Endometrial carcinoma; can biomarkers aid in the prediction of aggressive disease?

A study with focus on preoperative tumour markers

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Scientific environment

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Translational research in gynaecological cancer in general, and endometrial cancer in particular, has for several years had a solid foundation in Bergen led by Professor Helga Salvesen (principal investigator) at the Department of Clinical Medicine, University of Bergen and Department of Obstetrics and Gynaecology, Haukeland University Hospital. She initiated a systematic collection for a biobank from gynaecologic malignancies, at the Department of Obstetrics and Gynaecology, Haukeland University Hospital in 2001. After informed consent, freshly frozen tumour- and blood samples from women treated for gynaecological cancers were prospectively collected at our institution and in a multicentre setting (MoMaTEC).

Professor Lars Akslen at The Gade Institute, section for Pathology, University of Bergen and Department of Pathology, Haukeland University Hospital with his Tumour Biology Research Group has been a fundamental collaborator through these years. The *Tumor Biology Research Group* at The Gade Institute led by Professor Lars A. Akslen was established in 1995 and has aimed to perform translational cancer research at an international level identifying markers of aggressive cancers that can assist in prognostication and prediction of targeted treatment response. The biomarker studies have been especially related to angiogenesis and tumour-vascular interactions, and importance for the metastatic process, and tumour cell proliferation and cell cycle regulation. Studies have been performed across different tumour types (breast-, endometrial- and prostate cancer and melanoma) with long-time collaboration with clinical investigators, and also including international collaboration networks.

Professor Karl-Henning Kalland at the Gades Institute, University of Bergen, has been a long-term collaborator in microarray studies.

Several international collaborators are today involved in the *Studies of pathogenesis, prognostic markers and treatment in gynaecologic cancer,* led by professor Salvesen; Prof. Matthew Meyerson and Rameen Beroukhim, Harvard Medical School, Dana Farber Cancer Institute, Boston, USA, are involved in molecular studies and analyses of data.

Professor Ronald Simon, Prof. University Medical Center Hamburg-Eppendorf, Germany; is involved in FISH analyses of identified candidate genes in validation series.
Professor Roman Thomas, Max-Planck Institute for Neurological Research, Cologne, Germany, is involved in oncogen mutation screening (Oncomap).

Members of Nordic Society of Gynaecologic Oncology (NSGO) and European Society for Gynaecologic Oncology (ESGO) are recruiting patients for the prospective multicentre study, MoMaTEC.

This international prospective multicentre trial, MoMaTEC (Molecular Markers in Treatment of Endometrial Cancer, http://www.clinicaltrials.gov/ct2/show/NCT00598845), is an important basis of this present thesis. After a period of single centre prospective inclusion (Haukeland University Hospital), a multicentre approach was initiated in 2007 with 10 recruiting centres; Norwegian: Haukeland University Hospital, Bergen, St.Olav's Hospital, Trondheim (MD, PhD S.Tingulstad), Oslo University Hospital, Ullevål, Oslo (MD, PhD, Prof. A.Staff) Helse Førde Hospital, Førde (MD, PhD, J.Tjugum), Haugesund Hospital, Haugesund (MD, K.Oddenes) Hospital of Vestfold, Toensberg (MD, PhD, J.Rokne), Ålesund Hospital, Ålesund (MD, M.Lode), Akershus University Hospital, Oslo (MD, PhD, Prof. M.Engh).
Collaborating international centres: Gasthuisberg University Hospital, KULeuven, Belgium (MD, PhD, Prof. F.Amant) and Sahlgrenska Academy, University of Gothenburg, Sweden (MD, PhD, J.Marcickiewicz).

In relation to Professor Salvesen's *Endometrial cancer research group*, at present, four theses have been completed, four post-doc projects and six PhD projects (including this thesis) are ongoing.
The *Tumor Biology Research Group* at The Gade Institute led by Professor Akslen currently has 20 members, at present 12 PhDs completed; current supervision: 8 PhDs, 5 postdocs.

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Acknowledgements

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My supervisor, long-time colleague and good friend Helga Salvesen persuaded me, during several of our hiking-tours at Haugastøl, that conducting cancer research could be an interesting alternative to clinical hospital work, even for a dedicated gynaecologist. Her scientific and clinical knowledge, enthusiasm and quick response whenever I needed guidance have been invaluable! Without her thorough planning, international networking and co-operative skills recruiting participating centres, this large scale MoMaTEC study would not have been possible.

