proteins

Multiple cell cycle regulators have been reported in endometrial cancer, but only a few of them seem to be of clear prognostic value.[220, 221] Still, high cyclin A expression in one study exhibited an association to unfavorable prognosis.[221] The cell proliferation marker Ki67 is positive in all phases of the cell cycle except of and has been shown to be a robust marker for poor prognosis in endometrial cancer.[63]

Apoptosis related proteins

Bcl-2 seems to be more strongly expressed in hyperplasias and low grade endometrial carcinoma,[222, 223] which may indicate that Bcl-2 play a more prominent role in early

rather than in the late phases. Loss of Bcl-2 is shown to be a factor indicating poor prognosis,[224] and overexpression is thus correlated with good prognosis in endometrial cancer.[225]

Angiogenesis

Intratumoral microvessel density is thought to reflect the angiogenic activity of malignant tumors. High microvessel density (MVD), the total amount of microvessels in a defined area as well as immature vessels, relate to aggressive phenotypes and is of significant prognostic value in endometrial cancer.[63, 183, 226-228] Also, an increased MVD from endometrial hyperplasia to endometrial cancer has been observed.[229] MVD as a marker for aggressive tumors has been demonstrated in several other cancer types.[230, 231] High expression of the vascular endothelial growth factor VEGF is shown to indicate a poor outcome in endometrial cancer.[227, 232] Alterations in the microvasculature pattern termed glomeruloid microvascular proliferations (GMP) or glomeruloid bodies (GB) might also indicate an activated angiogenesis and is probably related to VEGF stimulation.[233, 234] Studies of different human tumors, among them endometrial cancer, show GMP to be a prognostic marker of survival.[235] Vascular proliferation is another very promising indicator of active angiogenesis and poor prognosis in endometrial cancer, even stronger than GMP and MVD.[183]

Molecules associated with cell adhesion and stromal invasion

Loss of β-catenin has been found to be an independent prognostic factor for unfavorable prognosis in endometrial cancer,[203, 236] especially in tumors with a favorable histological subtype. In contrast, another report did not find any association between β-catenin and prognosis.[237] Decreased E-cadherin expression is a marker of tumor progression, survival and distant metastasis.[237-239] P-cadherin as well as a switch from E- to P-cadherin expression, possible as an indication of EMT, is shown to be a prognostic factor in endometrial cancer.[236]

There is still a need for new and better prognostic and predictive markers, and there are several molecules that have so far not been investigated among endometrial cancer patients. Lipocalin 2 is a molecule that is shown to be associated with several cancers,[240-243] including ER- and PR-negative breast tumors.[244] Elevated levels of LCN2 have been observed in plasma and serum during various physiological and pathological conditions, such as metastatic breast and colorectal cancer, acute kidney injury, pancreatitis and preeclampsia.[245-247] Studies of breast and colon carcinoma cell lines propose that LCN2 is involved in the EMT process.[243, 248]

5. BACKGROUND AND AIMS OF THE STUDY

Presence of vascular invasion, *i.e.* tumor cells entering vascular channels, is a significant prognostic factor in several cancers.[181, 249-251] Stefansson *et al*. previously showed that vascular invasion was a strong prognostic marker in endometrial cancer.[30] We here wanted to further investigate the biology involved in vascular invasion. An improved understanding of this process might contribute to potential markers of metastatic spread and may provide clinically important information for better management of endometrial cancer. We also wanted to study a selection of tumor markers and genetic signatures with respect to the aggressive phenotype of endometrial cancer. Identifying new and sensitive molecular markers could provide a more optimal basis for individual treatment and increase our understanding of the tumor biology.

5.1 SPECIFIC AIMS

1. In **Paper I,** the aim was to evaluate the frequency of specific vascular invasion, *i.e*. lymphatic or blood vascular invasion, and their relation to clinico-pathological variables and prognosis in endometrial cancer.

2. In **Paper II,** the aim was to explore gene signatures identifying tumors with vascular invasion and to further validate selected candidate markers with immunohistochemical staining.

3. In **Paper III**, the aim was to investigate the relationship between candidate stem cell marker BMI-1, as well as a BMI-1 associated gene expression signature, with features of aggressive endometrial cancer including vascular invasion.

4. In **Paper IV**, the aim was to examine the prognostic implication of LCN2 expression in endometrial cancer in relation to EMT markers, angiogenesis, vascular invasion and patient survival.

6. MATERIALS AND METHODS

6.1 MATERIALS

Hordaland County has about 460 000 inhabitants representing around 10% of the total Norwegian population. Two independent populations based endometrial cancer series were used in this study. They have been collected at the Department of Gynecology and Obstetrics, Haukeland University Hospital and University of Bergen, Norway.

6.1.1 Retrospective series

The retrospective series, containing 316 patients, include all patients diagnosed with primary endometrial cancer during 1981-1990. Patients were followed from the time of primary surgery until death or last follow up in 2007. The median follow-up time for the survivors was 17 years (range 6-23 years). This series is well documented concerning clinico-pathological and follow-up information.[68, 252] The series consists of paraffin embedded material, both in standard blocks and tissue microarray (TMA) blocks, which have been used for immunohistochemical studies. Of all 316 patients, 12 were excluded due to a changed diagnosis and 5 due to a diagnosis based on cytological examination only with no available histological material.[63] Of the remaining 299 cases, sufficient tumor materials in primary blocks were available for 286 patients. In **Paper I**, whole sections with deeply infiltrating tumors were available for 276 tumors (97%). In **Paper II, Paper III** and **Paper IV**, 254-261 (89-91%), 264 (92%) and 256 (90%) tumors had sufficient quality and quantity for IHC registration present on TMA sections.

6.1.2 Prospective series

The prospective series contains 57 fresh frozen cases and in parallel paraffin embedded primary endometrial tumors that were prospective collected during 2001-2003. The patients were followed from time of primary surgery until September 2008 or until death. Median follow-up time for survivors was 5.1 years (range 0.6-7 years). Fresh tumor tissue was carefully dissected from the surgical specimens and divided in two parts: one part was immediately frozen in liquid nitrogen and stored for later use at -80^{o}C; the other half was fixed in formalin and paraffin embedded for histological examination. H&E stained sections were examined by a pathologist for the tumor fraction. The tissues contained a minimum of 50% tumor cells, but the majority had >80% tumor cells. These 57 samples were selected at random from a population based tissue bank of gynecologic cancers and have been used for gene expression studies in **Paper II** and **Paper III**. All patients were surgically staged according to the FIGO 1988 criteria.

