Molecular markers to predict prognosis and guide therapy in endometrial cancer

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Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

This project was carried out in the Bergen Gynecological Cancer Research Group at the Department of Clinical Science, University of Bergen. The group is headed by Professor Camilla Krakstad who is also the main supervisor of this project. Professor and consultant at Kvinneklinikken, Jone Trovik co-supervised the project. The group is closely linked to the Department of Obstetrics and Gynecology, Haukeland University Hospital. The research group consists of more than 20 members including senior members, post-docs, PhD-students, technicians, research nurses and medical students.

The Bergen Gynecological Cancer biobank has been prospectively collected since 2001 from patients with gynecological malignancies. The biobank contains blood samples and fresh frozen tumors from more than 5000 patients treated for gynecological malignancies at the Department of Obstetrics and Gynecology. Clinical data from the patients have been collected in parallel.

Bergen Gynecological Cancer Research Group is a part of the Centre for Cancer Biomarkers (CCBIO) which was awarded “Norwegian Centre of Excellency”. CCBIO focuses on translational research, predominantly by searching for new biomarkers to improve and individualize cancer treatment.

The group has a close collaboration with Professor and consultant at the Department of Radiology, Ingfrid Haldorsen.

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Abbreviations

^{18}F-FDG	Fluorodeoxyglucose
BMI	Body mass index
CA125	Cancer antigen 125
CEA	Carcinoembryonic antigen
CT	Computer tomography
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FDA	The U.S. Drug and Food Administration
FFPE	Formalin fixed paraffin-embedded tissue
FIGO	The International Federation of Gynecology and Obstetrics
GDF-15	Growth differentiation factor-15
GSEA	Gene Set Enrichment Analysis
HNPCC	Hereditary non-polyposis colorectal cancer syndrome
HSF1	Heat shock factor 1
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
IUD	Intrauterine devices
L1CAM	L1 cell adhesion molecule
MI	Myometrial infiltration
MIC-1	Macrophage inhibitory cytokine-1
MoMaTEC2	Molecular Markers in Treatment of Endometrial Cancer Study 2
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSH6	MutS homolog 6
MSI	Microsatellite instable

MSS	Microsatellite stable
OR	Odds ratio
pAKT	phosphorylated-Serine473-AKT
PARP	Poly ADP ribose polymerase
PCOS	Polycystic ovarian syndrome
PD-1	Programmed death receptor-1
PD-L1	Programmed death receptor ligand-1
PET-CT	Positron emission tomography-computer tomography
PI3K	Phosphinositide 3-kinases
POLE	Polymerase epsilon
PR	Progesterone receptor
ProMisE	Proactive Molecular Risk Classifier for Endometrial Cancer
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RR	Relative risk
SAM	Significance Analysis of Microarray
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TIL	Tumor infiltrating lymphocyte
TMA	Tissue microarray
TransPORTEC	Postoperative Radiation Therapy for Endometrial Carcinoma
TVU	Transvaginal ultrasound

Abstract

Background: Endometrial cancer is the most common gynecological malignancy in the Western world. The disease occurs in the epithelial lining of the uterus, called the endometrium. Although prognosis is good and most of the patients are diagnosed at an early stage, 15-20 % of patients experience recurrence. An accurate risk-stratification is lacking and as incidence is increasing due to the increased prevalence of obesity and extended life-expectancy, biomarkers for improved risk-stratification are needed.

Main objective: The main objective was to define biomarkers to better identify high-risk patients from low-risk patients in order to individualize therapy and targeted treatment.

Materials and methods: A prospectively and population-based series was collected and includes endometrial hyperplasias, primary tumors and metastases (**Paper I-IV**). Immunohistochemical staining was used for evaluation of HSF1, MSH6, PD-L1 and PD-1 (**Paper I, III and IV**). ELISA was performed for determination of plasma GDF-15 (**Paper II**). RNA microarray data were used for evaluation of mRNA levels (**Paper I, III and IV**).

Results: High expression of HSF1 associated with aggressive disease and poor survival in endometrial cancer. Protein level of HSF1 increased from primary tumors to metastasis. We found HSF1 to be an independent prognostic marker within ER-positive patients, a patient group with a presumed favourable prognosis. Gene expression analyses identified HSP90 inhibitors for targeted therapy (**Paper I**).

High plasma levels of GDF-15 associated with aggressive disease characteristics and poor prognosis, also in low-risk patients. GDF-15 can indicate recurrence during follow-up and was an independent marker for recurrence. We validated the role of GDF-15 as an independent marker for lymph node metastasis (**Paper II**).

PD-L1 and PD-1 are frequently expressed in endometrial cancer, 59% and 63%, respectively (**Paper III**). Expression was similar across MSS and MSI tumors. PD-L1 and PD-1 have no impact on survival, nor when stratified for MSI. In corresponding

metastatic lesions, expression was discordant and intra-variable compared to primary tumors.

High protein level of MSH6 identified aggressive endometrial cancer, also in low-risk patients (**Paper IV**). The prognostic value of MSH6 was validated both in curettage and hysterectomy specimen. MSH6 has independent prognostic impact preoperatively adjusted for age, histological risk-classification and hormone receptor status in the whole patient cohort. Also in a subgroup of patients with a putative low-risk disease, MSH6 demonstrated independent prognostic impact adjusted for age and hormone receptor status (**Paper IV**).

Conclusion: High expression of HSF1, GDF-15 and MSH6 predicts aggressive disease and poor survival (**Paper I, II and IV**). GDF-15 is an independent predictor of recurrent disease and lymph node metastasis (**Paper II**). PD-L1 and PD-1 are frequently expressed and expression pattern is similar across MSS and MSI tumors. Expression in corresponding metastatic lesions is discordant and intra-variable (**Paper III**).

List of Publications

I. **Engerud H**, Tangen IL, Berg A, Kusonmano K, Halle MK, Oyan AM, Kalland KH, Stefansson I, Trovik J, Salvesen HB, Krakstad C. High level of HSF1 associates with aggressive endometrial carcinoma and suggests potential for HSP90 inhibitors. **Br J Cancer** 2014, 111(1):78-84.

II. **Engerud H,** Hope K, Berg HF, Fasmer KE, Tangen IL, Haldorsen IS, Trovik J, Krakstad C. Plasma growth differentiation factor-15 is an independent marker for aggressive disease in endometrial cancer. **PLoS One** 2019, 14(1):e0210585.

III. **Engerud H,** Berg HF, Myrvold M, Halle MK, Bjorge L, Haldorsen IS, Hoivik EA, Trovik J, Krakstad C. High degree of heterogeneity of PD-L1 and PD-1 from primary to metastatic endometrial cancer. ***Submitted.***

IV. Myrvold M*, **Engerud H*,** Halle MK, Hoivik EA, Trovik J, Berg HF, Krakstad C. Added value of MSH6 as a prognostic marker in endometrial cancer. ***Manuscript.***

*Shared first author.

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Contents

1. Introduction

1.1 General introduction to endometrial cancer

Cancer is a leading cause of death worldwide and constitutes an enormous burden on society in both developed and less developed countries. Ovarian, cervical, vulvar and endometrial cancer are the main gynecological cancers. Among these cancer types, endometrial cancer is the most common gynecological malignancy in industrialized countries (1). It arises from the epithelial lining of the uterus, called the endometrium (Figure 1). Obesity is the main risk factor and incidence rates are rising, due to the higher prevalence of obesity and the prolonged life expectancy (2, 3). The overall prognosis is good and about three quarters of patients are diagnosed at an early stage (4). However, accurate risk-stratification is lacking and about 15-20% of patients experience recurrence. Treatment options for women with advanced, recurrent and metastatic disease are sparse and little improvement has been made the last decades. The disease has been largely under-studied and the potential for improvement of risk-stratification and therapy is substantial. In order to optimize and individualize treatment, there is a need for novel biomarkers to better define high-risk patients from low-risk patients (4). This thesis will focus on biomarkers that may aid in predicting prognosis and potentially guide therapy.

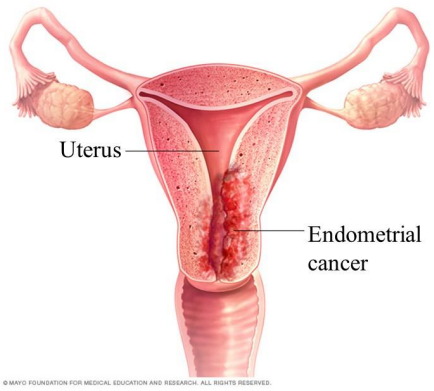

Figure 1: Endometrial cancer occurs in the epithelial lining of the uterine cavity. *Figure is cited from peoplebeatingcancer.org.*

1.1.1 Epidemiology

Incidence

Endometrial cancer is the 6th most common cancer among women worldwide and the most common gynecological malignancy in the Western world (5). The disease affected about 382 100 women worldwide in 2018 (5). In Norway there were approximately 750 new cases in 2017 (Figure 2) (6). The incidence has been steadily increasing over the past years, and the incidence is expected to further increase due to the prolonged life-expectancy and the increasing prevalence of obesity (4). Prediction models suggest between 1016-1257 new cases of endometrial cancer annually in Norway by 2025 (7).

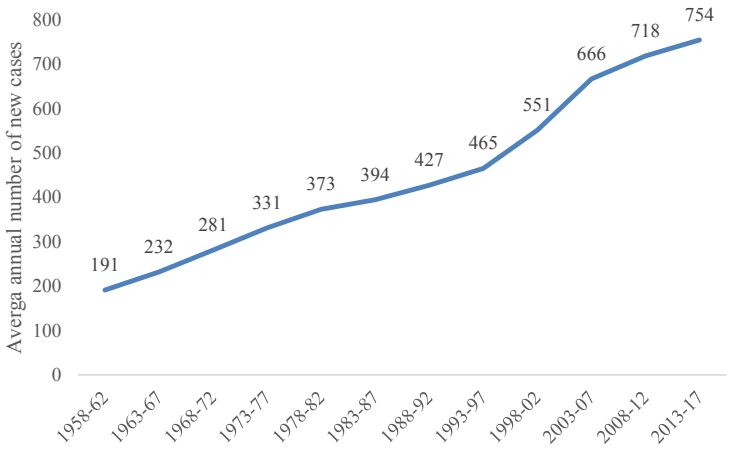

Figure 2: Incidence rates of uterine cancer in Norway by five-year period 1958-2017. *Based on data from Cancer Registry of Norway* (6).

Survival

Endometrial cancer is in general associated with a favorable survival. About 75 % of endometrial cancers are diagnosed at an early stage and the tumor is still confined to the uterine body (1). The overall prognosis is good with a 5-year relative survival of 84%. For patients with localized disease survival rates are 95%, however, survival drops to 59% when the patient have regional spread to the serosa of the corpus uteri, and/or adnexa, vaginal and/or parametrial involvement or metastatic pelvic nodes, and further drops to 40% if distant and metastatic spread have occurred (Figure 3) (6).

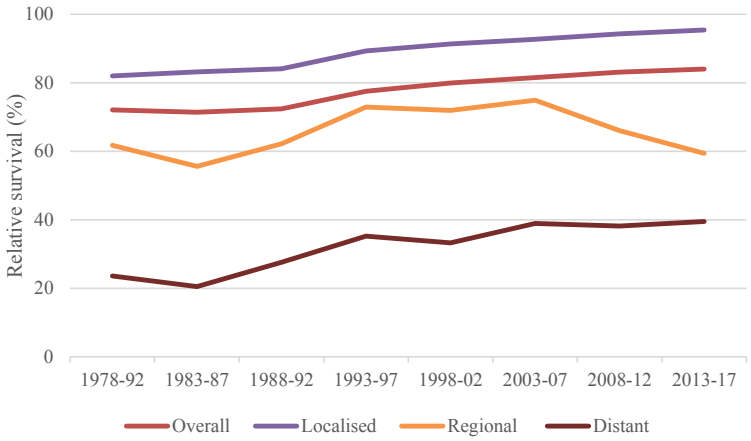

Figure 3: Five-year relative survival in Norway by stage and period of diagnosis, 1978-2017.

Based on data from Cancer Registry of Norway (6).

1.1.2 Risk factors

Acquired risk factors

Most endometrial cancers occur sporadic and the acquired risk factors are well known. Excessive estrogen production, obesity, physical inactivity, nulliparity, polycystic ovarian syndrome (PCOS), increasing age and history of breast cancer increase the risk of endometrial cancer (2, 8).

In endometrioid adenocarcinomas, counting about 80% of all endometrial cancers, excessive estrogen production is the main risk factor (1). Increased exposure of estrogens to the endometrium may cause increased proliferation and subsequently endometrial hyperplasia, and increased risk of developing endometrial cancer (9). Obesity results in inflammation and alteration of adipokine signaling. Further, it leads to secondary changes related to insulin signaling and lipid dysregulation that may foster cancer development (10). Higher risk of endometrial cancer by increased body mass index (BMI) has been demonstrated and overweight and obesity have been estimated to account for about 40% of cases of endometrial cancer in Europe (8, 11, 12). Also, the risk of death from endometrial cancer increases with higher BMI with a relative risk (RR) of 2.53 in women with BMI 30 to 34.9 kg/m^2 and a striking RR of 6.25 in women with BMI of at least 40 kg/m^2 (13). Sedentary behavior has demonstrated up to 66% increased risk of endometrial cancer (14). A potential beneficial effect of exercise and weight loss in postmenopausal and overweight women was demonstrated through decrease in free estradiol levels and increased levels of sex hormone-binding globulin (15). Further, physical activity, such as walking, showed a significant reduced risk of endometrial cancer (16, 17). Bariatric surgery demonstrated the effects of weight loss in reduction of circulating biomarkers for insulin resistance and inflammation (18). In addition, a reduction of endometrial Ki-67, phosphorylated-Serine473-AKT (pAKT), hormone receptors and restoration of glandular phosphatase and tensin homolog (PTEN) expression was displayed after surgery and subsequently weight loss (18).

Women with PCOS have a 2.7-fold increased risk for developing endometrial cancer (19). PCOS is thought to increase the risk of endometrial cancer through chronic

anovulation and long-term exposure of estrogens to the endometrium unopposed by progesterone (20, 21).

Hereditary risk factors

Endometrial cancer most often occur spontaneously, however, Lynch syndrome and Cowden syndrome are both hereditary syndromes that are associated with an increased risk of endometrial cancer (22-25). Identifying patients with one of these predisposition syndromes is important in order to provide individualized assessments of cancer risk, as well as tailored screening and prevention strategies.