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Anne C Staff, Professor at Ullevål University Hospital, generously included me in the project of testing GDF-15 as a serologic prognostic marker in endometrial cancer. Her working capacity and knowledge is impressive and abundant late-night e-mails fully compensate for the lack of physical proximity.

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I am very grateful for all contributions to the MoMaTEC trial, foremost from all women participating, but also clinicians at all centres taking time during busy clinical days to include, send specimens and fill in follow-up forms; Marie Engh, Harald Helland, Maragaret Lode, Klaus Oddenes, Janusz Marcickiewicz, Jan Rokne, Anne Staff, Solveig Tingulstad, Jostein Tjugum, Ingrid Vandenput and Henrica Werner.

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Karianne, Kamilla, Hans Kristian, Katrine, Kristina and Hans; work, clinical or academic, is but one part of life. Without the enjoying everyday family-life with you all it would not be worthwhile.

Introduction

Endometrial cancer is the most common gynaecological malignancy in the western world. Although the majority has a good prognosis, still almost 20% recur and one third of those dying from the disease were initially classified as early stage disease. Endometrial cancer is clearly under-researched in comparison to other cancer types, with several aspects regarding optimisation of risk-stratification and treatment, surgical as well as systemic, yet to be defined. Several molecular tumour markers have earlier been investigated in retrospective series and found to significantly influence prognosis, but this knowledge has not yet been incorporated in the clinic.

In this thesis we have examined markers in preoperative blood samples and routine curettage as well as hysterectomy specimens and relation to clinicopathological features and patients' survival. Also, findings in some of these formalin fixed paraffin embedded specimens have been related to comprehensive molecular profiling of freshly frozen tumour tissue collected in parallel for alterations in important signalling pathways related to carcinogenesis. We have investigated endometrial cancers both in a population based cohort setting from Hordaland County, Norway and in a large, prospective, international multicentre setting. Also alterations in disease characteristics as well as treatment strategies have been related to change in survival during a 30-years perspective for the population based cohort.

Our data validate that preoperative markers are significant related to aggressive, advanced disease, such as lymph node metastasis, and of potential value in the decision making regarding selecting patients for lymph node sampling and adjuvant treatment. Biomarkers correlated to activation of specific targetable tumour signalling pathways could be of importance for testing targeted therapies. Thus, relevant markers should be incorporated in the selection of patients for further randomized trials regarding individualised surgical as well as systemic treatment. Also, elucidating changes in survival over time in relation to characteristics for the patient population is important in the evaluation of treatment strategies.

Contents

Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog, Protein Kinase B
BMI	Body mass index
CA-125	Cancer antigen 125
CT	Computer tomography
DFS	Disease-free survival
DSS	Disease-specific survival
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
ERE	Estrogen response element
EBRT	External beam radiation therapy
FFPE	Formalin fixed paraffin embedded
FIGO	International Federation of Gynaecology and Obstetrics
GDF-15	Growth differentiation factor-15
GFP	Green Fluorescent Protein
GPER	G-protein coupled estrogen receptor
HE	Haematoxylin and eosin
HER2	Human Epidermal growth factor receptor 2
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HR	Hazard ratio
HRT	Hormone replacement therapy
ICD-10	International Classification of Diseases version 2010
IHC	Immunohistochemistry
LN	Lymph nodes
MDM2	Mouse Double Minute 2

MMR	Mismatch repair
MRI	Magnetic Resonance Imaging
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
OS	Overall survival
pAKT	Phospho AKT
PCR	Polymerase chain reaction
PFS	Progression-free survival
PI3Kinase	Phosphoinositide 3-kinase
PI3CA	Phosphoinositide 3-kinase catalytic alpha polypeptide
PI3R1	Phosphoinositide 3-kinase regulatory subunit
PR	Progesterone receptor
PTEN	Phosphatase Tensin homolog
SEER	Surveillance Epidemiology and End Results, USA National Cancer Institute's cancer statistics
STMN1	Stathmin 1, oncoprotein 18, Op18 or Metablastin
SNP	Single Nucleotide Polymorphism
TMA	Tissue MicroArray
TP53	Tumour protein 53, p53
VB	Vaginal brachytherapy