6.2 METHODS

6.2.1 Protein expression studies

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 5 μm sections of formalin-fixed and paraffin embedded tumor samples. The sections were deparaffinized in xylene and rehydrated in alcohol with decreasing alcohol concentration. During formalin-fixation, covalent chemical bonds between the proteins are created. These bonds can mask the target for antibody binding making detection difficult. Epitope retrieval can be achieved enzymatically (*e.g.* proteinase K, pepsin, trypsin, etc) or by heat. The method used in this study was microwave retrieval treatment in different buffers. Immunohistochemistry protocols for the different antibodies are listed in **Table 2**.

Table 2. *Immunohistochemical protocols*

Biomarker	Antigen retrievel	Dilution	Incubation	Detection
ANGPTL4 Sigma	MW[a] 20 min, citrate buffer[b] pH 6.0	1:15	60 min, RT	Envision
BMI-1 Upstate, 05-637, Clone F6	MW 15 min, TE buffer pH 9.0	1:800	O/N, 4°C	Envision
BMI-1 From Dr Arie P. Otte	MW 20 min, TE buffer pH 9.0	1:1	60 min, RT	CSA-kit[c]
CD-31 Dako, M0823	MW 20 min, TRS[d] pH 6.0	1:25	60 min, RT	Envision
Collagen type VIII Cosmo Bio LTD	MW 20 min, citrate buffer pH 6.0	1:250/1:100[e]	60 min, RT	Envision
D2-40 Dako, M3619	MW 15 min, TE[f] buffer pH 9.0	1:100	30 min, RT	Envision
IL8 R&D	MW 20 min, TRS pH 6.0	1:50	O/N, 4°C	PVP-HRP[g]
MMP3 Calbiochem	MW 20 min, citrate buffer pH 6.0	1:40/1:20[e]	O/N, 4°C	Envision
N-cadherin Dako, M3613	MW 20 min, TE buffer pH 9.0	1:25	60 min, RT	Envision
Lipocalin 2 R&D, MAB1757	MW 15 min, citrate buffer pH 6.0	1:25[h]	60 min, RT	1:300, goat anti-rat IgG HRP

[a]Microwave, [b]10 mM citrate buffer, [d]Target retrievel solution, [c]Catalyzed Signal Amplification system (Dako), [e]Dilution on regular slides, others TMA-sections, [f]Tris-EDTA (5 vs 0.5mM), [g]Power vision Poly-HRP anti-goat IgG, [h]Pre-blocked with goat serum diluted 1:4.

Tissue microarray (TMA)

A TMA block contains several cores of tissue that is punched from selected areas in a donor tissue block and then placed in a recipient TMA block. The TMA arrays used in **Paper II-IV** contains tissue cylinders of 0.6 mm in triplicate from each tumor. Whole tumor sections are stained by H&E, and areas with high cellularity and the

highest grade are identified by a pathologist and selected for the TMA block. This method was introduced by Kononen and collaborators in 1998.[253]

Staining index

Immunohistochemical staining was evaluated on whole tumor tissue slides and TMA sections using a semi-quantitative and subjective grading system taking into account both staining intensity and proportion of cells showing staining. Each slide was evaluated in a standard light microscope for immunohistochemical staining by 2 of the authors which were blinded for both clinico-pathological and follow-up information. A staining index (SI) was calculated as a product of the staining intensity (0; no staining, 1; weak staining, 2; medium staining and 3; strong staining) and positive area (1: <10%, 2: 10-50%, 3: >50%), giving a SI between 0-9. Cases were divided in two or three groups based on median or quartiles for the staining index, also considering the size of these groups, number of events and survival similarities.

Assessment of specific vascular invasion

Detection of tumor cells within vascular spaces is usually done on standard H&E stained sections. By using the antibodies D2-40 and CD31, it is possible to differentiate vascular invasion into blood or lymphatic vascular invasion. CD31 does not bind completely specific to blood vessel endothelia but might also show weak staining in lymphatic endothelium.[254] Blood vascular invasion was considered when the vessel with tumor cells showed positive staining for CD31, while the same vessel was negative for D2-40. Lymphatic vascular invasion was assessed when tumor cells had invaded a vessel positive for D2-40. The classification of specific vascular invasion was done on whole tumor sections showing the deepest infiltration of tumor cells. Two of the 102 positive cases based on H&E-slides, had different tumor blocks examined by IHC in **Paper I**.

Western blot

Western blot was used to investigate whether the antibodies used for immunohistochemistry indicated a specific staining.

6.2.2 Gene expression studies

Gene expression studies are of significant interest for many fields of biological research. The expression of genes might give insight into regulatory networks and lead to identification of genes relevant for biological processes.

cDNA and oligonucleotide microarray analysis

Total RNA is reversely transcribed into cDNA and thereafter amplified to cRNA (complementary RNA) with incorporation of fluorescently labeled ribonucleotides during the enzymatic amplification. The labeled cRNAs hybridize to complementary probes printed on the microarray slide with a frequency proportional to their relative abundance. After hybridization and stringent washing at optimized conditions, the amount of bound probes to each spot is scanned. Replicates of the microarray experiment was performed to show reliable and reproducible results.[255] Both one and two channel systems were used. In the two-channel system, the samples compared, *i.e.* tumor versus control, were labeled with two different fluorescent dyes. Cy3 and Cy5, often used for microarrays, emit green light and red light, respectively, when excited by incoming light of appropriate wavelengths. When Cy3-labeled and Cy5-labelled cRNAs from two different samples are mixed in equal amounts and hybridized to the microarray slide, the relative green and red light intensities generate a ratio that tells which gene is relatively up- or down-regulated.[256] In the one-channel system, only one dye is used, and only one sample is hybridized to each microarray slide. Here, the absolute level of gene expression is calculated based upon a defined background signal and computer based normalization procedures. In the two-channel

studies, either the Universal Human Reference RNA obtained from Stratagene or an in-house pool of RNA prepared from 18 different cell lines were used for reference. The largest available Agilent 44k oligonucleotide arrays were used and inter-array validation was achieved by the less comprehensive Agilent 21k and 22k microarrays.

qPCR

Candidate genes generated by SAM (Significance Analysis of Microarrays) in addition to hypothesis based genes were confirmed by real-time quantitative PCR (qPCR) with TaqMan Low Density Arrays (TLDA). We also adopted a supplementary approach to identify genes of interest from the microarray experiments. A list of 287 genes, compiled from the literature and having a known relationship with angiogenesis and invasion, was used. Individual genes were ranked by their combined associations with vascular invasion, mitosis, tumor cell necrosis, FIGO stage and metastatic phenotype. Genes with the lowest combined p-value (product of individual p-values) were further analyzed by qPCR. The idea when using this additional method was to identify genes associated with aggressive endometrial carcinoma subgroups. TLDA are microfluidic cards containing 384 wells per card. Each well contains specific, user-defined primers and probes, detecting a single gene. Of the 35 genes generated from SAM, 30 genes were identified with TaqMan assays at Applied Biosystems. A total of 87 genes in duplicate in addition to the control genes *ACTB* and *GAPDH* were analyzed with qPCR.