Lynch syndrome, also known as Hereditary non-polyposis colorectal cancer syndrome (HNPCC), is an autosomal dominant inherited cancer susceptibility syndrome and responsible for most heritable endometrial cancers. About 2-6% of all cases of endometrial cancer are linked to Lynch syndrome (22, 23). The syndrome is associated with having a germline mutation in any of the DNA mismatch repair genes *MSH2, MSH6, MLH1* or *PMS2* resulting in reduced ability of mismatch repair and increased microsatellite instability (MSI) (26, 27). Individuals with Lynch syndrome have an increased lifetime risk of developing colorectal and endometrial cancer, and are often diagnosed at an early age. Lifetime risk of developing endometrial cancer is estimated to be about 42-60%, and the risk of developing endometrial cancer exceeds the risk of developing colorectal cancer in women (28-30). Identification of Lynch syndrome typically indicates tumor mismatch repair deficiency, which have implications for prognosis and possible treatment with targeted therapy.

Cowden syndrome is an autosomal dominant syndrome associated with germline mutations in PTEN tumor suppressor gene (31). The syndrome is rare, affecting about 1 in 200 000 individuals (32). Cowden syndrome is associated with multiple benign hamartomas and increased lifetime risk for malignancies such as breast, thyroid and endometrial cancer. Studies report up to 30% increased lifetime risk for endometrial cancer (24, 25). Cowden syndrome-associated endometrial cancer is associated with endometrioid subtype and younger age at time of diagnosis (24).

1.1.3 Clinical features and diagnosis

Symptoms and diagnosis

The main symptom in patients with endometrial cancer is postmenopausal bleeding or irregular bleeding, present in about 90% of cases (1, 4). This facilitates early diagnosis and about 75% of cases are diagnosed at an early stage when the tumor is still confined to the uterus (4). As most cases present with postmenopausal bleedings and are diagnosed at an early stage, the evidence to support screening for endometrial cancer has been poor in the general population (1, 33). Patients with more advanced disease at time of diagnosis may present with pelvic pain and abdominal distension (1). Postmenopausal bleeding is an unspecific symptom, as only 5-10% of all women presenting with postmenopausal bleeding have cancer, but risk of cancer-associated postmenopausal bleeding increases with age and presence of risk factors (2). When suspecting endometrial cancer, the doctor will perform transvaginal ultrasound (TVU) to visualize any suspect tumor in the endometrium. Histological verification is obtained by endometrial biopsy. Further preoperative staging is performed by imaging e.g. computed tomography (CT) or magnetic resonance imaging (MRI) to map the extent of tumor and create recurrence risk groups, in order to plan the surgical procedure and adjuvant therapy.

Preoperative histology

As a part of diagnostics and the preoperative assessment, the doctor will perform an endometrial biopsy by pipelle or curettage to determine histological grade and type. A key challenge in treatment of endometrial cancer is to preoperatively identify high-risk patients from low-risk patients. The preoperative assessment aims to classify patients into low-, intermediate- or high-risk groups regarding lymph node invasion and recurrence and to help guide surgical staging to determine whether lymph nodes should be removed and to what extent. Preoperative histology has proven to be discordant with final postoperative type and grading (34, 35). Studies have shown that up to 25% of cases with a preoperative grade 1 histology are upgraded on final pathology (34, 35). Subsequently, this may have consequences for the surgical approach, and whether to assign to lymphadenectomy or not. Although lymphadenectomy has not shown

survival benefit, a complete surgical staging with lymphadenectomy has potential consequences for whether to assign the patient to adjuvant therapy, which may have implications on the patients' prognosis (36).

1.1.4 Preoperative imaging

In addition to a preoperative biopsy for histological typing and grading, the patient will undergo preoperative imaging as part of the preoperative work-up to plan the surgical procedure. The most important factors to be determined are myometrial infiltration (MI), cervical stroma invasion and evaluation of metastatic spread, either to lymph nodes, or neighboring or distant organs. The type of modalities that are used varies extensively between countries and hospitals, and the modalities that are used in Norway are mentioned in brief below.

TVU is a non-invasive method that is commonly available and affordable, and associated with a minimal discomfort for the patient. Ultrasound is helpful in deciding tumor location and determining tumor extent; MI and cervical stroma invasion. The method has, however, its limitations due to intervariable observations in between clinicians, especially among in-experienced doctors, and in the case of obese patients and limited possibility for evaluation of any retroperitoneal disease spread (37-40).

CT with intravenous contrast is widely available and less expensive than MRI. CT has a clear advantage when determining distant tumor spread and lymph node metastases in the pelvis, abdominal cavity and thorax. However, due to the little contrast difference between the tumor and myometrium, CT is not sensitive nor specific enough to assess the depth of myometrial infiltration or cervical stroma invasion (37, 40).

Pelvic MRI is a highly valuable imaging method for detection of deep myometrial invasion, cervical stroma invasion and metastatic lymph nodes. Although MRI is considered the best imaging method for preoperative staging in endometrial cancer, the diagnostic accuracy of MRI is reported to be variable (40).

Fluorodeoxyglucose (^{18}F-FDG) positron emission tomography-computer tomography (PET-CT) combines two imaging techniques and visualizes both morphologic and

metabolic tumor characteristics. The tracer ^{18}F-FDG visualizes glucose metabolism, which is often increased in tumor cells (41). Reportedly, ^{18}FDG PET-CT outperforms TVU and MRI in detecting lymph node metastases and distant spread, but is not suited for assessing depth of MI and cervical stroma invasion due to limitations of spatial resolution (40).

Histopathology

The final postoperative pathology report as well as the surgical staging, guide the clinician when assigning the patient for adjuvant treatment. Regardless of The International Federation of Gynecology and Obstetrics (FIGO) stage, endometrial cancers are classified according to their histological appearances, which is currently gold standard for patient risk-stratification. Endometrioid endometrial cancers are the most frequent histological type comprising about 80% of endometrial cancers. Endometrioid cancers are often estrogen-dependant and may be preceded by endometrial hyperplasia (9). Endometrioid carcinomas are typically composed of tubular glands lined by stratified or pseudostratified columnar cells with rounded nuclei and variably prominent nucleoli, and a varying degree of differentiation (2, 42). The non-endometrioid cancers are the most aggressive and constitute about 15-20% and serous and clear cell tumors are the most common types. Serous tumors presenting with a papillary growth pattern with highly pleomorphic tumor cells in which the tumor has frequent mitoses and necrosis, and clear cell tumors with a noticeable amount of clear cytoplasm (43, 44). The mixed tumors, carcinomas composed of more than one histological type with at least 10% of each component, are known to be challenging to properly classify for further treatment (45).

Grading of endometrial cancer is based on the amount of solid growth of the glandular component, and is of prognostic importance (46). However, non-endometrioid tumors are classified as high grade by definition, thus grade has no relevance in prognostic stratifications for this cancer type (2, 47). Endometrioid tumors are classified as follows.

Grade 1: well-preserved glandular pattern and less than 5% solid growth.

Grade 2: less well-defined glandular pattern and less than 50% of solid growth.

Grade 3: hardly recognizable glands and more than 50% of solid growth.

However, a major challenge in histopathological classification of endometrial cancer is tumor heterogeneity; small populations of cells with a different character within the same tumor that would have impact on diagnosis and treatment. In addition, inter-observer variability in distinguishing between high-grade endometrioid carcinomas and non-endometrioid carcinomas is significant. In order to improve risk-stratification and guide treatment, and to add value to standard histopathological stratification, molecular markers are needed to reduce inter-observer-variability and to better identify small populations of distinct cells within a tumor (44).

FIGO staging

Patients are surgically staged according to the FIGO staging system (Table 1) (48). The FIGO staging system was first introduced in 1988 (49) and revised in 2009 (48). Increasing FIGO stage implies increasing risk of recurrence and poorer prognosis, it is the strongest prognostic marker in endometrial cancer (47). In FIGO stage I and II, 5-year survival rates range from 74-91%. In FIGO stage III and IV, 5-year survival rates range from 57-66% and 20-26%, respectively (1, 2).

Table 1. FIGO staging system for endometrial cancer according to 2009 criteria.

Stage	**Description (48)**
I	Tumor confined to the corpus uteri
IA	Myometrial invasion <50%
IB	Myometrial invasion >50%
II	Tumor invades the cervical stroma, but does not extend beyond the uterus
III	Local/and or regional tumor spread
IIIA	Tumor invades the serosa of the corpus uteri, and/or adnexa
IIIB	Vaginal and/or parametrial involvement
IIIC1	Metastases to pelvic nodes
IIIC2	Metastases to para-aortic lymph nodes with or without positive pelvic lymph nodes
IVA	Tumour invasion of the bladder and/or bowel mucosa
IVB	Distant metastases including intra-abdominal metastases and/or inguinal lymph nodes

1.1.5 Treatment

Surgical treatment

The primary treatment of endometrial cancer is surgery and the cornerstone of treatment is hysterectomy with bilateral salpingo-oophorectomy with or without lymphadenectomy. In recent years, minimally invasive techniques such as laparoscopy (keyhole surgery) or robot-assisted surgery in low-stage disease have been increasingly used, reducing hospital stay and postoperative complications as opposed to laparotomy (open surgery) (50-52). For most patients simple hysterectomy is sufficient, but for patients with cervical stromal invasion radical hysterectomy is performed. Omentectomy is only performed in patients with high-risk histology; clear cell and serous (53).

The extent of lymphadenectomy varies worldwide and its role in the management of endometrial cancer is controversial. Lymphadenectomy is necessary for a complete surgical staging. However, no benefit in survival has been reported (54-56), and adverse effects such as lymphedema and lymphocele are frequently described (36, 56). Traditionally, patients with a putative intermediate and high-risk disease are commonly assigned to lymphadenectomy (53).

Sentinel lymph node mapping, a novel surgical technique replacing lymphadenectomy is becoming appreciated in many countries, as this may spare the patient from undergoing complete lymphadenectomy. The technique involves selective and limited removal of tumor-specific or organ-specific lymph nodes that are identified after injection of tracer dye into, or in proximity to, the primary tumor (57). Sentinel lymph node biopsy has proven equivalent to lymphadenectomy in staging of endometrial cancer and can potentially spare the patient from the unwanted side effects of lymphadenectomy, and still obtain complete surgical staging and reduce morbidity (58-60). However, the support in literature is still limited and the technique is not yet implemented in most countries (36, 61).

Adjuvant therapy

Adjuvant therapy may be beneficial to patients at high-risk of recurrence in order to reduce their risk of relapse. Combined chemotherapy with carboplatin and paclitaxel is the current standard first-line regimen, consisting of 6 cycles of 3-weekly paclitaxel and carboplatin and is administered to patients with high-risk of recurrence (62) (Table 2). In FIGO stage I this signifies that only grade 3 endometrioid cancers with MI ≥50 %, and all non-endometrioid cancers independent of MI are offered adjuvant chemotherapy, in addition to FIGO stage II-IV (33, 53). National guidelines in Norway are based on the ESMO/ESGO guidelines (63).

Table 2. Risk of cancer relapse in patients with FIGO stage 1 (53).

Low-risk	Stage 1A grade 1 and 2 endometrioid subtype
Intermediate risk	Stage 1A grade 3 endometrioid subtype
	Stage 1B grade 1 and 2 endometrioid subtype
High-risk	Stage 1B grade 3 endometrioid subtype, all stages with non-endometrioid subtype

Chemotherapy

A few trials have investigated the efficacy of chemotherapy. The evidence of benefit from adjuvant chemotherapy exists for patients with positive lymph-nodes, and the retrospective data is weak for the efficacy of chemotherapy in early-stage clear cell cancer (64). The Gynecologic Oncology Group 122 trial demonstrated improved progression-free survival and overall survival of doxorubicin and cisplatin versus whole abdomen irradiation in stage III and IV disease (65). However, an Italian trial and the Japanese Gynecologic Oncology Group trial did not demonstrate any survival benefit with chemotherapy compared to pelvic radiation (66, 67). These trials included mostly early-stage and low-grade disease, yet the difference in survival rates is not clear. A Cochrane review summarized a reduced risk of recurrence by adjuvant chemotherapy (68). Overall, endometrial cancer is considered a chemotherapy-sensitive tumor and taxanes, anthracyclines and platinum agents are generally active in

chemotherapy-naïve patients. However, response to second-line chemotherapy has been poor and only taxans have proven response rates of 20% (69).

Radiotherapy

Radiotherapy can be delivered vaginally as brachytherapy or externally to the pelvis. Adjuvant radiotherapy as treatment for early-stage disease has been widely debated as studies have shown to improve local control, however, no proven effect on survival (70-73). There is ample evidence in literature supporting a reduced locoregional recurrence rate from 12-20% if no additional treatment is provided, to 3-5% after adjuvant radiotherapy in high-risk patients (70-73). However, most studies agree that radiotherapy to early-stage endometrial cancer does not convey a survival benefit (70-73). Side effects after radiotherapy include chronic diarrhea, fecal leakage and reduced sexual functioning that can be debilitating and severely affect quality of life (74). In Norway, routine adjuvant radiotherapy in FIGO stage 1 and 2 patients was no longer offered after 2008 due to lacking evidence of survival benefit. The argument for this is that the majority of locoregional recurrences, if they occur, can be treated by therapeutic dosages of radiation, surgery and/or chemotherapy. In this way, unnecessary and long-term side effects can be avoided in most patients, preserving high quality of life, while still retaining high survival rates (75, 76).

Chemoradiation, combining chemotherapy and radiotherapy has not shown survival benefit (64). A recent study, the PORTEC-3 trial did show significantly improved 5-year recurrence-free and disease-specific survival with chemoradiotherapy compared to external-beam radiotherapy alone (77). However, no improvement in overall survival was demonstrated (77).

Hormonal therapy

Hormonal therapy is not recommended as adjuvant therapy, but may be considered in the metastatic setting, especially for tumors of low-grade endometrioid histology with a long time to recurrence (64). However, the response rates are modest and hormone receptor status is not always taken into consideration when assigning the patient to hormone treatment (78). Although response to hormonal therapy is more common

among patients with intact estrogen receptor (ER) and progesterone receptor (PR) expression, response rates and hormone receptor expression have proven discordant in prediction of response to hormonal therapy (79).

Conservative treatment

Conservative treatment may be of interest among patients with early stage endometrial cancer, that is stage 1A endometrioid grade 1 or 2, or endometrial hyperplasias. Especially among women of young age who want to preserve their fertility, or among women with comorbidity who are at high-risk of surgical complications. Oral progestin therapy with medroxyprogesterone or megesterol acetate has previously been the option, however, disease progression occurs in some cases and new therapeutic strategies and biomarkers to better select patients for treatment are needed (80).

Intrauterine devices (IUD) releasing progesterone have shown success in converting endometrial hyperplasias to normal epithelium, both among young women wanting to preserve their fertility, and among older women who are not suited for surgical treatment (81). In women with stage 1A endometrioid grade 1 and 2 cancers oral progestins such as megestrol acetate or IUDs have shown regression of disease (82).