Abstract

Background: Although endometrial cancer in general has a good prognosis, 15-20% recurs. Surgery is the main treatment with lymph node sampling increasingly advocated as compulsory for adequate staging. In metastatic disease, there is limited effect from systemic therapies including chemotherapy or antihormonal treatment. No other targeted therapies are yet available in a routine clinical setting. To improve and individualise therapy for this patient group, improved tools for identification of high-risk patients, to tailor surgery in particular, and identification of targetable molecular alterations for development of more effective systemic therapies, are urgently needed. Several biomarkers including hormone receptor status, TP53 and Stathmin expression have been found to be of prognostic importance in retrospective studies. The PI3Kinase signalling pathway is over-expressed in aggressive endometrial carcinomas and PI3kinase inhibitors are entering clinical trials for treatment of metastatic disease.

Main objectives: The main objective was to evaluate if biomarkers, particularly examined in a preoperative setting, could identify aggressive endometrial carcinomas, especially those with lymph node metastasis. An additional aim was to evaluate immunohistochemical markers potentially applicable as markers for response to antihormonal therapy and PI3Kinase-inhibitors. Also, we wanted to study changes in treatment strategy in relation to survival for endometrial carcinoma patients during a 30-year period in a population based setting.

Materials and methods: To evaluate potential biomarkers related to PI3Kinase signalling, a population based cohort was investigated for immunohistochemical expression of AKT, Phospho-AKT and Stathmin in hysterectomy specimens. These markers were also related to level of PI3Kinase signalling based on mRNA expression score in a prospective series of 76 patients (**Paper I**).

The prospective international multicenter study MoMaTEC; Molecular Markers in Treatment of Endometrial Cancer, recruited clinical data, tissue and blood samples from 1192 endometrial cancer patients treated at 10 different centres during 2001-

2010. Preoperative curettage specimens and blood samples have been investigated for expression of a panel of potential biomarkers; Stathmin, Estrogen Receptor (ER), Progesterone Receptor (PR), TP53 and GDF-15 (**Paper II, III and IV**).

Changes in clinicopathological features and treatment were related to survival in a population based cohort of endometrial cancer patients from Hordaland County, Norway over the last 30 years (**Paper V**).

Results: Stathmin overexpression in hysterectomy specimens was strongly correlated with characteristics for aggressive disease and poor survival. PI3Kinase signalling activation was significantly associated with overexpression of Stathmin. Neither AKT nor phospho-AKT expression showed any significant correlations with clinicopathological factors nor PI3Kinase signalling levels (**Paper I**).

Overexpression of Stathmin validated to be correlated with aggressive disease in the large prospective multicentre setting (**Paper II**). Stathmin staining in curettage specimens was an independent predictor of lymph node metastases and overexpression of Stathmin estimated in curettage and hysterectomy specimens were both independent predictors of poor survival.

High preoperative plasma GDF-15 level was significantly associated with aggressive disease. Adjusting for age and histological risk factors detected in preoperative biopsies, plasma GDF-15 independently predicted risk of lymph node metastasis. GDF-15 level also independently predicted poor prognosis (**Paper III**).

Pathologic expression of ER, PR and TP53 in preoperative curettage specimen correlated significantly with high age at diagnosis, high FIGO stage, non-endometrioid histology, high grade, metastatic nodes and poor prognosis in a large prospective multicenter setting. Double negative ER-PR independently predicted lymph node metastasis and poor survival. Even for the most favourable group of lymph node negative endometrioid tumours, ER-PR negative status influenced survival independent of tumour grade (**Paper IV**).

The number of endometrial cancer patients from Hordaland County increased significantly from 1981 through 2010 (**Paper V**), with a simultaneous increase in body mass index and decrease in disease stage at diagnosis. Routinely performed pelvic lymph node sampling increased, adjuvant radiotherapy was reduced and survival increased significantly during the same period.

Conclusions: Stathmin immunohistochemical staining is superior to AKT and phospho-AKT staining in detecting PI3Kinase signalling activation and endometrial carcinomas with poor outcome (**Paper I**).

Stathmin staining has been validated to identify endometrial carcinomas with aggressive clinic-pathological features in a large multicenter setting. Immunohistochemical staining for Stathmin in preoperative biopsies (curettage) independently predicts lymph node metastasis and poor survival (**Paper II**).