Bioinformatics

Microarray experiments give rise to expression data of thousands of genes, and it might be challenging to extract meaningful biological information.[257] The expression data must be pre-processed, and background intensity and spots with low signals that can not be distinguished from the background must be removed. Normalization must be done to eliminate systematic variation in intensity, which is not due to actual differences in gene expression.[258] The lowess normalization method **(Paper II)**

corrects for dye-specific effects and assume that most genes would have unchanged expression levels and are expected to be centered around zero.[259] Genes that do not show reliable values in more than 70% of the samples and have an intensity of less than 2SE (standard error) over the background, or have saturated spots were filtered. Missing values in the filtered dataset were predicted using LSimpute adaptive.[260] This method uses correlations between genes to replace missing values, e.g. cellular co-regulation of genes in functional processes. In **Paper II,** differences in gene expression of 57 tumors related to vascular invasion were investigated. An appropriate significance threshold value was needed. We used a threshold value with a minimum fold change of 2.0 and the Significance Analysis of Microarrays (SAM) to identify changes in gene expression that are biologically and statistically significant.[261] Briefly, SAM uses a gene specific t-test, and each gene is assigned a score due to its change in gene expression relative to the standard deviation of repeated measurements for that gene.

The predictable strength of the constructed gene signature in relation to vascular invasion was tested using Leave-one-out-cross-validation (LOOCV). One sample is kept out in each round and a classifier is made of the remaining samples. The classifier changes each round due to the different samples in the training set. The classifier is tested on the outsider, and the predicted result is compared to the true status and a false discovery rate is constructed. Forward selection and backward elimination are two statistical methods used for constructing condensed predictor gene sets out of the originally gene signature.

Cell cultures

In vivo, tumor cells are known to influence blood and lymphatic vessels during the metastasis process. Many of the interactions may take place by soluble factors such as cytokines, including several pro- and anti-angiogenic mediators. In **Paper II,** we wanted to investigate if endothelial cells *in vitro,* stimulated by conditioned medium

from endometrial tumor cells, showed any up- or down-regulated genes with special focus on candidates from the vascular invasion signature. Seven different endometrial cancer cell lines were cultured, the media were centrifuged and the supernatant was referred to as conditioned media. Endothelial cell lines HUVEC and HMVEC were then exposed to conditioned media for 18 hours. RNA was purified and gene expression was detected by microarray analysis. The focus of this study was to examine possible alterations in the expression levels of our up-regulated candidate genes from the vascular invasion signature, induced by the influence of tumor cells on the endothelium.

6.2.3 Statistical methods

Comparison of categorical variables was done with Pearson's Chi-square test. Univariate survival analysis was performed by the product-limit method (Kaplan-Meier method), using the log-rank test for differences between subgroups. Multivariate survival analysis was performed with Cox' proportional hazards method and the likelihood ratio test (Lratio). The time of primary operation was used as the entry date, and death from endometrial cancer was the end-point. All statistical analyses performed in **Paper I-IV** were performed with the SPSS software package version 15.0 or PASW statistical software package version 17. Statistical analyses in **Paper II-III** related to gene expression were done with the software J-Express or SDS 2.2.

7. MAIN RESULTS

Paper I

Specific vascular invasion, *i.e.* whether tumor cells are present in lymphatic or blood vessels, was determined by using antibodies CD31 and D2-40 on 276 endometrial cancers in the retrospective series. Univariate survival analysis revealed that patients with blood vessels invaded by tumor cells seem to have the worst prognosis, whereas patients with lymphatic vessels invaded have an intermediate prognosis. Patients without vascular invasion had the best prognosis. The same was seen when using recurrence free survival. Multivariate survival analysis showed blood vascular invasion to be a strong and independent prognostic factor together with the standard variables histological type, histological grade and FIGO stage. This was seen among all cases as well as for the endometrioid subtype. Our data suggest that haematogenous spread indicates a more aggressive subgroup of endometrial cancers.

Paper II

Gene expression patterns in 57 endometrial cancers from our prospective series were analyzed with microarrays and qPCR in relation to vascular invasion. A vascular invasion signature (VIS), expressing differences with respect to vascular invasion, was found to be prognostically significant by univariate analysis, although not by multivariate analysis. Published gene signatures relevant for tumor progression were also examined. By hierarchical clustering, signatures for endothelial cells, wound response, TGF-β and a VEGF-signature were significantly related to vascular invasion.

Single gene candidates including *ANGPTL4*, *COL8A1*, *IL8* and *MMP3*, all being upregulated with vascular invasion, were examined by IHC. Weak or no expression for ANGPTL4 and IL8 was associated with reduced survival. Collagen type VIII and

MMP3 were co-expressed in tumor cells and were both associated with vascular invasion at the protein level. Endothelial cells stimulated with conditioned media from endometrial tumor cells showed an up-regulation of *ANGPTL4* and *MMP3*.

Paper III

BMI-1, a candidate stem cell marker, is a member of the polycomb group and has been reported to be elevated in several cancers, both at protein and mRNA levels. A BMI-1 driven signature consisting of 11 genes has also been reported to be a prognostic signature for many cancers.[123] Our microarray data contained 9 of these 11 genes. Low *BMI-1* mRNA expression was significantly associated with the presence of vascular invasion and high histological grade Also, a significant correlation between low mRNA levels of *BMI-1* and loss of ERα and PR expression was shown. Tumors with a lower BMI-1 protein expression were associated with the presence of vascular invasion, deep myometrial infiltration and loss of ER and PR staining. Importantly, *BMI-1* mRNA levels were significantly associated with BMI-1 protein expression, whereas the 9-gene signature showed an inverse correlation to *BMI-1*, *ERα* and *PR* mRNA expression. The signature was significantly associated with non-endometrioid subtype, high histological grade, vascular invasion and poor patient prognosis in our endometrial cancer series.