The link between endometrial cancer and metabolic syndrome has made metformin interesting as treatment or adjunctive treatment for early endometrial cancer. Metformin has shown results in treating endometrial hyperplasia, especially among patients with PCOS and early stage endometrial cancer with reduction in tumor markers such as Ki-67, pAKT and ER (80, 83).

When choosing conservative treatment, the patient should be informed of the risk of an inadequately staged/treated disease, an inherited genetic cancer risk and the potential risk of a synchronous/metachronous ovarian cancer.

Targeted therapy

Targeted therapy, a treatment modality that is directed against a specific molecular target identified in the patients' cancer or tumor microenvironment, is the cornerstone of precision medicine (84). One well-known example of targeted therapy is anti-HER2 (trastuzumab) which has demonstrated good response rates in HER2-positive breast cancer patients (85). To date, no targeted therapies are established in clinical use for endometrial cancer patients. The lack of predictive markers to select patients for treatment has limited the potential of targeted therapies. Previously, temsirolimus (mTOR-inhibitor), trastuzumab and bevacizumab (VEGF-A-inhibitor) have been investigated; however, the response rates have been modest (86-88). Targeting aberrations of the phosphinositide 3-kinases (PI3K) pathway have also yielded modest results in clinical trials, however combined poly ADP ribose polymerase (PARP) and PI3K inhibitors in mouse models have shown synergistic effects (9).

The identification of the four molecular subtypes in endometrial cancer by TCGA contributed to a shift of paradigm and gave momentum to further research for targeted therapy in endometrial cancer (89). Four distinct molecular subtypes were identified, each with distinct impact on survival. One of the molecular subtypes, MSI has emerged as a promising predictive biomarker for response to immunotherapy in solid tumors, due to the increased number of neo-antigens (90-92). Treatment with immune checkpoint inhibitors has become an appreciated treatment with promising response rates in the recurrent and metastatic setting in solid MSI-high tumors (93-95). Results have demonstrated less toxicity than chemotherapeutic regimens and a potential for durable response (93-95). As U.S. Drug and Food Administration (FDA) granted approval to pembrolizumab (PD-1-inhibitor) for treatment of advanced and recurrent MSI-high endometrial cancer, treatment with immune checkpoint inhibitors has become an option also for endometrial cancer patients (96). The KEYNOTE-028 study with treatment of pembrolizumab to PD-L1 positive, advanced MSI-high endometrial cancer has demonstrated promising results (97). A recent phase II study with avelumab (PD-L1-inhibitor) demonstrated promising results in MSI-high patients regardless of PD-L1 expression, but demonstrated no efficacy in MSS endometrial cancers (98).

However, treatment with immune checkpoint inhibitors may not be exclusively for patients with MSI-tumors. Several recent clinical trials indicate that patients with microsatellite stable (MSS) tumors may benefit from immune checkpoint inhibitors in combination with a second drug. In a trial based on biomarker unselected, advanced endometrial cancer patients, an objective response to combination therapy with pembrolizumab and lenvatinib (a multikinase inhibitor of VEGFR1, VEGFR2, VEGFR3) was recorded in 16 out of 45 patients with MSS-tumors, compared to two out of four patients with MSI-tumors (99). Recently, FDA approval was granted to lenvatinib in combination with pembrolizumab, for the treatment of patients with advanced endometrial cancer regardless of MSI, who have disease progression following prior systemic therapy and are not candidates for curative surgery or radiation. Promising results by combination therapy with PD-1 blockade and cytotoxic T-lymphocyte–associated protein-4 (CTLA-4) to MSS tumors have also been described (100). The PD-L1/PD-1 pathway is explained in detail in section 1.2 Tumor Biology.

1.2 Tumor biology

1.2.1 General tumor biology

The human genome gives rise to hundreds of cell types with multiple functions. Cancer is by definition uncontrolled division of abnormal cells in any part of the body. For normal cells to evolve progressively to an abnormal and neoplastic state they acquire several capabilities in order for the cell to become malignant.

Hanahan and Weinberg summarized several decades of intense cancer research in the two "Hallmarks of cancer" papers, further contributing to the understanding of cancer biology (101, 102). The hallmarks include *sustaining proliferative signalling* which is the most fundamental trait of cancer cells. By *evading growth suppressors,* tumor cells successfully circumvent powerful programs which negatively regulate cell proliferation. *Activating invasion and metastasis* for tumor cells to progress to higher pathological grades of malignancy. *Enabling replicative immortality,* which is in marked contrast to normal cells that have a limited number of growth-and-division cycles. *Inducing angiogenesis* by neovascularization to provide oxygen and nutrients. *Resisting cell death* in which apoptosis is attenuated in tumor cells and they succeed in becoming high-grade malignant. Two more emerging hallmarks have emerged, including *avoiding immune destruction* in which the immune system plays a role of resisting or eradicating formation and progression of early-stage neoplasias or late-stage tumors and *deregulating cellular energetics* in which tumor cells upregulate glycolysis. Two enabling characteristics have also emerged, *tumor-promoting inflammation,* which is driven by cells of the immune system and serve as promoters of tumor progression and *genome instability and mutation,* which is responsible for random mutations and chromosomal rearrangements (101, 102).

In recent years focus has been on detecting genomic alterations in the tumor. For precision oncology, it is crucial to identify molecular cancer drivers. A recent study identified 299 cancer drivers in a pan-cancer study comprising 33 cancer types, among the most frequent in endometrial cancer was tp53, PTEN, PIK3CA and MAP3K1 (103).

1.2.2 Endometrial tumor biology

Signalling pathways relevant for endometrial cancer and this project especially is discussed in brief in the paragraphs below.

The heat shock response

The heat shock response plays a central role in promoting survival and increased proliferation (104, 105). The heat shock response is the most conserved cellular protective mechanism and responsible for cellular homeostasis, by combatting the negative effects caused by stressors such as increased temperature, oxidative stress and inflammation (106). The transcriptional activator, heat shock factor 1 (HSF1), mediates the regulation of the heat shock gene transcription (107). HSF1 triggers massive transcription of genes, such as heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70), which facilitate normal protein folding and protect the proteome from misfolding and aggregation that could cause lethal damage. The role of HSF1 in cell survival has been linked to carcinogenesis, and the role of HSF1 to modulate oncogenesis was demonstrated in HSF1 knockout-mice, which had reduced susceptibility to tumor formation (108). Also, elevated levels of HSF1, heat shock protein 60 (HSP60) and HSP90 in aggressive prostate carcinoma cell lines have been demonstrated (109). Increasing evidence supports that HSF1 plays a crucial role in tumor formation, but the exact role is not fully understood (106). However, HSF1 in cell cultures demonstrated to support malignant transformation by increased proliferation, survival, protein synthesis, and glucose metabolism (105, 108).

The Transforming Growth Factor (TGF)-β signalling pathway

The TGF-*β* superfamily has a role in inflammatory and apoptotic pathways in injured tissues and during disease processes. The TGF-*β* pathway has been found to be redirected away from suppressing cell proliferation and instead become a tumor promoter (101). Growth factor differentiation factor-15 (GDF-15), also called macrophage inhibitory cytokine-1 (MIC-1), is a part of the TGF-*β* superfamily. It was first identified in activated macrophages (110). GDF-15/MIC-1 is involved in tumor pathogenesis and is associated with cell cycle arrest and apoptosis (111). Expression

of GDF-15/MIC-1 is mediated by p53 and studies have shown that measurement of circulating tumor-derived MIC-1 is a good *in vivo* indicator of p53 pathway activation (112).

Immunosurveillance and checkpoint inhibitors

The role of the immune system in tumor formation has been an unresolved issue and widely debated. However, in recent years, research has increasingly supported that the immune system can indeed prevent tumor formation. The theory of cancer immunosurveillance proposes that cells and tissues are monitored by an ever-alert immune system, which is responsible for recognizing and inactivating potentially dangerous mutant cells that can lead to tumor formation (101, 113). The impact of intratumoral lymphocyte infiltrates on clinical outcome have been demonstrated in several solid cancers, such as ovarian and colorectal, in which tumors with heavily infiltrated cytotoxic T-cells and natural killer cells have a better prognosis than patients that lack the abundance of these cells (114). In endometrial cancer, the presence of tumor infiltrating lymphocytes (TILs), specifically the presence of CD8+ T-lymphocytes was demonstrated to be an independent predictor of improved overall survival, also in the subgroup of type II endometrial cancer patients (115). Consequently, immunotherapy has made its step into cancer care by treatment with immune checkpoint inhibitors, which have emerged as a major treatment modality in oncology and precision medicine. Potential targets for immune checkpoint inhibitors are e.g. PD-L1 and PD-1. PD-L1 is expressed on tumor cells and binds to the receptor PD-1 on cytotoxic T-cells. This binding causes suppression of the T-cell, as a negative feedback system that represses the immune system, and is a strategy for tumor cells to escape from the anti-tumor activity of T-cells (96). Antibodies to PD-1 or PD-L1 block the binding of PD-L1 on tumor cells to PD-1 receptors on T-cells, and allow the T-cells to induce the immune response against tumor cells (Figure 4).

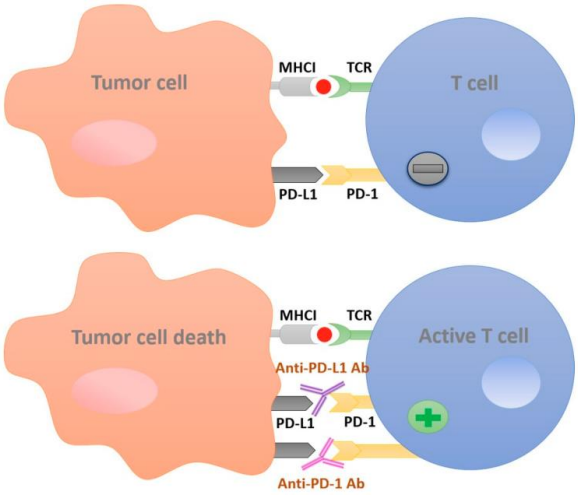

Figure 4: The PD-L1/PD-1 pathway. The figure shows how antibodies to PD-L1 and PD-1 block the PD-L1/PD-1 pathway and activates the T-cell in order to fight tumor cells.

Figure is reprinted with permission from Cancers (116).

The two types of endometrial cancer

Traditionally, endometrial cancers have been divided into type I and type II (117, 118). Type I tumors are associated with obesity, metabolic syndrome and tumors are highly estrogen-dependent with a positive ER and PR status. Approximately 80% of the tumors are low grade (grade 1 and 2) while 20% are high grade (grade 3) (118). Type II tumors on the other hand, are not associated with metabolic syndrome, are less estrogen-dependent and comprise mostly serous tumors. Type II tumors are associated with aggressive clinical features such as deep MI and lymph-node metastasis, and thus have a poorer prognosis as opposed to type I tumors (118).

The Cancer Genome Atlas project

The Cancer Genome Atlas project is a joint effort between the National Cancer Institute and the National Human Genome Research Institute and began in 2006. The cancer genomics program has molecularly characterized over 20 000 primary cancers from 33 cancer types with matched normal samples. In endometrial cancer, TCGA identified four molecular subgroups by performing integrated genomic, transcriptomic, and

proteomic characterization of 373 endometrial cancers (89). The four molecular subgroups were ultramutated polymerase epsilon (POLE), hypermutated MSI, copy number abnormalities-low and copy number abnormalities-high (Table 3). The TCGA publication has led to a shift of paradigm in endometrial cancer research, gaining more insight to the molecular landscape of endometrial cancer and slowly leaving behind the more traditional way of stratifying endometrial cancers into type I and II. There has been an increasing interest in integration of molecular markers and it has gained momentum to further research on the molecular level and targeted therapy especially.

Table 3: TCGA classification of endometrial cancers

Subgroup	**Selected characteristics (89):**
POLE ultramutated	Very high number of mutations
	Favorable PFS
	Frequent mutations in *PTEN, PIK3CA* and *KRAS*
MSI	High number of mutations
	Frequent MLH1 promoter hypermethylation
	Low number of SCNAs
	Few mutations in *TP53*
Copy number high	High number of SCNAs
	Frequent *TP53* mutations
	Low degree of MSI
	Poor PFS
Copy number low	Low mutation rate
	Microsatellite stable

POLE: polymerase PFS: progression-free survival. MSI: microsatellite instable SCNA: somatic copy number

A classification tool for clinical use

A more pragmatic and less expensive classification tool of the four molecular subgroups by identification of surrogate markers has been proposed by the ProMisE (Proactive Molecular Risk Classifier for Endometrial Cancer) and TransPORTEC (Postoperative Radiation Therapy for Endometrial Carcinoma) initiatives (119, 120). TCGA data were conducted mainly on low-risk endometrial cancers and serous cancers, however clear cell cancers were lacking. The TransPORTEC has validated a simple molecular classification on high-risk endometrial cancer, resulting in four distinct molecular subgroups "POLE mutated", "microsatellite unstable", "TP53 mutated" (surrogate marker for copy number-high) and "no specific molecular profile" (120, 121). ProMisE has defined "microsatellite unstable", "POLE mutated", "p53 wild type" and "p53 abnormal" (119, 122-124). Central in the defining of MSI by immunohistochemistry is the two mismatch-repair proteins MSH6 and PMS2. Lack of nuclear expression of either two proteins depicts microsatellite instability (119, 124), and the protocol has been applied in **Paper III** and **IV**. The application of more clinically applicable methods on formalin-fixed paraffin-embedded (FFPE) samples, using sequenzing and immunohistochemistry, serves as a potential routine clinical classifier (120, 125, 126).

1.3 Biomarkers in endometrial cancer

In endometrial cancer, prognostic markers are needed in order to better differentiate high-risk patients from low-risk patients (4). In order to improve clinical decision making and treatment strategies, predictive markers for response to therapy are highly needed (127). In spite of rigorous research the last years, few biomarkers have reached clinical practice (4).

The Biomarkers Definitions Working Group has defined a biomarker as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, patho-genic processes, or pharmacologic responses to a therapeutic intervention"(128). Biomarkers can be measured in serum, plasma or urine, but more invasive techniques requiring tumor tissue, such as immunohistochemistry and DNA/RNA analyses are widely used (129). Anything that is quantifiable in a patient may potentially serve as a biomarker. For a biomarker to be of clinical interest it must add information to what is already known from established clinicopathological variables or predictors. Figure 5 illustrates the areas in the diagnostic work-up where a biomarker could be helpful in patient care.

Biomarkers are in general categorized in two groups 1) Prognostic markers as tools for diagnosis, disease staging or indicator of disease outcome. 2) Predictive markers anticipating the likely response to a specific therapy.