Plasma GDF-15 has been documented as elevated in two independent patient cohorts of endometrial cancer patients compared to controls. High preoperative GDF-15 plasma level was significantly correlated with aggressive subtypes and a significant and independent predictor for lymph node metastasis and poor survival (**Paper III**).

Double negative hormone receptor status (ER and PR negative) in preoperative endometrial cancer curettage has been validated to identify patients with poor prognosis in a prospective multicenter setting. ER-PR status independently predicts lymph node metastasis (**Paper IV**).

During the 30-year period 1981 through 2010, a reduction in adjuvant radiotherapy and increase in routine pelvic lymphadenectomy and curative surgery with advanced disease, are associated with improved disease-specific- and overall survival in a population-based study of endometrial carcinoma patients with steadily increasing body mass index (**Paper V**).

List of publications

This thesis is based upon four publications and one manuscript submitted for publication, referenced in the text by their respective roman numerals:

I. **Trovik J**, Wik E, Stefansson I, Carter SL, Beroukhim R, Oyan AM, Kalland KH, Akslen LA, Salvesen HB (2010). Stathmin is superior to AKT and phospho-AKT staining for the detection of phosphoinositide 3-kinase activation and aggressive endometrial cancer. *Histopathology 57: 641-46*

II. **Trovik J**, Wik E, Stefansson IM, Marcickiewicz J, Tingulstad S, Staff AC, Njolstad TS, Vandenput I, Amant F, Akslen LA, Salvesen H (2011). Stathmin overexpression identifies high risk patients and lymph node metastasis in endometrial cancer. *Clin Cancer Res 17: 3368-77*

III. Staff AC, **Trovik J**, Eriksson AG, Wik E, Wollert KC, Kempf T, Salvesen HB (2011). Elevated plasma growth differentiation factor-15 correlates with lymph node metastases and poor survival in endometrial cancer. *Clin Cancer Res 17: 4825-33*. **A.C. Staff and J. Trovik contributed equally to the work.**

IV. **Trovik J**, Wik E, Werner HMJ, Krakstad C, Helland H, Vandenput I, Njølstad TS, Stefansson IM, Marcickiewicz J, Tingulastad S, Staff AC, Amant F, Akslen LA, Salvesen HB (2012). Biomarkers in endometrial cancer curettage predict lymph node metastasis, recurrence and poor survival in the MoMaTEC prospective multicenter trial. *Manuscript submitted.*

V. **Trovik J**, Mauland KK, Werner HMJ, Wik E, Helland H, Salvesen HB (2012). Improved survival related to changes in endometrial cancer treatment, a 30-year population based perspective. *Gynecol Oncol 125: 381-87*

1. Introduction

1.1 Epidemiology

Endometrial cancer is a tumour originating from the endometrial lining of the uterus. It is the seventh most common malignancy amongst women worldwide, with nearly 200.000 new cases each year, comprising 4% of all cancers in females. The incidence is varying throughout the world being the forth most common cancer in industrialised regions after breast, colorectal and lung cancers. Still it is more rare in developing countries, supporting that environmental factors contribute to development of the disease.[1] In developing countries cervical cancer is by far the dominant gynaecological malignancy with 453 300 new cases pr year, followed by endometrial cancer with 144 900 cases. [1] In western societies endometrial cancer is the most common gynaecological cancer, with 142 200 new cases estimated each year and with a life-time risk of approximately 1,6 %[1], with comparable figures for Norway [2], as illustrated in figure 1. The highest incidence worldwide is seen in USA with more than 20/100 000 women each year followed by Europe with 11-14/100 000.[3]

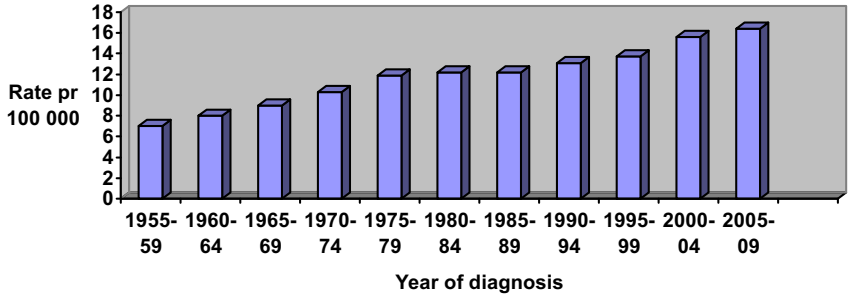

Figure 1 Age-adjusted incidence rate (world standard) of endometrial cancer in Norway per 100 000 per 5-year period of diagnosis.