Paper IV

In our retrospective series, strong LCN2 expression was associated with non-endometrioid endometrial carcinomas, nuclear grade 3, >50% solid growth and ER/PR negativity. There was no association with EMT-markers (P-cadhein, N-cadherin, E-cadherin and β-catenin). Of the angiogenesis markers, VEGF-A showed a significant relationship with LCN2 expression. Regarding prognosis, cases with no LCN2 staining had the best survival, cases with medium staining showed an intermediate survival, while the small subgroup of patients showing strong LCN2 expression had a significantly worse prognosis.

8. DISCUSSION

8.1 DISCUSSION OF MATERIALS AND METHODS

8.1.1 Patient series

As described in the Materials and Methods section, the population based retrospective series used in **Paper I-IV** includes all women diagnosed with primary endometrial carcinomas in Hordaland County during 1981-1990. This is a well documented series with a long follow-up time, and the series has been used in approximately 30 published research articles. The tumor material in this series was retrospectively collected from the archives at the Department of Pathology, Haukeland University Hospital. This archive contains formalin-fixed and paraffin embedded tissue blocks and original slides. This makes it possible to collect large series with long follow-up, which is invaluable in research. Variation in perioperative tissue handling, *e.g.* delay of fixation and fixation time may affect the sensitivity of immunohistochemical methods. For instance, delayed formalin fixation has a negative effect on ER and PR staining in breast cancer.[262]

The prospective series contains 57 fresh frozen endometrial carcinomas, 22 with vascular invasion and 35 without, and randomly collected during the period 2001-2003. To account for a possible selection bias in the prospective series, a panel of standard variables was compared with the retrospective population based patient series. No significantly differences were found for vascular invasion, histological subtype, histological grade, necrosis, mitosis and FIGO stage **(Table 1)**.

For gene expression studies done in **Paper II-III,** mRNA of good quality was needed. Tissue handling of fresh material is important, since mRNA starts to degrade by RNase enzymes within the first hour after surgical removal if the tissue is not frozen rapidly.[263]All endometrial tumors used for expression studies in **Paper II-III**

contained at least 50% tumor cells, and the majority of them contained more than 80%.

Table 1. *Patient characteristics for the prospective and retrospective series*

Variable		Prospective series N (%)	Retrospective series N (%)	p-value[a]
Vascular invasion	Absent	35 (61)	183 (64)	NS[b]
	Present	22 (39)	103 (36)	
Histological subtype	EEC[c]	51 (89)	257 (90)	NS
	NEEC[d]	6 (11)	29 (10)	
Histological grade	1 and 2	44 (77)	177 (62)	NS
	3	13 (23)	109 (38)	
Necrosis	Absent	22 (39)	119 (42)	NS
	Present	35 (61)	167 (58)	
Mitosis[e]	Low	42 (74)	216 (76)	NS
	High	15 (26)	70 (24)	
FIGO stage[f]	I/II	48 (84)	230 (81)	NS
	III/IV	9 (16)	55 (19)	

[a]Pearson Chi-Square, [b]NS= no significant difference between the prospective test series and the retrospective validation series for the respective variable, [c]Endometrioid endometrial cancer, [d]Non-endometrioid endometrial cancer, [e]Median values used as cut-off point, [f]Data for one patient is missing in the retrospective validation series

8.1.2 Gene expression studies

Microarray analysis is a powerful method allowing investigation of gene expression patterns of thousands of genes at the same time. To obtain a successful microarray experiment, it is important that all processes from the beginning to the end are optimimal.[264] The purity of RNA is important to avoid non-specific signals, and fresh

material is absolutely preferable compared to fixed tissues.[265] Formalin fixed and paraffin embedded material usually contains degraded mRNA which is difficult to recover in a quantitative way.[266] When making cDNA (complementary DNA), total RNA or purified mRNA can be used. However, only 5% or even much less of the total RNA is mRNA. Therefore, non-specific cross hybridization might be expected when total RNA is used as a source of labeled target nucleic acids in microarray hybridizations. A study comparing either purified total RNA and mRNA (poly(A)RNA) in both two- and one-channel detection platforms demonstrated, nevertheless, that using total RNA as input to microarray hybridizations generated equally good results as using poly(A)RNA.[267] This observation was important to save both materials and labor during microarray studies. Total RNA was purified and reversely transcribed into cDNA by using random hexamers and the M-MLV enzyme. Oligo (dT) primers, specific primers and random hexamers are the most common primers used in the reverse transcriptase synthesis of cDNA. Random hexamers have been shown to give the best representation of all mRNA sequences.[268]

Confirmation of microarray gene expression

Confirmation of microarray gene expression results is desirable, and we consider qPCR to be the method most relevant for small-scale validation. Many commercial assays are available, and the method is not too time consuming and does not require large amounts of RNA. The linear dynamic range is much higher for qPCR than for microarray analysis, and as a result more compressed fold changes are usually obtained based upon microarray data compared to qPCR data. P-value, FDR and fold change can be used to validate gene expression data, but questions still remain regarding which values should be used. If qPCR data do not validate the microarray results, should one assume that qPCR gives a more true result than microarrays and eliminate that gene?[269-272] We here decided to use qPCR analysis as end-point, and genes not significant for vascular invasion were excluded. The same cut-point as we used for SAM was also applied for qPCR (fold change ≥ 2.0; $p<0.05$).

Normalization is needed to compensate for differences in the amount of biological material and can be done by several methods. The most common technique is to use an internal reference gene that is assumed to be expressed at a constant level. The problem is to find a gene with small variations between samples. A reference gene with stable expression in one organ may not be suitable for normalization of gene expression in another.[273] Thus, for the qPCR validation study, we used two internal control genes, *ACTB* and *GAPDH*.

A summary score of the vascular invasion gene signature was found for each patient by summarizing the normalized expression values for up-regulated genes and subtracting the sum of down-regulated genes. Linear regression was used to test for correlations between the microarray generated versus qPCR generated vascular invasion signatures. The two gene expression techniques, with *GAPDH* as reference gene for qPCR, were strongly correlated (r=0.93). The strong correlation indicates that *GAPDH* was a suitable reference gene for our endometrial cancer samples. Probably, the most optimal would have been to use a set of internal control genes and test them on a subset of the samples to see which gene gave less variation between samples.