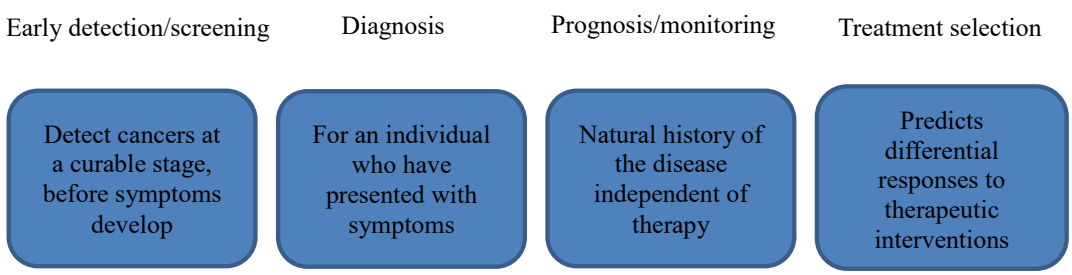

Figure 5: Examples of areas in the diagnostic work-up where a biomarker could be helpful in patient care.

1.3.1 Prognostic biomarkers

Prognostic markers provide information about the patients' prognosis, regardless of therapy (129). To improve risk-stratification and tailor therapy, prognostic biomarkers are crucial in treatment of endometrial cancer. Prognostic biomarkers are in clinical use for several cancer types, such as prostate specific antigen (PSA) in prostate cancer (130), cancer antigen 125 (CA125) in ovarian cancer (131) and serum-derived carcinoembryonic antigen (CEA) in colon cancer (132). Established prognostic markers in endometrial cancer are all of clinicopathological origin and constitute e.g. FIGO stage, lymphovascular space invasion, histological grade and type (4). Although molecular biomarkers with prognostic impact are identified in endometrial cancer, none of them have reached clinical practice yet.

Aneuploidy, overexpression of p53 and *k-ras* (*KRAS)* amplification have independently been associated with poor survival in endometrial cancer (133-135). L1 cell adhesion molecule (L1CAM) protein expression has been proposed as a strong prognostic biomarker and predictor of lymph node metastases (136-138). The presence of TILs, specifically the presence of CD8+ T-lymphocytes were demonstrated to be an independent predictor of improved overall survival, also in type II endometrial cancer patients (115).

However, a small step into clinical implementation is the identification of loss of both ER and PR in prediction of lymph node metastasis and poor outcome (139), which has led to The Molecular Markers in Treatment of Endometrial Cancer study 2 (MoMaTEC2, NCT02543710). The trial is a phase IV implementation trial for optimized stratification of surgical treatment. Low-risk patients (endometrioid tumors grade 1 or 2, or grade 3 with <50% MI, with no sign of extrauterine disease) with positive hormone receptor status for both ER and PR will omit lymphadenectomy. High-risk endometrial cancer with either negative ER or PR will undergo lymphadenectomy.

1.3.2 Predictive biomarkers

Predictive markers give information about the anticipated effect of a certain therapeutic intervention (129). Predictive markers are crucial in order to assign patients for targeted treatment. In several cancer types, predictive markers and treatment with targeted therapy have made progress the recent years, such as BRAF-inhibitors in treatment for metastatic malignant melanomas (140), and HER2-inhibitors in breast cancer (85). So far, in endometrial cancer, few predictive markers have emerged and consequently treatment with targeted therapy is limited.

In endometrial cancer, ER and PR have been suggested to predict improved response to hormonal treatment (78, 141), and high expression of Stathmin has demonstrated to predict poor response to paclitaxel (142).

MSI has emerged as a predictive marker for response to immunotherapy in solid tumors, and is an emerging marker for response to immunotherapy in endometrial cancer as well (90-92, 143). After FDA approved pembrolizumab (PD-1-inhibitor) for treatment of MSI-high recurrent and metastatic endometrial cancer, treatment with immune checkpoint inhibitors have become an option also for endometrial cancer patients (96). Programmed death receptor 1 (PD-1) and programmed death receptor ligand 1 (PD-L1) are established predictive markers and targets for treatment with immune checkpoint inhibitors in solid tumors (90, 143). The KEYNOTE-028 study with pembrolizumab to PD-L1 positive, advanced MSI-high endometrial cancer has demonstrated promising results (97). Targeted therapy with immune checkpoint inhibitors is further explained in section 1.1.4 Targeted therapy.

2. Aims of the study

2.1.1 Background and general aims

An increasing number of patients are being diagnosed with endometrial cancer, and due to the prolonged life-expectancy and the increasing prevalence of obesity the numbers are expected to further increase. The overall prognosis is good, however 15-20 % of patients experience recurrence. The survival rates for recurrent, metastatic and advanced disease are poor and little improvement has been made the last decades. Thus, it is urgent to find biomarkers that better select high-risk patients from low-risk patients to tailor therapy and develop targeted treatment. The overall aim of this study was to define biomarkers that better predict prognosis and guide therapy.

2.1.2 Specific aims

Paper I: Evaluate the prognostic impact of HSF1 in endometrial cancer. Also, we aimed to investigate the transcriptional alterations related to HSF1 protein level by microarray analysis.

Paper II: Identify GDF-15 as a marker for recurrent disease. Additionally, we aimed to validate GDF-15 as a prognostic marker for aggressive disease and as an independent marker for lymph node metastases.

Paper III: We aimed to determine the expression patterns of PD-L1 and PD-1 in both primary tumors and corresponding metastatic lesions, stratified for microsatellite instable and microsatellite stable cancers.

Paper IV: The aim of this study was to determine the prognostic value of MSH6 both in preoperative curettage specimen and in hysterectomy specimen.

3. Materials and methodological considerations

3.1.1 Patient series

All samples included in the study were retrieved from the Bergen Biobank for Gynecological Cancer (REK number 2014/1907). Patients included in the study are diagnosed and treated for endometrial cancer at Haukeland University Hospital, Bergen, Norway. All patients are prospectively included from 2001 to 2015. Haukeland University Hospital is a referral hospital for Hordaland County and covers approximately 10 % of the population. The cohort is considered population-based as incidence rates, patient- and disease characteristics are representative of the entire Norwegian population (6).

All patients gave written informed consent prior to study inclusion. Patients were treated according to current national guidelines. Blood samples and urinary samples were collected for research purpose preoperatively. Tumor tissue was collected perioperatively and snap frozen in liquid nitrogen ("fresh frozen") and stored at -80 degree celcius. Clinicopathological information regarding age, parity, menopausal status, FIGO stage, histological grade and type, and type of treatment provided was obtained from medical journals and registered and de-identified by a unique patient ID. Histopathological diagnosis was obtained from routine pathology reports for final hysterectomy specimen. Follow-up data were collected for at least 5 years. An overview of biological samples and applied methods is shown in Figure 6.

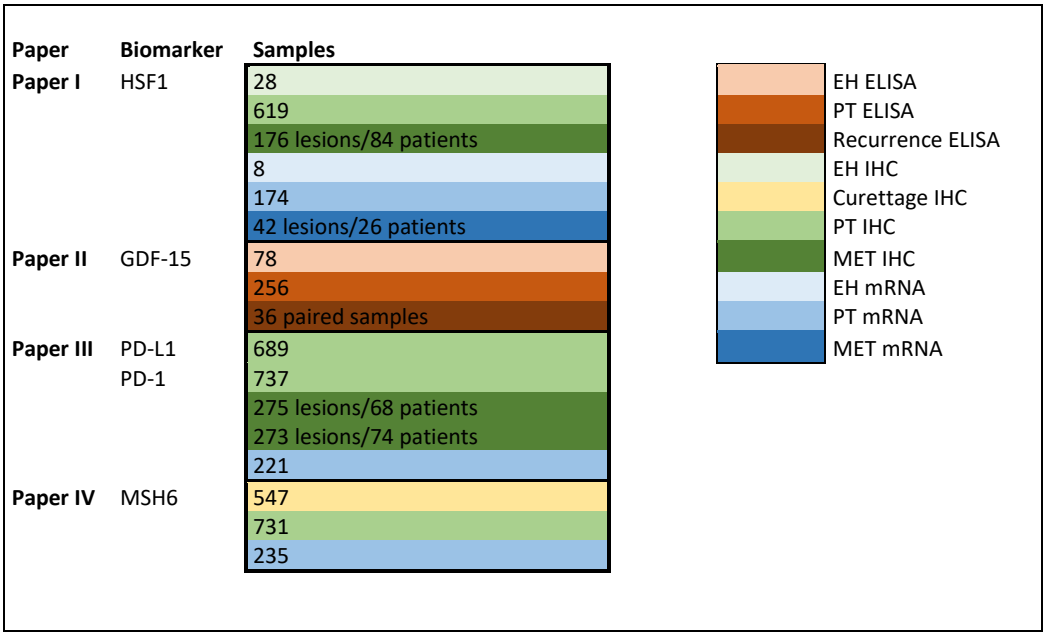

Figure 6: The figure gives an overview of how many patient samples, what kind of patient samples and which methods that were applied in each paper. Patient samples have been prospectively collected from 2001-2015. EH: endometrial hyperplasia PT: primary tumor MET: metastasis IHC: immunohistochemistry.

The Bergen Biobank for Gynecological Cancer contains tissue samples from patients with endometrial hyperplasia, primary tumors and corresponding metastases. Thus, the cohort is suitable for investigation and comparison of molecular alterations that may be important for cancer initiation and tumor progression. However, the biobank does not contain normal endometrial tissue and comparison is made between endometrial hyperplasias, primary tumors and metastatic lesions.

3.1.2 Immunohistochemistry

Tissue microarrays

Tissue microarrays (TMAs) were prepared from formalin fixed and paraffin embedded (FFPE) tissue. The area with the most representative tumor was selected from hematoxylin and eosin stained slides. In case of heterogeneity the least differentiated and densest area was selected. Using a custom made precision instrument (Beecher Instrument, Silver Spring, MD, USA) three tissue cylinders (0.6 mm) were punched out and mounted in a recipient paraffin block. For metastases and endometrial hyperplasias, only one tissue cylinder was punched out.

TMAs are a time-, tissue- and cost-effective method which also reduces the batch-effect. However, a major concern of the use of TMAs is that a tissue cylinder of 0.6 mm in diameter may not be representative of the entire tumor. Especially regarding tumor heterogeneity, full sections have been considered gold standard and as for all biomarkers clinical implementation relies on validation on full sections (144). However, previous studies have demonstrated a good concordance between the staining of ER, PR, p53 and epithelial membrane antigen (EMA) in full sections and TMAs when using three tissue cylinders (145, 146). Also, a good sensitivity for detection of PD-L1 (**Paper IV**) in TMAs when using three tissue cylinders has been demonstrated (147).

Immunohistochemistry

TMA slides (5 μm) were cut and dewaxed in xylene and rehydrated in ethanol before antigen retrieval (citrate buffer pH 6 or Tris EDTA pH 9) in microwave for 15 min. Peroxidase block was applied for 8 minutes before incubation with primary antibody (Table 4). The respective secondary antibody (anti-mouse or anti-rabbit) was applied for 30 minutes. Following diaminobenzidine (DAB) for 3-8 minutes and counterstaining with hematoxylin before dehydration in alcohol and mounting.

Table 4: Staining protocol for immunohistochemistry.

Target	Primary antibody	Buffer	Dilution	Incubation
HSF1	4356, Cell Signaling, Danvers, MA, USA	citrate pH 6	1:100	30 min RT
PD-L1	E1L3N, Cell Signaling, Danvers, MA, USA	citrate pH 6	1:100	1 hour RT
PD-1	D4W2J, Cell Signaling, Danvers, MA, USA	Tris EDTA pH 9	1:300	1 hour RT
PMS2	PMS2-L-CE, Leica Biosystems, Wetzlar, Germany	Tris EDTA pH 9	1:25	1 hour RT
MSH6	MSH6-L-CE, Leica Biosystems, Wetzlar, Germany	Tris EDTA pH 9	1:25	1 hour RT

RT: room temperature.

Staining evaluation

The immunostained sections were reviewed by light microscopy and scored visually by a semiquantitive and subjective method. Evaluation of staining was performed by two independent observers and blinded for the clinical characteristics and outcome. A staining index was calculated as a product of staining intensity (0–3) and area of positive tumour cells (1<10%, 2=10%–50% and 3>50%) (148, 149).

Cut-off for biomarkers

In **paper I** for subsequent statistical analyses, indexes were grouped in tertiles, considering the size of the subgroups and the number of events in each category. Tertile division was selected according to similarity in survival within each tertile. Index 0–4 was considered low, index 6 intermediate and index 9 was considered high. HSF1 staining was nuclear for all cases with positive staining. The κ-value was calculated to be 0.72 for HSF1 by two independent observers.

In **paper III** indexes were grouped in quartiles for PD-L1, considering the size of the subgroups and the number of events in each category. Quartile division was selected according to similarity in survival in each quartile. The lower quartile corresponded to negative (staining index=0) expression only, quartile 2 to 4 were merged together and subsequently cut off was negative/positive. Staining for PD-L1 was glandular and

mainly cytoplasmic. For PD-L1 the κ-value was calculated to be 0.74 by two independent observers. For PD-1, expression was evaluated as positive when >5% of stromal staining was detected and the cut-off was negative/positive. The κ-value was calculated to be 0.72 for PD-1 by two independent observers. Loss of nuclear staining of one of the two mismatch-repair proteins, MSH6 and PMS2 were identified as MSI. Positive stromal staining was used as internal control. For MSH6 and PMS2, staining was defined as negative when less than 10% glandular staining was observed. In case of negative cases with no stromal staining (lack of positive control), full sections were stained to determine status as previously described (150). Cases were defined as negative and thus MSI if either MSH6 or PMS2 was negative,

In **paper IV,** for both curettage and hysterectomy specimen two different cut-offs were used for MSH6. Either by two groups, into low=SI 0-4 and high=SI 6-9 or indexes were grouped in tertiles, considering the size of the subgroups and the number of events in each category, low=SI 0-4, intermediate=SI 6 and high=SI 9. Table 5 gives an overview of all cut-offs that have been used in each paper.

Table 5 Cut-off for biomarkers.

	SI Low exp	SI Intermediate exp	SI High exp
HSF1 (Paper I)	0-4	6	9
PD-L1 (Paper III)	0		1-9
PD-1 (Paper III)	negative		positive
MSH6 (Paper IV)	0-4	6	9
MSH6 (Paper IV)	0-4		6-9

SI: staining index. Exp: expression.

Commercially available and validated antibodies for HSF1, PD-L1, PD-1, PMS2 and MSH6 were used in previously developed staining protocols, however with optimization of antibody dilution and antigen retrieval. The sensitivity and specificity of antibodies are debated, however the protocol that is used in our lab has been validated and optimized multiple times. For HSF1, PD-L1, PD-1, PMS2 and MSH6, we used validated antibodies that have previously been used in publications (119, 124,

151-153). In both **paper I** and **IV** a significant correlation between mRNA and protein level was observed and supports that IHC reflects mRNA level. Due to the possibility of unspecific staining, antibodies to detect prognostic and predictive biomarkers are debated. To optimize the reliability one can use positive controls and optimize staining protocol such as primary antibody dilution, incubation temperature and time. IHC allows for spatial and subcellular evaluation of protein expression, however, the method is not suitable for presice and objective quantification of protein levels. Other methods such as reverse phase protein arrays (RPPA) and mass spectrometry would achieve a more accurate measurement of protein levels. However, IHC is less costly, and a highly clinical applicable and robust tool to guide treatment when proper antibody optimization and validation have been conducted.