Adapted from Cancer Statistics Norway 2009; http://www.kreftregisteret.no

Endometrial cancer is very rare in young women as more than 90% of cases occur after 50 years of age.[3] In the Norwegian Cancer Registry median age at primary diagnosis is 66 years with no cases reported before the age of 25, peaking at 110/100 000 in the 70-74 years age group[2], as illustrated in figure 2.

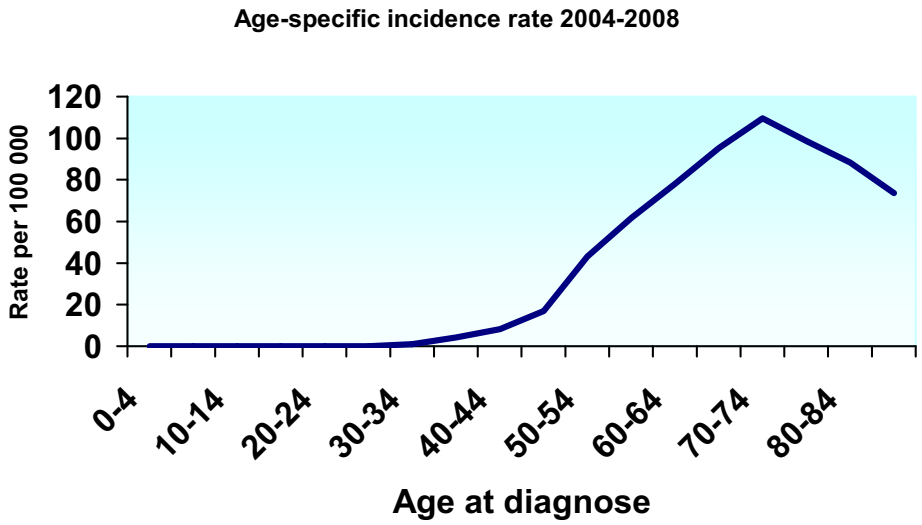

Figure 2 Age-adjusted incidence rate of endometrial carcinoma per 100 000 person years according to patient age in 5-years groups.

Adapted from Norwegian Cancer Registry 2008. [2]

The overall incidence of endometrial cancer is rising[4, 5], as shown for Norway in figure 1 and for four of the Nordic countries in figure 3. In Norway, the age-adjusted incidence per 100 000 person-years was 13.4 in 1999 and has continued to rise to 17.4 in 2008.[2] Based on observed trends, this is predicted to increase even further the next decenniums.[6] The overall increase can be explained partly by increased life expectancy but possibly also as a consequence of the worldwide epidemic of obesity.

Endometrial cancer generally has a good prognosis with 5 years survival approaching 80% in developed and 70% in developing countries.[3] In Europe, by large, a stable, 90% 1-year and 76% 5-years survival has been noted for patients treated 1990-99.[7, 8]

But for the period 1990-2004, the increase in survival ranged from 0.8 to 14.2% in data from 10 out of 11 European cancer registries.[4] In contrast, the American SEER database, reported increased mortality in their population from 1988-2001.[9]