We wanted to generate a gene signature characteristic for tumors showing vascular invasion and by that signature identify tumors having an aggressive behavior. Such a signature might provide clinically important information for better management of the patients. Also, the vascular invasion signature would possibly provide an improved understanding of the biology involved in tumor progression and metastatic spread.

8.1.3 Protein expression studies

Immunohistochemistry is widely used to study protein expression, distribution and localization in human malignancies. In **Paper II,** we wanted to investigate the protein

expression of up-regulated genes identified in the signature**.** Many of these genes do not have corresponding antibodies that are well documented, and their use requires validation. In most cases it is difficult to tell by IHC if an antibody binds specifically or not. We here used western blot to investigate the specificity of an antibody, and a distinct band with the predicted protein mass gives an indication that the antibody is specific. We also used sections of tissues known to express the investigated protein as positive controls, and often multi-tissue blocks containing different cancers and normal tissues. In **Paper III**, the BMI-1 protein expression was investigated using two different BMI-1 antibodies, one commercial and one non-commercial. The commercial BMI-1 antibody has been examined by our group by western blot.[274] Both BMI-1 antibodies gave similar results on IHC which supports the reliability of the results. We stained TMA sections in **Paper II-IV**. Using TMAs, with several different tumors on one slide, decreased variation in the treatment between tumors is ensured. Paraffin blocks with several different cases are tissue, money and time saving. Cores in triplicate have been shown to be representative for the whole tumor section for several antigens.[275-277]

The staining index method, including both the staining intensity and the proportion of tumor cells showing positive staining, was established in our laboratory and has been used on many cancer types and in different studies.[236, 274, 278, 279] Dividing the patients into subgroups by using this staining index is distinctive for each antibody. Cut-points used are often based on median or quartiles for the staining index together with the size of the subgroups, number of events and survival similarities. There is no clear consensus on how to divide patients into subgroups, sometimes making it difficult to compare results from different studies. Reporting recommendations for tumor marker prognostic studies (REMARK) have been proposed by the National Cancer Institute and European Organisation for Research and Treatment of Cancer.[280]

8.1.4 Cell cultures

Tumor-endothelial interactions can be studied by different methods. Cell and mouse models with endothelial cells, smooth muscle cells and matrix proteins make it possible to study vessel formation under the influence of external stimuli.[281, 282] There are also systems where cells can be co-cultured and separated by a porous membrane that allows the passage of soluble factors.[283] We decided to study tumor-endothelial interactions by exposing endothelial cell lines HUVEC and HMVEC to conditioned media from endometrial cancer cells, and study changes in gene expression by microarray analysis **(Paper II)**. This is a complex experiment and there are several critical aspects. We used 7 different endometrial cancer cell lines, and the information about these cells is limited. For instance, different assays could have given us more information regarding the detailed phenotypes of the cancer cells. Also, there are critical time aspects, considering time for cancer cells to create the conditioned media, and the time span in which endothelial cells are incubated with the media. These time points were chosen based on available literature.

8.2 DISCUSSION OF RESULTS

Endometrial cancer is the most common malignant tumor in the female genital tract among women in the western world, and the incidence is increasing.[42] The majority of endometrial cancers is diagnosed at an early stage and has a good prognosis, but 15-20% recurs and show limited response to treatment.[284] Endometrial carcinoma is a heterogeneous disease, both histologically and clinically. One of the major challenges is to identify histopathological features or tissue-based biomarkers that can predict aggressive subgroups. Multiple genetic changes occur during progression from normal to malignant cells, and these changes are largely uncharacterized. Good predictive and prognostic markers are important for optimal treatment and follow-up of the patients.

8.2.1 Vascular invasion

Vascular invasion is used as a marker to identify aggressive tumors, and this feature is regarded as an indicator of metastatic spread already evident in the primary tumor.[285] This unfavorable prognostic factor should be reported in a routine setting,[24] however, less is known about the molecular pathogenesis and characteristics of these early steps of metastatic dissemination. In our studies, vascular invasion has shown to be an adverse prognostic factor, both by univariate and multivariate analysis. In subgroup analyses among endometrioid tumors, vascular invasion was significantly associated with poor survival **(Figure 5)**.

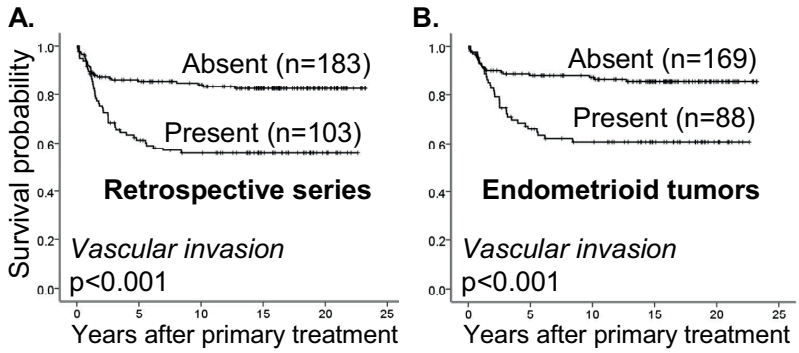

Figure 5.

*Vascular invasion is shown by univariate survival analysis to be associated with poor survival in **A:** the retrospective series (1981-1990) and **B:** among the endometrioid tumors in the same series.*

In multivariate survival analysis of vascular invasion together with standard clinico-pathological variables, this feature was an independent prognostic factor among the endometrioid tumors. Details of the multivariate analysis are given in **Table 3**.

Table 3. *Multivariate survival analysis (Cox' proportional hazards regression model) among the endometrioid endometrial cancers in the retrospective series (n=256).*

Variables	Categories	n	HR[a]	p-value[b]
Vascular invasion	Absent	168	1	0.001
	Present	88	2.5	
Histological grade	1-2	172	1	<0.001
	3	84	10.8	
FIGO stage	I/II	213	1	0.001
	III/IV	43	2.4	

[a]Hazard Ratio, [b]Lratio test

In the prospective series (n=57), vascular invasion was also significantly associated with decreased patient survival, both by analyzing the whole series as well as the endometrioid subgroup. **(Figure 6)**. In multivariate survival analysis of vascular invasion together with standard clinico-pathological variables, vascular invasion did not reach independent prognostic importance, but this is most likely due to lack of statistical power. Despite this, we consider vascular invasion to be a strong indicator of aggressive endometrial cancers.