3.1.3 Enzyme-linked immunosorbent assay (ELISA)

In **paper II**, human enzyme linked GDF-15 Quantakine ELISA kit (#DGD150, R&D Systems, Minneapolis, USA) was used to measure GDF-15 in plasma. According to the manufacturer´s instructions, 50 µL plasma sample or standard was added in a 96-well microplate, coated with a monoclonal antibody specific for human GDF-15, and incubated for 2h in room temperature. 200 µL human GDF-15 conjugate was added after washing, and incubated for 1 hour in room temperature. The wells were washed again before 200 µL of substrate solution were added and incubated for 30 min in room temperature protected from light, followed by 50 µL of stop solution. The absorbance was measured in a microplate reader at the wavelength of 450 nm, and plasma concentration of GDF-15 calculated.

All plasma samples were retrieved from EDTA-blood vials after centrifuged 2000 rounds per minute for 10 minutes and stored in -80℃ until analyzed. No correlation between storage time and plasma levels of GDF-15 was demonstrated. Analysis was performed in duplicate for n=102 and blinded for clinical characteristics and outcome. The endometrial cancer samples were analyzed in the same run, reducing inter-assay variation. The assay has a detection limit of 20 ng/L, an intraassay imprecision of 10.6% or less, and an interassay imprecision of 12.2% or less (154).

ELISA is a commonly used biochemical assay which is robust and thoroughly validated. The assay has an adequate intraassay and interassay imprecision and is not considerable influenced by other biological substances, such as hemoglobin, heparin, albumin and bilirubin (154). Overall, ELISA is a relatively inexpensive and clinical applicable method.

3.1.4 Messenger ribonucleic acid (mRNA) microarray analysis

Gene expression alterations in relation to HSF1 expression (**Paper I**) were investigated in microarray gene expression data already available from 8 endometrial hyperplasias, 174 primary endometrial cancers and 42 metastatic endometrial cancer lesions, the latter from 26 individual patients. For PD-L1 and PD-1 (**Paper III**), gene expression alterations were also investigated in microarray gene expression data already available. A number of 221 of these patients with available gene expression data from primary tumors overlapped with protein expression data and were used in subsequent analysis. The microarray analysis has been performed prior to this thesis. Briefly, tissue samples were snap-frozen in liquid nitrogen stored -80℃ for RNA extraction. Hematoxylin and eosin stained slides were used to identify areas with high tumor cell content (preferably>80%, minimum 50% tumor purity). RNA was extracted from unstained slides using the RNeasy Mini Kit (Qiagen, Hilden, Germany), hybridized to Agilent Whole Human Genome Microarrays 44k (cat. no. G4112F) according to the manufacturer's instructions, and scanned using the Agilent Microarray Scanner Bundle (Agilent, Santa Clara, CA, USA). Expression data were normalized using quantile normalization. Median spot signal was used as intensity measure. Normalisation of raw data and expression analyses were performed using the J-Express software (Molmine, Bergen, Norway).

Tissue with the highest tumor purity was intentionally selected for RNA extraction. However, an association with high tumor purity and aggressive endometrial cancers has been demonstrated (155). This may lead to a selection bias of the most aggressive cancers for gene expression analysis, and gene expression data may not be transferable to a routine clinical setting. It therefore needs to be emphasized that gene expression analysis performed by RNA extraction needs to be verified by other methods and in

true population-based series prior to potential implementation in the clinic. However, the Bergen Biobank for Gynecological Cancer contains population-based and prospectively collected patient samples with detailed clinical records, FFPE sections and a large number of overlapping fresh tissue samples, which gives a unique opportunity for investigation of overlapping samples.

Gene expression analyses

Significance Analysis of Microarray (SAM) was used in **Paper I and III** to identify genes differentially expressed between groups. Gene Set Enrichment Analysis (GSEA) to identify gene sets differentially expressed between groups was applied in **Paper III** and was performed applying gene sets from Molecular Signatures Database (MSigDb, version 6.2). All analyses were performed using the J-Express software (Molmine, Bergen, Norway).

Connectivity Map

Connectivity Map (http://www.broadinstitute.org/cmap/) is a publically available database that aims to establish a relation among disease, physiological processes, and the action of drugs (156). The drug signatures are generated before and after treating cell lines with different drugs. By applying gene signatures representing a biological state to the Connectivity Map database, a "connectivity score" is provided with the top ranked compound signatures correlated and anti-correlated to the gene signature that was applied. The method was used in **Paper I.**

Connectivity Map provide useful information, however, not without limitations. The changes in expression are based on cell lines from breast cancer, prostate, leukemia and melanoma (156), thus it may affect the results that the cell lines are not of endometrioid origin. Also, a fundamental limitation is that cell lines constitute a more simple system compared to a complex cancer *in vivo*, and the gene expression caused by the microenvironment is not taken into consideration (157). Overall, Connectivity Map is time-efficient and useful as a hypothesis-generating tool.

3.1.5 Statistical methods

Statistical analysis was conducted applying Statistical Program for the Social Sciences, version 21, 24 and 25 (IBM Inc. Chicago, IL, USA). All p-values were two sided and p-value of less than 0.05 was considered statistically significant. Pearson Chi-square and Fisher exact test were used for comparison between categorical data. Univariate survival analysis was performed using the Kaplan-Meier method and log-rank test, grouping low versus high concentration. Disease-specific survival was defined as time from primary treatment to death from endometrial cancer. Patients who died from other causes or were lost to follow-up were censored at the date of death/last follow-up. Differences in survival between groups were estimated by the log-rank (Mantel–Cox) test. Variables were visually examined by a log-minus-log plot to check the assumptions about proportionality over time, and tested for potential interactions before inclusion in the multivariate proportional hazards regression models (Cox analyses). Unadjusted and adjusted hazard ratios were calculated as measures of effect. Significance of change in protein expression from primary tumors to corresponding metastatic lesions was evaluated using Fisher's exact and Wilcoxon's signed-rank tests. Non-parametric tests Mann Whitney U and Wilcoxon Signed Rank were used for comparison of continuous data between study groups. Binary logistic regression was used to evaluate the odds ratio (OR) for lymph node metastases and recurrence.

3.1.6 Approvals

The Norwegian Data Inspectorate, Norwegian Social Sciences Data Services (15501) and Western Regional Committee for Medical and Health Research Ethics (REK 052.01, Paper I; 2009/2315 and 2014/1907, Paper II-IV and 2018/594, Paper IV).

4. Summary of results

Paper I

We explored 28 endometrial hyperplasias, 619 primary tumors and 176 metastatic lesions from 84 corresponding primary tumors for protein expression of HSF1 by immunohistochemistry in formalin fixed paraffin-embedded (FFPE) tissue. Transcriptional alterations related to HSF1 protein level were investigated by mRNA microarray analysis for 224 freshly frozen samples. We found that high expression of HSF1 of protein and mRNA levels in endometrial cancers reflect aggressive phenotype. High expression of HSF1 was significantly associated with high age, non-endometrioid histological type, high grade and aneuploidy (all p-values <0.02). Among the ERα-positive patients, high HSF1 was significantly associated with non-endometrioid type, high grade and aneuploidy (all p-values <0.004). The same pattern was seen for the ERα-negative patients. In ERα-positive patients, HSF1 was an independent prognostic marker. HSF1-related gene signatures were associated with poor survival and increase during disease progression. HSP90 inhibitors were suggested as targeted therapy.

Paper II

In this study, we included plasma samples from 78 patients with hyperplasias, 235 with endometrial carcinomas and 36 corresponding patients with recurrence. We demonstrated that high plasma level GDF-15 is associated with poor prognosis and shorter time to recurrence. High plasma level was significantly associated with high age, high FIGO-stage, non-endometrioid type, high grade and myometrial infiltration (all p-values <0.003) Also, in patients with a presumed low-risk stratification high plasma levels GDF-15 predict aggressive disease. In plasma samples from patients at time of primary treatment, the preoperative level of plasma GDF-15 was significantly higher for patients who later experienced recurrence than for patients who did not develop recurrent disease. For these patients, with available paired samples, plasma levels of GDF-15 at recurrence were significantly higher than plasma levels of GDF-

15 measured at time of primary diagnosis. In regression analysis, GDF-15 is an independent marker for lymph node metastases and recurrence.

Paper III

We explored the expression patterns of PD-L1 and PD-1 in FFPE tissue from 689 and 737 primary tumors, respectively. 275 corresponding metastases from 68 patients were explored for protein expression of PD-L1 and 273 corresponding metastases from 74 patients were explored for protein expression of PD-1. In primary tumors, PD-L1 and PD-1 are expressed in 59% and 63%, respectively, but have no impact on survival, nor when stratified for MSS and MSI. Expression patterns of PD-L1 and PD-1 are similar across MSS and MSI tumors. Available corresponding metastatic lesions show heterogeneous expression of PD-L1 and PD-1 compared to primary tumors, and a considerable intra-variable expression. Gene expression analysis was performed in an already available dataset with 221 patients revealing upregulation of several genes related to immunological activity, including *CD274* (encoding for PD-L1), in PD-1 positive tumors.

Paper IV

Elevated levels of mRNA *MSH6* demonstrated poor survival where patients with high *MSH6* had a 5-year survival of 65% compared to 93% in patients with low *MSH6* ($p<0.001$). To further confirm the prognostic value of MSH6, 547 curettage specimen and 731 hysterectomy specimen were investigated for protein expression of MSH6. High expression of MSH6 was associated with high age, FIGO stage III-IV, non-endometrioid type, high grade and lymph node metastasis in hysterectomy. In survival analysis, high expression of MSH6 was associated with poor survival ($p<0.001$). The same pattern was seen in curettage specimen. In curettage specimen high expression of MSH6 was associated with high age, FIGO stage III-IV, non-endometrioid type, high grade, lymph node metastasis and deep myometrial infiltration. High expression of MSH6 was associated with poor survival ($p<0.001$). MSH6 was an independent prognostic marker preoperatively, adjusted for age, histological risk-classification and

hormone receptor status. Also, in patients with a putative low-risk stratification preoperatively, high protein expression of MSH6 depicts poor survival.

5. Discussion of results

A key challenge in tailoring cancer treatment is to identify high-risk from low-risk patients. In order to do so, biomarkers are crucial. The incidence of endometrial cancer is increasing, and in spite of an overall favorable prognosis, 15-20% of patients experience recurrence (4). Prognostic markers in order to better predict prognosis, and predictive markers for response to treatment and targeted therapy are of paramount importance to improve treatment of endometrial cancer. For a biomarker to be of clinical interest, it must add valuable information in addition to what is already known from established clinicopathological variables or predictors. Biomarkers can be measured in serum, plasma or urine, but more invasive techniques requiring tumor tissue, such as immunohistochemistry and DNA/RNA analyses are widely used (129). The Bergen Biobank for Gynecological Cancer contains tissue samples from patients with endometrial hyperplasia, primary tumors and corresponding metastases. The large biobank gives a unique opportunity to study biomarkers in a large patient cohort.

In this thesis, which contains four papers, the overall aim was to explore biomarkers, both tissue- and plasma markers that better predict prognosis and guide therapy. We aimed to evaluate the prognostic impact of the tissue marker HSF1 (**Paper I**), identify plasma GDF-15 as a marker for recurrent disease and to validate GDF-15 as a prognostic marker for aggressive disease and as an independent marker for lymph node metastases (**Paper II**). We also aimed to determine the expression patterns of PD-L1 and PD-1 in tissue from both primary tumors and corresponding metastatic lesions, stratified for microsatellite instable and microsatellite stable cancers (**Paper III**). Further**,** we aimed to define the prognostic value of MSH6 in tissue from curettage and hysterectomy specimens (**Paper IV**). We here present prognostic value of HSF1, GDF-15 and MSH6, and demonstrate a frequent expression of PD-L1 and PD-1 with a discordant expression in metastatic lesions. Overall, our results contribute to a step towards improved risk-stratification in endometrial cancer in order to improve clinical decision-making.

5.1 Prognostic markers in order to better identify high-risk patients

High HSF1 protein level associates with aggressive endometrial cancer, also in ER-positive patients

High protein level of HSF1 demonstrated poor survival and was associated with aggressive clinical characteristics in endometrial cancer patients (**Paper I**). However, the exact role of HSF1 in endometrial cancer is still not fully elucidated and its role as a prognostic marker needs to be further explored. In cancer in general, the role of HSF1 has become increasingly relevant, and it is evident that HSF1 plays a role in cell-proliferation, anti-apoptosis, epithelial-mesenchymal transition, migration and invasion of cancer cells, and metastasis (106, 158). Increasing evidence has supported that HSF1 plays a role in tumorigenesis, by expression of heat shock proteins to protect proteins from degradation that are essential to tumorigenesis (104). Other findings suggest that HSF1 supports malignant transformation by controlling core cellular functions such as proliferation, survival, protein translation and glucose metabolism (108). The relevance of HSF1 as a prognostic biomarker has been demonstrated in several cancers, such as hepatocellular carcinoma and colorectal cancer (159, 160). Interestingly, in breast cancer, a cancer type with many similarities to endometrial cancer, HSF1 has been suggested as a key regulator of carcinogenesis, and high levels of HSF1 protein have been related to poor survival (105). We found a significant increase of protein expression of HSF1 from primary to metastatic lesions. This supports previous findings that HSF1 plays a role in promoting migration and invasion of cancer cells and metastasis (158).

High HSF1 was associated with poor survival in both ER-positive and ER-negative patients. Although more research is needed to fully understand the interplay between HSF1 and ER, this may suggest that HSF1 can identify a subgroup of patients with ER-positive tumors who could benefit from adjuvant therapy, despite being regarded as having a favorable prognosis. Interestingly, HSF1 has been proposed as an inhibitor of estrogen-dependent transcription which supports our findings of HSF1 as an independent prognostic marker within ER-positive patients (161). However, high

HSF1 is also associated with poor survival in ER-negative patients, which is in line with cell line studies where depletion of HSF1 was found to reduce the malignant state of the cell regardless of ER status (108).

High plasma level GDF-15 identifies patients with poor prognosis also in putative low-risk patients

Biomarkers derived from blood samples are easier to obtain compared to tissue biomakers. The sampling is less invasive, and the biomarker can be measured repeatedly during the course of the disease. In endometrial cancer, there is a need to preoperatively identify patients with aggressive disease. We demonstrate independent prognostic impact of GDF-15 measured in plasma from preoperative blood samples (**Paper II**). Previous findings support the role of GDF-15 as a biomarker in endometrial cancer and demonstrate the role of GDF-15 as a marker for aggressive disease and lymph node metastases (162). Our findings are in line with this, however we also demonstrate prognostic impact in patients with putative low-risk disease. Further, GDF-15 is an independent predictor of recurrent disease, when adjusting for age, histology and MI. Previously, GDF-15 has been proposed as a marker for discrimination between uterine sarcomas and benign leiomyomas, further emphasizing the association with GDF-15 as an indicator of malignancy (163, 164). The role of GDF-15 has been widely studied in other cancers and previous findings have shown overexpression in malignant melanomas, prostate-, pancreatic- and colonic cancers (165-168). Although the function of GDF-15 in cancer is not fully understood, it is known that GDF-15 can affect cell proliferation, differentiation, apoptosis, invasion and metastases (169). However, GDF-15 has been deemed as an unspecific marker and has also been suggested as a predictor of poor outcome in cardiovascular disease and after cardiac arrest (170, 171). Interestingly, in breast cancer recent findings reveal downregulation of GDF-15 by silencing of *ras suppressor-1 (RSU-1)* and other proteins related to invasion and metastasis (172). Further, GDF-15 has been suggested to reflect the characteristics of the tumor microenvironment and has been proposed as a marker for cytokine production and immune infiltration at the tumor site in breast cancer (173).

A molecular marker for lymph node metastasis, which can guide which patients to assign for lymphadenectomy is useful when planning the surgical procedure. To spare a patient from the morbidity of undergoing lymphadenectomy if unnecessary is valuable. Interestingly, GDF-15 was superior to preoperative histological risk classification in predicting metastatic lymph nodes (**Paper II**). Our results suggest that GDF-15 can predict lymph node metastasis, which is supported by previous findings (162). The sentinel lymph node technique as alternative to traditional lymphadenectomy has become increasingly interesting in endometrial cancer, demonstrating a high degree of diagnostic accuracy in detecting lymph node metastases (60). However, the technique is still not implemented in most countries and there are limitations related to obesity. Hormone receptor status is currently studied (in the clinical implementation study MoMaTEC2, NCT02543710), this is one example of the clinical relevance of molecular markers to guide lymphadenectomy. PET-CT has demonstrated a satisfying accuracy in predicting lymph node metastases (40, 174), but this imaging method is not yet incorporated in routine clinical use. A plasma marker to assist preoperative risk-stratification, such as GDF-15 could thus be useful in the preoperative work-up.

5.2 Markers for targeted therapy to tailor treatment

HSP90 inhibitors as targeted therapy in patients with high HSF1 protein level

Predictive markers are needed to tailor therapy and targeted treatment. We suggested HSP90 inhibitors as potential targeted therapy in patients with high HSF1 protein level (**Paper I**). Targeting HSP90 in cancer has become increasingly interesting and has demonstrated promising response rates and durable results (175). HSP90 inhibitors have been suggested as treatment in combination with carboplatin and paclitaxel for recurrent and advanced ovarian cancer (176). A recent study revealed the potential of HSP90 inhibitors to platinum-resistant endometrial cancer that overexpress the phosphoglycerate kinase 1 (PGK1), which mediates DNA repair and methylation through the HSP90/ERK pathway (177). Combined inhibition of AKT and HSF1 has demonstrated efficacy *in vitro* and *in vivo* in breast cancer (178). However, HSP90 as

a druggable target in endometrial cancer needs to be further explored in bigger and more robust studies both *in vitro* and *in vivo*.

High expression of MSH6 identifies aggressive endometrial cancers

The identification of the four molecular subtypes with prognostic significance in endometrial cancer by TCGA has led to an improved understanding of the molecular landscape of endometrial cancer (89). A more pragmatic approach to applying molecular biomarkers for risk stratification has been proposed, and both the TransPORTEC and the ProMisE initiatives have developed a more clinical applicable method (119, 120, 124, 179). The increased interest of MSI as one of the molecular subgroups for risk-stratification, and the defining of this subgroup by the mismatch repair proteins MSH6 and PMS2, has also led to the relevance of MSH6 as a prognostic marker in endometrial cancer. We demonstrate an association with high expression of MSH6 and poor survival in endometrial cancer (**Paper IV**). Supporting the role of MSH6 as a prognostic marker in endometrial cancer, are previous findings demonstrating independent prognostic impact of MSH6 in a cohort of 243 endometrial cancer patients (180). High expression of MSH6 has demonstrated a role of increased proliferation, migration and invasion in glioblastoma (181). Co-overexpression of MSH2 and MSH6 has resulted in several genome instability phenotypes, causing increased mutation rates, elevated loss of heterozygosity and increased sensitivity to DNA replication inhibition and DNA-damaging agents (182). Interestingly, high expression of MSH6 has been linked to poor survival in oral squamous cell carcinoma and prostate cancer (183, 184). In malignant melanoma, elevated levels of both mRNA and protein expression have been associated with aggressive clinical features and poor prognosis (185, 186). This support our results, where high gene- and protein expression of MSH6 were to associate with aggressive disease. We demonstrate independent prognostic impact of MSH6 preoperatively, adjusted for age, preoperative risk-stratification and hormone receptor status. Also, in patients with a putative low-risk stratification MSH6 identifies patients with a poor prognosis. The prognostic impact was validated in both curettage and hysterectomy specimen. mRNA levels corresponded to protein levels determined by IHC. This demonstrates an added value

of determining MSI by immunohistochemically staining of MSH6 and PMS2 according to the ProMisE classifier which is on the verge of clinical implementation (119, 124). However, to identify patients with aggressive disease, evaluation of staining intensity of MSH6 by scoring index is crucial to identify patients with poorer survival and need for adjuvant treatment or closer follow-up.

Predictive markers to guide therapy with immune checkpoint inhibitors

To date, no targeted therapy is implemented in clinical practice for endometrial cancer patients. The PD-1 antibody, pembrolizumab was granted FDA approval in 2017 for treatment of MSI-high cancers regardless of tumor type (143), and PD-L1 and PD-1 expression in endometrial cancer has been of increasing interest. Therapy with pembrolizumab to MSI-high recurrent and metastatic endometrial cancer has demonstrated promising results (97). The PD-1/PD-L1 pathway has been extensively studied in cancer in general (187). However, the expression pattern of PD-L1 and PD-1 has not been thoroughly explored in endometrial cancer. The reported fraction of PD-L1 and PD-1 positive tumors has been inconsistent and investigated in small cohorts (188-190). We identified high expression rates of PD-L1 and PD-1 in endometrial cancer, 59% and 63%, respectively (**Paper III**). We show similar expression pattern of PD-L1 and PD-1 across MSS and MSI tumors indicating that not only patients with MSI-high tumors may be eligible for treatment with immune checkpoint inhibitors. Previous studies have shown more frequent expression of PD-L1 in MSI tumors compared to MSS tumors (190, 191). However, recent clinical studies have indicated that MSI-status may not be definite for response to immune checkpoint inhibitors. A recent trial demonstrated efficacy when combining pembrolizumab and lenvatinib multikinase inhibitor of VEGFR 1-3 in metastatic endometrial cancer, and an objective response was recorded in 16/45 patients with MSS-tumors compared to two out of four MSI-tumors (99). Also, a study testing combination therapy with dostarlimab (PD-1-inhibitor) and chemotherapy (carboplatin and paclitaxel), regardless of MSI-status (NCT03981796) is in the pipeline and the results from this trial will hopefully indicate if also MSS patients are responders. Still, the robustness of PD-L1 and PD-1 as predictive markers for response to immune checkpoint inhibitors is debated, as studies

have shown a variable predictive value of PD-L1 (97, 192). Interestingly, gene expression analyses of all patients with expression of PD-1, regardless of MSI, identified upregulated genes related to immune activity, including the gene *CD274* (encoding for PD-L1), further emphasizing the immunological activity in patients with PD-1 positive tumors.

Expression of PD-L1 and PD-1 in corresponding metastases is intra-variable and discordant to primary tumors

Evaluation of expression of PD-L1 and PD-1 in metastases is of particular importance as treatment with immune checkpoint inhibitors is of foremost relevance in metastatic endometrial cancer. Interestingly, we reported a considerable intra-variable expression in metastatic lesions and a discordant expression from primary tumors to metastatic lesions (**Paper III**). Previous studies have found frequent expression of PD-L1 in metastatic colorectal cancer compared to primary tumors and an increase of PD-L1 during disease progression (193). Also, discordant expression between primary tumors and corresponding metastatic lesions has previously been demonstrated in breast cancer, malignant melanoma and head and neck cancers (194-196). Although treatment with pembrolizumab to PD-L1 positive, advanced endometrial cancer patients has previously demonstrated durable antitumor activity (97), a variation in response was noted and heterogeneity was deemed as a possible explanation in cases where there was a lack of response. The observed variation in PD-L1 and PD-1 expression in the metastases might thus be relevant for the response to treatment, also in endometrial cancer. Further studies to explore PD-L1 and PD-1 expression in metastatic lesions prior to treatment would be interesting to determine the ability of these biomarkers to predict response to checkpoint inhibitors.

Implementation of a biomarker from basic research, to pre-clinical development and clinical development is a demanding and time-consuming process (127). In endometrial cancer, the process has been slow and although several biomarkers have been identified in endometrial cancer, few of them have changed clinical routines (197). However, a small step is the local initiative MoMaTEC2 (NCT02543710).

Based on the findings from MoMaTEC1, an international multicenter study, which demonstrated that combined loss of ER and PR in pre-operative curettage predicted lymph node metastases and poor survival (139). MoMaTEC2 includes low-risk patients with positive hormone receptor status for both ER and PR to omit lymphadenectomy. High-risk endometrial cancer with either negative ER or PR will undergo lymphadenectomy. Until recent years, randomized trials have been the gold standard of clinical research, however the need for a large sample size, long study duration and the costs have been some of the shortcomings (198). Histological type has primarily been the known determinant of drug responsiveness and cancer drugs have previously been developed separately for different histological types of tumors. However, this focus has in recent years been supplemented with focus on genomic alterations in the tumor. To investigate the efficacy of targeting genomic alterations that occur across a wide variety of tumor types, basket trials are best suited (199, 200). Umbrella trials maintain the focus on single histology, as for traditional clinical trials, however, umbrella trials stratify treatment based on prespecified genomic biomarkers (200). These biomarker-guided clinical studies are important tools to continue making progress in the field.

In this thesis, the overall aim was to explore biomarkers that better predict prognosis and guide therapy. HSF1 demonstrated prognostic impact and was interestingly an independent marker for poor prognosis within ER-positive patients, a patient group with a presumed favorable prognosis. For patients with high expression of HSF1, we suggested HSP90 inhibitors for targeted therapy (**Paper I**). However, for disease monitoring during follow-up, GDF-15 demonstrated independent impact as a predictor of recurrent disease. GDF-15 also identified patients with poor prognosis within a subgroup of patients with a putative low-risk stratification. Interestingly, GDF-15 was superior to histological risk classification in predicting metastatic lymph nodes and can be helpful in the diagnostic work-up when deciding when to assign the patient for lymphadenectomy (**Paper II**). However, GDF-15 is an unspecific marker, potentially biased by cardiovascular disease. The identification of patients with MSI-tumors and expression of PD-L1 and PD-1 opens doors to treatment with immune checkpoint

inhibitors (**Paper III**). The demonstrated discordant and intra-variable expression of PD-L1 and PD-1 in metastatic lesions and evaluation of PD-L1 and PD-1 prior to assigning the patient to treatment with immune checkpoint inhibitors are important for further evaluation of the predictive value of PD-L1 and PD-1. Evaluation of MSI-status in endometrial cancer by IHC staining for PMS2 and MSH6, reveals potential of MSH6 as a prognostic marker in endometrial cancer when using staining index for evaluation of protein expression. MSH6 adds value as an independent prognostic marker preoperatively both in the overall patient group, and can also identify patients with poorer survival within a subgroup of patients with a putative low-risk disease (**Paper IV**).

Validation in large patient cohorts and clinical studies to determine the potential of HSF1, GDF-15 and MSH6 is needed. The relevance of PD-L1 and PD-1 as predictive markers for response to immune checkpoint inhibitors in endometrial cancer needs to be further evaluated in clinical trials. However, the identification of these biomarkers raise hope for an improved risk-stratification in endometrial cancer.

6. Conclusions

Paper I: High expression of HSF1 is associated with aggressive disease and predicts poor survival. We suggest HSP90 inhibitors for targeted therapy.

Paper II: High plasma levels of GDF-15 is associated with poor survival and is an independent marker for recurrent disease and lymph node metastases.

Paper III: PD-L1 and PD-1 are frequently expressed in primary tumors and expression is similar across MSS and MSI-tumors, but PD-L1 and PD-1 do not have prognostic impact. We demonstrate a considerable intra-variable expression in corresponding metastatic lesions and a discordant expression form primary tumors to corresponding metastatic lesions.

Paper IV: High mRNA level and protein expression of MSH6 are associated with aggressive disease and predict poor survival in both curettage and hysterectomy specimen. MSH6 also identifies patients with poor prognosis in patients with a putative low-risk stratification preoperatively. MSH6 is an independent marker for poor prognosis in curettage specimens.

7. Future perspectives

Novel and robust biomarkers for clinical use is an urgent need in order to better detect high-risk from low-risk patients. Predictive markers to better predict response to therapy are crucial to improve disease management and individualize therapy. To date no prognostic markers have been implemented for routine clinical use. In this thesis we have suggested some possible solutions. The following points are suggested follow-up studies.

By gene expression analysis we suggested HSP90 inhibitors as targeted therapy. The role of HSP90 inhibitors in endometrial cancers should be determined in studies *in vitro* and *in vivo*.

GDF-15 can add value to clinical practice in order to monitor disease development during follow-up. Studies to further determine the ability of GDF-15 in predicting and monitoring recurrence would be of high interest. The role of GDF-15 as a highly clinical applicable plasma marker should be determined in clinical studies.

Efforts should be made to implement MSI-status in clinical practice for endometrial cancer, both due to the value of MSI as a risk-stratifier and the potential predictive value of MSI for response to immune checkpoint inhibitors. To add to the clinical relevance of MSI-status for endometrial cancer, staining of MSH6 should be evaluated by staining index to further define the role of MSH6 as a marker for poor prognosis. Further studies exploring PD-L1 and PD-1 expression in corresponding metastatic lesions compared to the primary tumor prior to treatment, would be interesting to determine the heterogeneity of PD-L1 and PD-1. The predictive value of PD-L1 and PD-1 and the efficacy of PD-L1 and PD-1 inhibitors in endometrial cancer should be evaluated in clinical studies. More research is needed to determine if combination therapy yields better results than monotherapy.