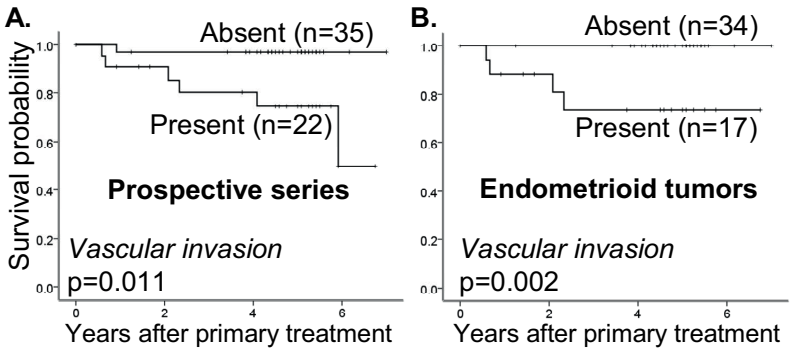

Figure 6.

*Univariate survival analysis of **A:** the whole prospective series, n=57 and **B:** the endometrioid endometrial tumors in relation to vascular invasion, n=51.*

In **Paper I**, we show that lymphatic vascular invasion occurs more frequently than blood vessel invasion (31% versus 18%). Our data indicate that both of these characteristics (LVI and BVI) are biologically important for clinical progress of endometrial cancer, but hematogenic spread as indicated by BVI appears to characterize more aggressive tumors. In cervical carcinoma, BVI has been reported to be associated with more aggressive phenotypes, and found to be an independent prognostic factor.[286] Blood vessel invasion has been reported to be an independent factor for overall and relapse-free survival in other cancer types like node-negative breast cancer, colorectal cancer and urothelial carcinoma.[181, 287, 288] Further, LVI has showed a correlation to lymph node metastasis in breast cancer, gastric cancer and bladder transitional cell carcinoma.[289-291]

8.2.2 Genes related to vascular invasion and tumor progression

Cancer cells originate from multiple genetic alterations and cellular changes. Several genes are known to be involved in tumor progression, but the underlying molecular mechanisms that determine the metastatic potential are not fully characterized.[292]

Various diseases and pathological conditions may be reflected by gene expression profiles, and derived signatures may be useful for prognostic consideration and for sub-grouping of patients.[293, 294] Global gene expression patterns might improve disease classification and give higher efficiency in the field of cancer diagnosis.

A gene signature consisting of 18 genes in relation to vascular invasion was identified in **Paper II**. It would have been an advantage to validate the gene signature on separate series of endometrial cancer, but such data were not available at the time. A future goal would be to expand the series significantly and derive independent signatures specific for BVI and LVI, with sufficient statistical power for reliable subgroup analysis.

Several studies have used different models to characterize aggressive tumors and learn more about the underlying biological mechanisms. For instance, signatures have been constructed for epithelial-mesenchymal transition (EMT),[295] normal tissues have been compared with tumor tissues,[296] and metastatic and non-metastatic cancer tissues have been examined.[297]

Vascular involvement in our material was related to predefined gene sets for epithelial-mesenchymal transition, wound response, endothelial cells and VEGF activity.[298-301] Taken together, these data support a relationship between activated angiogenesis, stroma remodeling and vascular spread as an indicator of metastatic disease.

Subsequently, published gene signatures related to tumor progression were mapped to our data set. Our vascular invasion signature was associated with the VEGF signature ($r=0.74$, $p<0.001$), the BMI signature in **Paper III** ($r=0.71$, $p<0.001$) and the wound response signature ($r=0.87$, $p<0.001$), supporting that our vascular invasive signature may identify aggressive cancers **(Figure 7)**.

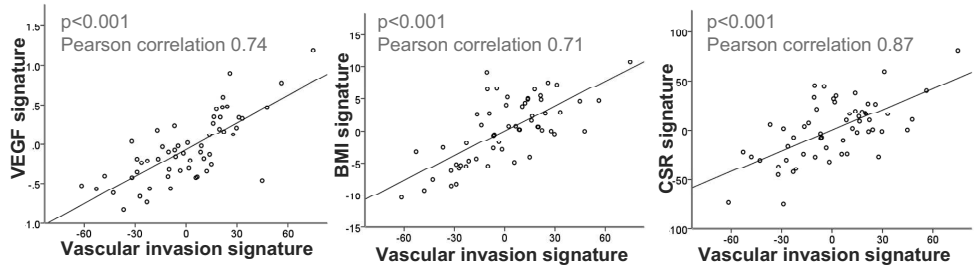

Figure 7.

The vascular invasion signature shows correlations to the published signatures for VEGF, BMI-1 and wound response (CSR).[123, 298, 300]

Our vascular invasion signature was then examined in an external dataset on breast cancer from Lu and collaborators,[292] including information on tumor type, grade, tumor size, lymphatic vascular invasion, node status, ER and HER2 expression (n=129). A summary of the vascular invasion signature was done, and patients were divided into two groups by the median value. Patients with a high signature score showed associations to tumor type, histological grade, ER and HER2 status (**Table 4**). Survival data were not available.

Table 4. *Associations between the vascular invasion signature (VIS) and clinico-pathological features in 129 breast cancer patients.*[292]

Variables	Categories	VIS ≤ median N (%)	VIS >median N (%)	p-value
Histological type	Ductal	42 (44)	53 (56)	0.047
	Lobular	14 (74)	5 (26)	
	Mixed	9 (60)	6 (40)	
Histological grade	1	20 (74)	7 (26)	0.002
	3	19 (59)	13 (41)	
	3	26 (37)	44 (63)	
ER status	Negative	19 (36)	34 (64)	0.006
	Positive	46 (61)	30 (39)	
HER2 status	Negative	57 (58)	41 (42)	0.002
	Positive	8 (26)	23 (74)	
Tumor size	≤2 cm	31 (57)	23 (43)	NS
	>2 cm	34 (45)	41 (55)	
LVI	Absent	40 (50)	40 (50)	NS
	Present	25 (51)	24 (49)	
Node status	Negative	31 (48)	33 (52)	NS
	Positive	34 (52)	31 (48)	

We then examined a public data set (NCBI GEO: GSE2109) containing 111 endometrial cancers with information about histological type, grade and FIGO stage (data not available for all tumors). We mapped our 18 genes from the vascular invasion signature, made a summary signature and divided patients into two groups by the median value. Patients with a high signature score showed significant associations

to histological grade (p=0.019) and a trend regarding FIGO stage (p=0.071) (**Table 5)**.