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Paper II

II

PLOS ONE

RESEARCH ARTICLE

Plasma growth differentiation factor-15 is an independent marker for aggressive disease in endometrial cancer

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Abstract

Objective

Better biomarkers are needed in order to identify patients with endometrial carcinoma at risk of recurrence and who may profit from a more aggressive treatment regimen. Our objective was to explore the applicability of plasma growth differentiation factor 15 (GDF-15) as a marker for recurrent disease, as well as a marker for poor prognosis and lymph node metastases.

Methods

EDTA-blood samples were obtained from 235 patients with endometrial cancer before primary surgery. For 36 of these patients, matching blood samples were collected at time of recurrence. Blood samples were also collected from 78 patients with endometrial hyperplasia. Plasma GDF-15 was measured by an enzyme-linked immunosorbent assay (ELISA). Preoperative pelvic MRI scans for 141 patients were investigated in parallel for imaging variables.

Results

Preoperative plasma level of GDF-15 was significantly higher for patients who experienced recurrence (1780 ng/L; 95% CI; 518–9475 ng/L) than for patients who did not develop recurrent disease (1236 ng/L; 95% CI; 307–7030 ng/L) ($p<0.001$). Plasma levels of GDF-15 at recurrence (2818 ng/L, 95% CI 2088–3548 ng/L) were significantly higher than plasma levels of GDF-15 measured at time of primary diagnosis (1857 ng/L, 95% CI; 1317–2398 ng/L) ($p = 0.001$). High plasma level GDF-15 independently predicts recurrent disease (OR = 3.14; 95% CI 2.10–4.76) and lymph node metastases (OR = 2.64; 95% CI 1.52–4.61). Patients with high plasma level of GDF-15 had significantly larger tumor volume ($p = 0.008$).

Competing interests: The authors have declared that no competing interests exists.

Conclusion

Elevated plasma level of GDF-15 is associated with aggressive disease and lymph node metastasis in endometrial carcinoma. GDF-15 may be helpful in indicating recurrent disease.

Introduction

Endometrial cancer is the most common gynecologic malignancy and the fourth most common cancer among women in industrialised countries. Incidence is increasing worldwide, mostly due to the obesity epidemic. The prognosis is good with an overall 5-year survival of 80%, however 15–20% of patients with a presumed low risk disease experience recurrence [1]. Better biomarkers are thus needed in order to identify patients at high risk of recurrence who may profit from a more aggressive treatment regimen. To date there are no biomarkers for prognosis routinely available or in widespread use in the clinic and the majority of suggested markers have been developed for immunohistochemistry based detection in patient tissue biopsies. However, there has been little focus on identifying markers in preoperative blood samples [2–4]. Such markers are less invasive than those from biopsy, easily obtainable and could also be measured repeatedly during the course of the disease. A robust prognostic plasma biomarker would therefore potentially be highly valuable in the clinic.

Growth differentiation factor-15 (GDF-15), is a distant member of the transforming growth factor (TGF)-beta superfamily, also named macrophage-inhibitory cytokine -1 (MIC-1), and was originally identified in activated macrophages [5]. The TGF-beta superfamily has a role in regulating inflammatory and apoptotic pathways in injured tissues and during disease processes. GDF-15 is associated with cell cycle arrest and apoptosis [6]. Expression is dramatically increased in diseased states, such as acute injury, inflammation and cancer [7]. The prognostic value of GDF-15 is previously explored in cardiac disease, during pregnancy and in cancer. It has been suggested as a prognostic biomarker where increased GDF-15 is associated with increased risk of death at 1 year in patients with non-ST-elevation acute coronary syndrome [8] as well as in patients with ST-segment elevation and myocardial infarction [9]. In the placenta GDF-15 is physiologically highly expressed [10] and low expression of GDF-15 is associated with miscarriages [11]. Elevated levels, however, are associated with diabetes mellitus and pre-eclampsia [12]. In cancer, GDF-15 overexpression has been reported in malignant melanomas, prostate-, pancreatic- and colonic cancers [13–16]. Furthermore, elevated serum levels of GDF-15 are linked to cancer-associated anorexia and weight loss in prostate cancer [17]. In gynecological malignancies GDF-15 is reportedly an independent marker of aggressive disease in ovarian cancer [18]. For uterine sarcomas, elevated GDF-15 may aid in discriminating aggressive sarcomas from benign leiomyomas [19], whereas for endometrial cancer increased GDF-15 expression has been reported to predict lymph node metastases and poor survival [20].

In the present study we wanted to explore the applicability of GDF-15 in predicting endometrial carcinoma recurrence. In addition, using an extensive panel of clinicopathological variables including survival, our aim was to validate GDF-15 as a prognostic marker in endometrial carcinoma and as a possible predictor of lymph node metastases.

Materials and methods

Patient samples

EDTA-blood samples were obtained preoperatively from 235 patients with endometrial cancer before primary surgery. During time of follow-up 48 patients developed recurrence and blood

samples were collected from 36 of these patients at time of recurrence. In addition, EDTA-blood was collected from 78 patients diagnosed with endometrial hyperplasia. All patients have been diagnosed at Haukeland University Hospital, Norway between 2003 and 2014 and clinical data as well as blood samples were prospectively collected. Patients signed informed consent. Regional Committees for Medical and Health Research Ethics approval: 2009/2315 and 2014/1907. The median follow-up in this cohort is 43 months (range 1–189). Blood samples were centrifuged at 1600 g for 15 min and the plasma was stored at– 80 ˚C until measurement of GDF-15. Distribution of measured GDF-15 plasma level was not influenced by storage time.

Additionally, data regarding GDF-15 plasma levels were also locally available from an independent cohort of 466 endometrial cancer patients previously published [20]. In order to improve statistical power when predicting lymph node metastases and recurrence, the data were merged together with the current cohort when performing regression analyses. Data were missing for recurrence from 47 patients, histological type from 47 patients and myometrial infiltration from 49 patients, and the resulting cohort included 603 patients. Regarding lymph node status, data were missing for 190 patients and preoperative histology missing for 65 patients, and the cohort included 495 patients.

GDF-15 measurements

GDF-15 in plasma was measured by the Human enzyme linked GDF-15 Quantakine ELISA kit (#DGD150, batch #P153423, R&D Systems, Minneapolis, USA). The ELISA was performed according to the manufacturer´s instructions. Briefly, 50 μL plasma sample or standard was added in a 96-well microplate coated with a monoclonal antibody specific for human GDF-15, and incubated for 2h in room temperature. Following washing, 200 μL human GDF-15 conjugate was added and incubated for 1 hour in room temperature. The wells were washed again before 200 μL of substrate solution were added and incubated for 30 min in room temperature protected from light, followed by 50 μL of stop solution. The absorbance was measured in a microplate reader at the wavelength of 450 nm, and plasma concentration of GDF-15 calculated. To confirm reproducibility, a subset (n = 102) were measured in duplicates. Clinical data were blinded while performing and evaluating laboratory investigations. The assay has a detection limit of 20 ng/L, an intraassay imprecision of 10.6% or less, and an interassay imprecision of 12.2% or less [21].

Preoperative magnetic resonance imaging (MRI)

In parallel, preoperative pelvic MRI scans for 141 patients were assessed to derive the following imaging variables: endometrial tumor size, signs of deep myometrial invasion, cervical stroma invasion and lymph node metastases. MRI was conducted on a whole body 1.5-T MRI system (Siemens Avanto running Syngo v. B17, Erlangen, Germany) using a six channel body coil applying a standardized imaging protocol [22]. To reduce motion artefacts 20 mg of butylscopolamine bromide (Buscopan; Boehringer, Ingelheim, Germany) was administered intravenously. Mean time (range) between MRI examination and surgical staging was 1.5 (0–12) weeks.

Statistical analyses

Statistical analyses were conducted applying Statistical Program for the Social Sciences (SPSS), version 24 (IBM Inc. Chicago, IL, USA). All p-values were two sided and p-value of less than 0.05 was considered statistically significant. Pearson Chi-square or Fisher exact test were used for categorical data. Univariate survival analysis was performed using the Kaplan-Meier

method and log-rank test, grouping low versus high concentration. Cut-off values for categorization were based on tertiles according to the size of the subgroups and the number of events in each category. The two lower GDF-15 tertiles were merged due to similar survival. Cut-off value based on this method was found to be near identical to our previous study [20] (cut-off in previously published cohort: 1400 ng/L, cut-off in this cohort 1418 ng/L). For analyses where the cohorts were merged, cut-off value was 1418 ng/L. Low-risk patients were defined as endometrioid histologic type and grade 1 and 2 disease, and high risk patients as endometrioid grade 3 and non-endometrioid. Disease-specific survival was defined as time from primary treatment to death from endometrial cancer. Patients who died from other causes or were lost to follow-up were censored at the date of death/last follow-up. Non-parametric tests Mann Whitney U or Wilcoxon Signed Rank were used for comparison of continuous data between study groups. Binary logistic regression was used to evaluate the odds ratio (OR) for lymph node metastases and recurrence.

Results

Plasma GDF-15 associates with poor survival, also in low-risk patients

To validate previous observations that plasma level of GDF-15 is a biomarker for poor prognosis in endometrial cancer, plasma level of GDF-15 was determined in an independent patient population including 235 patients with primary endometrial carcinoma. High level of plasma GDF-15 was associated with reduced disease-specific survival ($p = 0.001$, Fig 1A) with 5-year survival rate of 72.9% compared to 94.1% in patients with low GDF-15. In addition, high GDF-15 indicated reduced recurrence-free survival ($p<0.001$, Fig 1B) with 5-year recurrence-free survival rate of 61.2% compared to 89.3% in endometrial cancer. GDF-15 level was significantly higher in patients aged >66 years, and in patients with advanced FIGO stage, non-endometrioid histologic subtype, high grade and with deep myometrial infiltration (all p-values ≤ 0.003, Table 1). These findings are in line with previous findings from a separate cohort from our hospital [20]. To further explore the usefulness of plasma GDF-15, we performed analyses in the low-risk subgroup of patients, defined by endometrioid histology and grade 1 or 2 disease on preoperative curettage specimen (n = 148). Also in this patient subgroup, high level of plasma GDF-15 was associated with poor prognosis ($p = 0.002$, Fig 1C) with a 5-year survival rate of 72.8% compared to 97% in patients with low GDF-15. High age, high grade

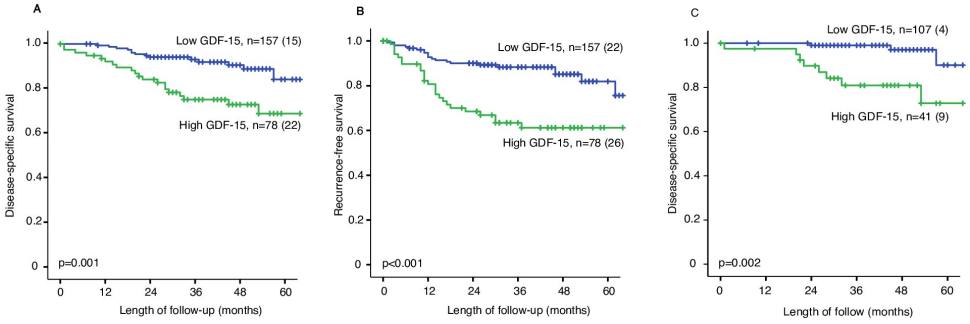

Fig 1. Disease-specific survival (A) and recurrence-free survival (B) in 235 patients illustrated by Kaplan Meier curves. Disease-specific survival in patients with a putative low-risk disease preoperatively, that is endometrioid grade 1 or 2 (C). P-values are calculated by the Mantel-Cox log rank test. Low GDF-15 is the two lower tertiles, 1. and 2. combined. High GDF-15 is the 3. tertile. Number of cases are given in each category and in parenthesis number of disease-specific deaths or recurrence.

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Table 1. GDF-15 measured in plasma samples from 235 patients with endometrial cancer in relation to clinico-pathological factors.

	Low, n (%)	High, n (%)	P-value*
Age, y			<0.001
<66	86 (84)	16 (16)	
≥66	71 (53)	62 (47)	
FIGO			0.003
I/II	140 (71)	58 (29)	
III/IV	17 (46)	20 (54)	
Histologic type			0.002
Endometrioid	126 (72)	48 (28)	
Non-endometrioid	31 (51)	30 (49)	
Non-endometrioid types			0.003
Clear cell	4 (40)	6 (60)	
Serous	20 (61)	13 (39)	
Carcinosarcomas	3 (25)	9 (75)	
Undifferentiated	4 (67)	2 (33)	
Histologic grade**			0.002
Grade 1	76 (84)	15 (16)	
Grade 2	27 (55)	22 (45)	
Grade 3	18 (64)	10 (36)	
Myometrial infiltration			0.001
<50%	101 (75)	33 (25)	
≥50%	55 (56)	44 (44)	

*P-values are calculated by Chi-Square test or Fisher exact test.

**Endometrioid included only.

Low = 1. and 2. tertile

High = 3. tertile

FIGO: International Federation of Gynecology and Obstetrics

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and deep myometrial infiltration were all associated with high levels of GDF-15 (all p-values ≤0.007, Table 2) in low-risk patients. BMI did not correlate with increasing plasma concentrations of GDF-15 (R^2 0.003).

Plasma GDF-15 does not distinguish between hyperplasias and endometrial cancer

As the plasma level of GDF-15 has been reported to be elevated in cancers compared to healthy controls [20], we investigated if plasma GDF-15 also is a marker for progression from hyperplasia to endometrial cancer. Plasma concentrations of GDF-15 were compared between 78 patients with endometrial hyperplasias and 235 patients with primary endometrial cancer. There was no significant difference between plasma levels of GDF-15 in endometrial hyperplasias (1502 ng/L; 95% CI, 1219–1785 ng/L) and primary tumors (1611 ng/L; 95% CI, 1446–1776 ng/L) (p = 0.807). When performing a more detailed analysis of endometrioid endometrial cancers only, we observed that the increase of GDF-15 occurs between grade 1 and grade 2 (p = 0.003, Fig 2A).

Elevated plasma level of GDF-15 is associated with recurrent disease

Given the association with aggressive disease, we investigated if GDF-15 could be a marker for recurrence. When analyzing blood samples from endometrial cancer patients at time of

Table 2. GDF-15 in plasma samples in relation to clinicopathological factor in patients with preoperative low-risk staging*.

	Low, n (%)	High, n (%)	P-value#
Age, years			0.005
<66	59 (83)	12 (17)	
≥66	48 (62)	19 (38)	
FIGO			0.36
I/II	98 (74)	35 (26)	
III/IV	9 (60)	6 (40)	
Histologic type			0.218
Endometrioid	103 (74)	37 (26)	
Non-endometrioid	4 (50)	4 (50)	
Non-endometrioid types			0.242
Clear cell	0	1 (100)	
Serous	3 (50)	3 (50)	
Carcinosarcomas	1 (100)	0	
Histologic grade**			0.007
Grade 1	70 (82)	15 (18)	
Grade 2	24 (57)	18 (43)	
Grade 3	5 (56)	4 (44)	
Myometrial infiltration			0.007
<50%	73 (80)	18 (20)	
≥50%	34 (60)	23 (40)	

*Low-risk patients defined as endometrioid histology and grade 1 or grade 2 disease on preoperative curettage.