Table 5. *Associations between the vascular invasion signature (VIS) and clinico-pathological features in 111 endometrial cancer patients.*

Variables	Categories	VI sign ≤median N (%)	VI sign >median N (%)	p-value
Histological type	EEC[a]	48 (53)	43 (47)	NS
	NEEC[b]	8 (40)	12 (60)	
Histological Grade	1	12 (80)	3 (20)	0.019
	2	14 (42)	19 (58)	
	3	12 (38)	20 (62)	
FIGO stage	I-II	30 (55)	25 (25)	0.071
	III-IV	9 (33)	18 (67)	

[a]Endmetrioid endometrial cancer, [b]Non-endometrioid endometrial cancer

Finally, gene expression data from 230 grade 1-3 breast cancers were also examined in relation to our vascular invasion signature.[302] We mapped our 18 genes from the vascular invasion signature, made a summary signature and divided patients into two groups by the median value. Patients with a high signature score showed significant associations to histological grade (p <0.001), ER (p <0.001), PR (p=0.001) and response to preoperative chemotherapy (p=0.001). A trend regarding HER2 (p=0.082) was also seen (**Table 6**).

Table 6. *Associations between the vascular invasion signature (VIS) and clinico-pathological features in 230 breast cancer patients.*

Variables	Categories	VI sign ≤median N (%)	VI sign >median N (%)	p-value
Histological Grade	1	10 (77)	3 (23)	<0.001
	2	59 (63)	35 (37)	
	3	46 (37)	77 (63)	
ER status	Negative	27 (30)	62 (70)	<0.001
	Positive	88 (62)	53 (38)	
PR status	Negative	50 (40)	76 (60)	0.001
	Positive	65 (63)	39 (37)	
HER2 status	Negative	100 (53)	90 (47)	0.082
	Positive	15 (38)	25 (62)	
Response to pre-op.	pCR[a]	14 (29)	34 (71)	0.001
chemotherapy	RD[b]	101 (56)	81 (44)	

[a]pCR: pathological complete response, no residual invasive cancer, [b]RD: residual invasive cancer

The associations between our gene signature (VIS) and publicly available datasets from breast and endometrial cancers, and associations with aggressive clinico-pathological phenotypes **(Table 4, 5 and 6, Figure 4)** provide further evidence that our signature might manage to identify aggressive tumors, not just endometrial cancers.

A BMI-driven signature containing 11 genes, generated from a *BMI-1*$^{+/+}$ versus *BMI-1*$^{-/-}$genetic background, is suggested to have prognostic impact in several cancers.[123] In **Paper III**, we show that the BMI-1 signature also had a prognostic impact in endometrial cancer, with a significant association to vascular invasion. Interestingly, our results in **Paper III** demonstrate an inverse correlation between *BMI-1* mRNA expression and the BMI driven signature. This inverse correlation might indicate that *BMI-1* is not directly responsible for driving the 11-gene signature in endometrial cancers. Low *BMI-1* gene expression shows an association to vascular invasion and other aggressive phenotypes in **Paper III.** Contrary, studies in head and neck squamous cancer cells showed that overexpression of *BMI-1* enhances tumorigenic properties.[303]

8.2.3 Genes expressed by endothelial cells

Tumor-vascular interactions are important for tumor progression. Results from clinical trials indicate that the use of bevacizumab, a monoclonal antibody directed against VEGF, improves the outcome of breast cancer.[304] The tumor microenvironment consists of tumor stroma with blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells. Interactions between tumor cells and their environment are bidirectional, with tumor cells often dominating.[305, 306] With focus on endothelial cell gene expression, endometrial tumor cells were cultured, and their medium was added to the HUVEC and HMVEC endothelial cells **(Paper II)**. Microarray analysis showed two of our upregulated candidate genes, *ANGPTL4* and *MMP3*, to be clearly upregulated in stimulated endothelial cells. ANGPTL4 has previously been described as upregulated in endothelial cells during hypoxia,[307] but its role in tumor progression is uncertain. During angiogenesis, endothelial cells are expressing MMPs that cleave components of the stroma, thus making it possible for endothelial cells to migrate and form new vessels.[308]

8.2.4 Prognostic factors

Several prognostic factors for endometrial cancer have been published, and the impact of age, histological type and grade, ploidy, hormone receptor status and FIGO stage is well established.[2] A combined panel of prognostic markers might improve the identification of endometrial cancers with increased risk of recurrence. In **Paper II,** Collagen 8 and MMP3 showed associations with vascular invasion by protein expression, and ANGPTL4 and IL8 were related to survival by univariate analysis. By multivariate models, ANGPTL4 was an independent prognostic factor, also in the endometrioid subgroup. Surprisingly, lack of ANGPTL4 protein expression was associated with the worst survival, while strong ANGPTL4 expression was related to the best prognostic outcome. This is in contrast to our expectations based on mRNA data. There are several possible explanations for our finding: primers and antibody may detect different variants of the ANGPTL4 gene and protein, and there might be post translational regulations of *ANGPTL4* mRNA. Also, vascular invasion and survival are different end-points. Studies on breast cancer models show that tumor cell derived ANGPTL4 enhances tumor cell metastasis to the lungs by disrupting endothelial cell-cell junctions and increasing the permeability of lung capillaries [301]. Also, a study of Kaposi's sarcoma showed ANGPTL4 to promote angiogenesis and vascular permeability.[309] On the contrary, a report on 3LL and B16F0 cell lines indicated that ANGPTL4 prevented the metastatic process by inhibiting vascular activity.[310] The study showed that ANGPTL4 inhibited both tumor intravasation and extravasation. Overexpression of ANGPTL4 in melanoma cells gives a lower capacity for adhesion (to fibronectin, laminin, vitronectin and BSA), migration and invasion.[310] In oesophageal squamous cell carcinoma, ANGPTL4 showed a correlation with both LVI and BVI and seems to play an important role in metastasis through lymphovascular invasion. Strong ANGPTL4 expression is correlated with poor prognosis in this type of cancer.[311] ANGPTL4 has also been shown to be important in keratinocytes during wound healing,[312] and knock-down of ANGPTL4 gave an impaired migration. Thus, whether ANGPTL4 promotes or inhibits vascular leakiness and cancer metastasis remains unclear and might possible represent a tissue specific

response. Still, these experimental studies clearly support that ANGPTL4 is involved in cancer progression.