**Endometrioid included only.

#P-values are calculated by Chi-square test or Fisher exact test.

Low = 1. and 2. tertile

High = 3. tertile

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primary treatment, the preoperative level of plasma GDF-15 was significantly higher for patients who later experienced recurrence (1780 ng/L; 95% CI; 518–9475 ng/L) than for patients who did not develop recurrent disease (1236 ng/L; 95% CI; 307–7030 ng/L) ($p < 0.001$, Fig 2B). This might indicate a potential for GDF-15 in predicting recurrence. To investigate this further, plasma samples were collected at time of recurrence from 36 patients, and compared to corresponding plasma samples collected at time of primary treatment. For these patients, with available paired samples, plasma levels of GDF-15 at recurrence (2818 ng/L, 95% CI 2088–3548 ng/L) were significantly higher than plasma levels of GDF-15 measured at time of primary diagnosis (1857 ng/L, 95% CI; 1317–2398 ng/L) ($p = 0.001$, Fig 2C). This may suggest a role for GDF-15 in monitoring recurrence.

Plasma GDF-15 independently predicts lymph node metastases and recurrence

A prediction model for recurrence was calculated from our merged cohort of 603 patients (described in materials). The cut-off value grouping high and low GDF-15 was 1418 ng/L. Patients with high plasma level of GDF-15 had significantly higher risk of recurrent disease (OR = 3.14; 95% CI 2.10–4.76) in univariate analysis. In the multivariate model, after adjusting for age, postoperative histology and depth of myometrial infiltration, the predictive value

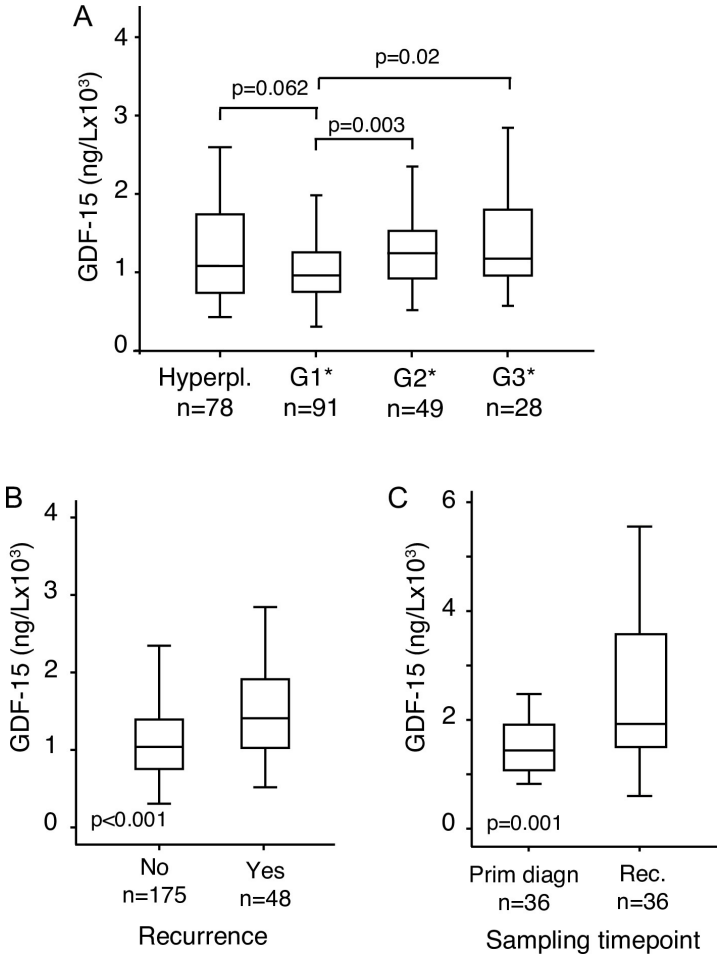

Fig 2. Box plots showing plasma level of GDF-15 in hyperplasias and grade 1–3 (A), in patients who experienced recurrence during their follow-up and in patients who did not (B) and plasma level of GDF-15 in paired samples at time of primary diagnosis and at time of recurrence (C). Number of cases are given. P-values are calculated by the Mann Whitney U test in independent samples and Wilcoxon Signed Rank test in related samples. *Endometrioid included only.

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remained significant with an adjusted OR of 1.99 (95% CI 1.23–3.22, Table 3). Furthermore, in the merged cohort of 495 patients with lymph node status (also described in materials and cut-off value of 1418 ng/L), high plasma level of GDF-15 was significantly associated with lymph node metastases (OR = 2.64; 95% CI 1.52–4.61) in univariate analysis. In multivariate analysis, adjusting for preoperative histological risk (high; endometrioid grade 3 or non-endometrioid versus low; endometrioid grade 1 or 2) the predictive value of GDF-15 remained significant with an adjusted OR of 2.49 (95% CI 1.42–4.37, Table 4). Age was not significant in in

Table 3. Prediction of recurrence in 603 patients with endometrial cancer, univariate and multivariate logistic regression.

Variable	N	Univariate OR	95% CI	P	Multivariate OR	95% CI	P
Age				<0.001			0.043
	603	1.05	1.03–1.07		1.02	1.00–1.05	
Histology				<0.001			<0.001
Endometrioid	492						
Non-endometrioid	111	4.50	2.86–7.09		3.72	2.29–6.05	
Myometrial infiltration							<0.001
<50%	390			<0.001			
≥50%	213	2.67	1.77–4.04		2.27	1.45–3.55	
GDF-15				<0.001			0.005
Low	419						
High tertile	184	3.14	2.07–4.76		1.99	1.23–3.22	

Variables significant in univariate analyses were used in the final multivariate model.

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univariate analysis and was therefore not included in the multivariate analysis. Statistical power was too low to perform the analysis in the independent cohort of 235 patients.

High tumor volume detected by MRI is associated with high plasma GDF-15

In order to further validate GDF-15 as a preoperative marker of prognosis, we compared plasma levels of GDF-15 with imaging variables from routine preoperative MRI (n = 141). Patients with high plasma level of GDF-15 had significantly larger tumor volume; with mean tumor size of 17 ml (95% CI: 12–22 ml) in patients with low GDF-15 as opposed to mean tumor size of 27 ml (95% CI: 13–40 ml) in patients with high GDF-15 (p = 0.008). Also, high plasma level of GDF-15 was associated with MRI assessed deep myometrial infiltration (p = 0.05) and cervical stroma invasion (p = 0.03, Table 5).

Discussion

Biomarkers derived from blood samples are easily available and has been less explored compared to tissue biomarkers in endometrial cancer. GDF-15 in plasma has previously been proposed as a biomarker in endometrial cancer [20], and has also been suggested as a serum biomarker in patients with prostate, pancreatic and colon cancers [13, 14, 16]. In addition,

Table 4. Prediction of lymph node metastases in 495 patients with endometrial cancer, univariate and multivariate logistic regression.

Variable	N	Univariate OR	95% CI	P	Multivariate OR	95% CI	P
Curretage histology				0.014			0.036
Low risk*	373						
High risk**	122	2.06	1.16–3.66		1.87	1.04–3.37	
GDF-15				0.001			0.001
Low	346						
High tertile	149	2.64	1.52–4.61		2.49	1.42–4.37	

Variables significant in univariate analyses were used in the final multivariate model.

*Low-risk patients defined as endometrioid histology and grade 1 or grade 2 disease on preoperative curettage.

**High-risk patients defined as endometrioid histology and grade 3 disease or non-endometrioid histology on preoperative curettage.

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Table 5. Clinical characteristics on preoperative MRI in 141 patients in relation to plasma level of GDF-15.

	Low n (%)	High n (%)	P- value*
Myometrial infiltration			0.05
<50%	63 (85)	11 (15)	
≥50%	48 (72)	19 (28)	
Cervical stroma affection			0.03
no	90 (84)	17 (16)	
yes	7 (58)	5 (42)	

*P-values are calculated by Chi-Square test.

Low = 1. and 2. tertile

High = 3. tertile

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GDF-15 has been proposed as a general predictor of cardiovascular disease [23] also in apparently healthy women [24]. In this study, we validate that high level of plasma GDF-15 is associated with clinical characteristics depicting aggressive disease and poor survival in endometrial cancer. In previous studies by Staff et al. elevated plasma level of GDF-15 was associated with aggressive histologic types, lymph node metastases, reduced recurrence-free survival, and death due to endometrial cancer [20]. Our results are in line with these findings and validate GDF-15 as a prognostic marker in endometrial carcinoma.

Plasma biomarkers might be useful for screening if a marker could detect early stages of disease. However, we did not find that plasma levels of GDF-15 distinguish between hyperplasias and grade 1 endometrioid endometrial cancers. Previous reports have identified an increase in plasma GDF-15 from healthy controls to cancer [14, 16, 20]. It is interesting that hyperplasias show similarly high plasma GDF-15 compared to grade 1 endometrioid endometrial cancers. This may indicate that elevation of GDF-15 is an early event and occurs simultaneously with development of hyperplasias. It has previously been reported that serum GDF-15 levels progressively increase from premalignant colonic lesions to cancer initiation with a further increase of plasma levels GDF-15 at time of metastasis [16]. It is known that GDF-15 can induce various pleiotropic effects during cancer progression by negatively or positively modulating cell proliferation, differentiation, apoptosis, invasion, and metastases, dependent of cancer cell types, disease stage, and tumor microenvironment [25]. However, the function of GDF-15 is not yet fully understood. A more thorough investigation of GDF-15 in early stages of disease should include large cohorts of controls and hyperplasias, preferentially also with repeated sampling of individual patients.

For endometrial cancer, there is a need to preoperatively identify patients with aggressive disease to stratify for optimal treatment. Presently, preoperative diagnosis relies on risk classification based on a preoperative biopsy and at some centers, preoperative imaging, preferentially MRI, is included. Addition of an easily obtainable serum biomarker could add relevant information. Importantly, we find that in patients with putative low risk based on preoperative histology (endometrioid grade 1 or 2), high level of GDF-15 predicts poor prognosis and is associated with aggressive features in endometrial cancer. In contrast, low-risk patients with low levels of GDF-15 have a 5-year survival of 97%. This suggests a promising role for GDF-15 in confirming preoperatively that some putative low-risk patients have an excellent prognosis, supporting the clinical value of plasma GDF-15 in endometrial cancer treatment.

Additionally important for treatment of endometrial cancer patients is the ability to identify patients with risk of recurrence. We here find that in preoperative samples, plasma levels of GDF-15 are higher for patients that later experience recurrent disease compared to patients

that do not experience recurrence. Further analyses showed the same for paired samples, where level of GDF-15 increased in samples from primary treatment to samples obtained at time of recurrence. When adjusting for age, histology and myometrial infiltration, high plasma level of GDF-15 was an independent marker for predicting recurrence. To the best of our knowledge we demonstrate for the first time that elevated levels of GDF-15 may be helpful in follow-up of patients to detect recurrence. Although the sample size of 36 paired samples is low, the results are promising and indicating a role for GDF-15 in monitoring recurrence. The increase of GDF-15 in aggressive disease and recurrence has been reported in gene- expression signatures from circulating tumor cells [26], further emphasizing our findings. Measuring GDF-15 in plasma from patients with endometrial cancer may be helpful when selecting women who are likely to profit from adjuvant therapy after primary treatment. Monitoring plasma levels of GDF-15 during follow-up can potentially also guide the recommended frequency of follow-up examinations including diagnostic imaging such as MRI or Computer Tomography (CT) after primary treatment.

Interestingly, GDF-15 was superior to histological risk classification in predicting metastatic lymph nodes. GDF-15 could therefore be helpful as an indicator of lymph node metastases and when selecting women for lymphadenectomy. The value of lymph node sampling is controversial and studies are not convincing when evaluating survival benefit and short- and long term complications for the patients who undergo lymphadenectomy [27]. Thus, markers for better prediction of lymph node metastasis may be valuable in the clinic. Novel techniques, such as sentinel lymph node mapping in endometrial carcinoma is increasingly acknowledged [28], however it has limitations among others in relation to obese patients and is still not implemented in most countries, thus a marker in blood samples is still clinically relevant.

Using imaging methods as an adjunct to preoperative serum or tumor biopsy risk stratification may be a useful clinical tool. We demonstrated correlation between plasma GDF-15 and MRI determined tumor size and cervical infiltration. MRI has been reported to outperform that of endocervical curettage for preoperative prediction of cervical stromal invasion [29]. Also, for differentiation of low grade endometrial cancer from endometrial hyperplasia, preoperative MRI and FDG-PET yield promising imaging markers [30]. Imaging markers may thus be better than plasma GDF-15 in detecting early disease since the elevation of GDF-15 seems to occur prior to cancer development. However, the patient population with available imaging data should be larger to validate these findings.

Endometrial cancer is associated with both obesity and high age, factors known to increase the risk of comorbidity such as cardiovascular disease. Cardiovascular disease is not systematically reported in our cohort, and could potentially have biased our results due to its association with high plasma GDF-15 [23, 24]. However, the use of disease-specific survival in our survival analyses and the identified association with both high grade and myometrial infiltration, which is independent of comorbidity and high age, support that GDF-15 specifically detects aggressive endometrial cancer in our cohort. Also, given the association that high plasma level of GDF-15 decreases time to recurrence, further emphasizes that GDF-15 is increased due to the patient's cancer status as reduced time to recurrence is not likely to be influenced by cardiovascular disease.

Few biomarkers have so far been identified from plasma [2–4] and blood derived markers would be useful in clinical practice as they are less invasive for the patient, are relatively inexpensive and may prove helpful both for preoperative prognostication and in detecting recurrent disease during follow-up. Monitoring plasma levels of GDF-15 during follow-up can potentially guide clinicians when to refer patients to renewed diagnostic imaging by MRI and Computer Tomography (CT) or FDG PET-CT to detect recurrence. Robust plasma markers

could thus represent a valuable tool in clinical practice. However, further and larger studies are needed to further evaluate GDF-15 as a plasma marker for recurrence.

We conclude that elevated levels of plasma GDF-15 is associated with an aggressive clinical phenotype and lymph node metastasis in endometrial carcinomas. Elevated GDF-15 is also a marker of recurrent disease at time of recurrence. The clinical value of plasma GDF-15 for prediction of lymph node metastases, prognostication and as a marker of recurrent disease, however, needs to be validated in larger patient cohorts and in clinical trials prior to potential implementation in the clinic.

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