IL8 is known as an angiogenesis inducer, and studies have shown that IL8 stimulates endothelial proliferation and capillary tube formation *in vitro*.[313] Surprisingly, our results showed high IL8 protein expression to be associated with a favorable prognosis. This could possible indicate that IL8 is involved in a subgroup of low-grade endometrial carcinomas.

In **Paper III,** we showed that low BMI-1 expression was related to aggressive features in endometrial cancer. For instance, BMI-1 mRNA and protein showed a negative correlation to vascular invasion. Whereas several cancers have shown high BMI-1 expression to be associated with increased risk for metastasis, the exact mechanism for this relation is not known.[125, 314-316] A study of colon cancers showed BMI-1 to be associated with distant metastasis but not with vascular invasion,[317] and a study of normal nasopharyngeal epithelial cells showed that high *BMI-1* expression induces an EMT-like phenotype, with PTEN as a direct target.[318] *BMI-1* is considered to be an oncogene,[319] while our study indicates that *BMI-1*could have a suppressor function in certain tissue contexts.

LCN2 is known to be up-regulated in response to inflammation,[320] and increased levels of LCN2 has been observed in several cancers.[240, 241, 243] We show in **Paper IV** that LCN2 appears to be associated with tumor progression in endometrial cancers. Different studies have indicated LCN2 to be involved in the EMT process. In our series, however, LCN2 did not show any significant associations to any of the EMT markers included. Of the vascular markers, only VEGF-A expression showed a significant association with LCN2. Thus, the exact role for LCN2 in the EMT process seems unclear, since some studies indicate LCN2 to promote EMT,[243, 248] while others show LCN2 to inhibit this process.[321] Regarding metastasis, mammary tumor mouse models show conflicting results concerning the role of LCN2 in the process. One

study suggests LCN2 to be a potential candidate for targeted therapy, while another study reported that LCN2 was not a promoter for lung metastasis.[322, 323] In our material, LCN2 expression was increased among endometrial tumors with distant metastasis.

9. CONCLUSIONS

1. Invasion of tumor cells into the vascular systems occurs more frequently in lymphatic vessels than in blood vessels in endometrial cancer **(Paper I).**

2. Specific blood and lymphatic vascular invasion were of independent prognostic importance in multivariate survival analysis in our endometrial cancer series **(Paper I)**. Among the endometrioid tumors, blood vessel invasion was independently significant.

3. A vascular invasion signature derived in **Paper II** showed significant associations with clinico-pathological phenotype and survival. The signature correlates to a published VEGF signature that identifies aggressive tumors in several different cancer types.

4. Published signatures showed correlations to vascular invasion in our data set **(Paper II)**. Two TGF-β signatures, known to be involved in EMT, an endothelial signature and a wound response signature were associated with vascular invasion.

5. ANGPTL4 and IL8 expression showed associations to patient survival in **Paper II**. ANGPTL4 was prognostically significant by multivariate survival analysis and also in the endometrioid subtype.

6. The "BMI-1 driven" signature showed an association to patient survival and correlations to aggressive features of endometrial cancer **(Paper III)**. The signature also showed an inverse correlation to BMI-1 gene and protein expression **(Paper III).**

7. Loss of BMI-1 mRNA and protein expression was significantly associated with vascular invasion and ER/PR negative tumors in endometrial cancer **(Paper III)**.

8. LCN2 expression was associated with aggressive features of endometrial cancer including patient survival **(Paper IV)**.

10. FUTURE PERSPECTIVES

We plan to continue our work with focus on vascular invasion together with angiogenesis and epithelial mesenchymal transition (EMT) using cell and mouse models.

Tumor growth and metastasis requires angiogenesis, a process with growth of new blood vessels from already existing vascular structures. Most tumors without angiogenesis would remain in a dormant state. This makes angiogenesis an important target for the control of tumor expansion and progression. The tumor microenvironment consists of proliferating tumor cells, tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells. Interactions between tumor cells and their environment are bidirectional. Many of the steps of metastasis rely on activities of non-tumor cells, like endothelial cells and fibroblasts. Interrupting tumor-host interactions that stimulate tumor growth and metastatic spread is of importance in cancer treatment.

Several groups have developed *in vitro* and *in vivo* systems that mimic the formation of capillary networks showing many features of *in vivo* angiogenesis.[282, 324, 325] These cell culture systems are composed of endothelial cells that form vascular channels, and interactions with other cells are studied.[324] Using mouse models, tumor cells are implanted into immunocompromised NOD-SCID mice together with endothelial cells and smooth muscle cells.[282, 325] After a certain time period these implants develop a functional vasculature.

By using these models, it is possible to study tumor-endothelial interactions both *in vitro* and *in vivo* in more detail. Our future line of research would be to investigate how different factors affect tumor cell migration, endothelial cells and their tube formation, intravasation of tumor cells and metastatic spread. Of particular interest

are different regulators of EMT in tumor cells and their role in angiogenesis and metastasis. We would also explore the effects of vascular regulators like ANGPTL4, COL8A1 and MMP3 (from **Paper II)** on EMT, tumor-vascular interactions and metastatic spread. Identifying new molecular markers and investigating their effects in tumor progression could be helpful in developing improved targeted therapy.

11. ERRATA

Corrections in bold:

Introduction: Page 57, Table 5: FIGO stage I-II within VI sign >median, N (%): 25 (25) should read: 25 (**45**).

Paper I: Page 175, Materials and Methods, paragraph 3, line 3: "The median follow-up period for the survivors was 9 years (range 5-15 years)" should read: The median follow-up period for the survivors was **17** years (range **6**-**23** years).

Paper I: Page 175, Materials and Methods, paragraph 3, line 6: "Among the 117 patients who died during the follow-up period, 70 patients died from endometrial carcinoma, while 47 died from other causes" should read: Among the **165** patients who died during the follow-up period, **74** patients died from endometrial carcinoma, while **91** died from other causes.

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13. PAPERS I-IV