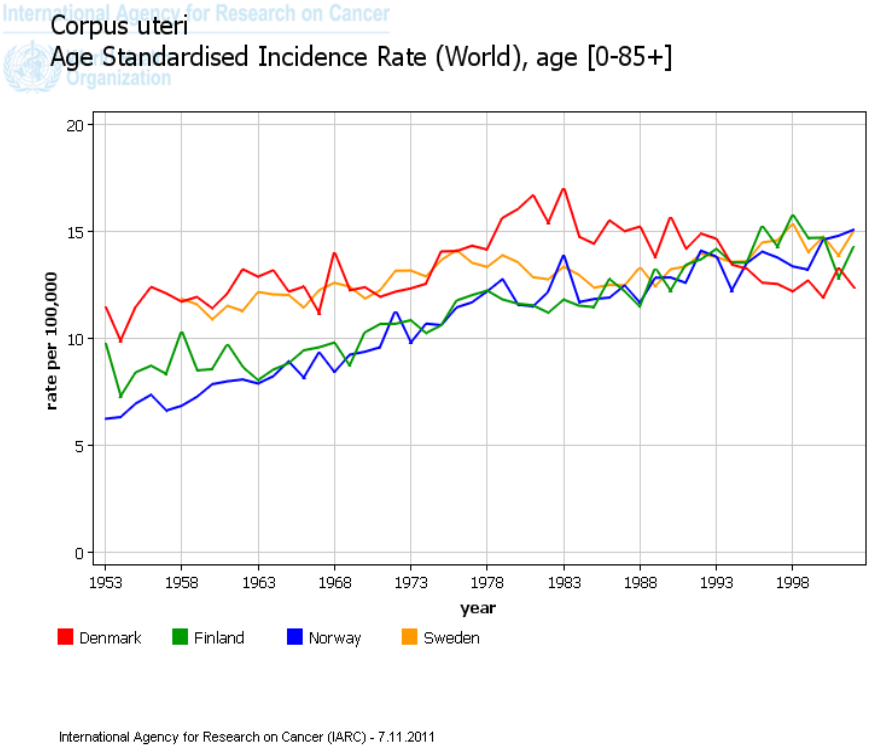

Figure 3 Age standardised incidence rates of endometrial cancer in the Nordic countries per 100 000 according to year of diagnosis.

Adapted from International Agency for Research on Cancer (IARC) http//www.iarc.fr

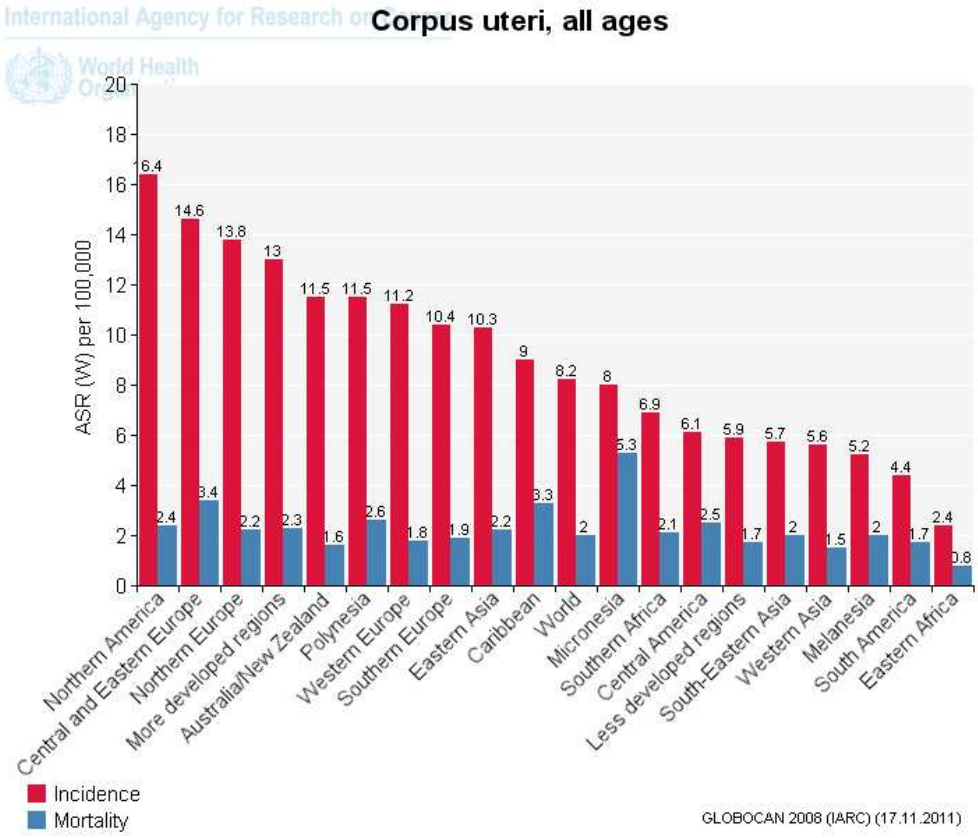

Figure 4 Age-adjusted world standard incidence rate and mortality for uterine cancer per 100 000 from different areas of the world.

Adapted from Globocan, IACR; http//globocan.iarc.fr

1. 2 Etiology and risk factors

The majority of endometrial carcinomas are sporadic. Unopposed long-lasting estrogen stimulation, not counterbalanced by progesterone, is considered to be a major contributing factor to disease development.[3, 10, 11] This is supposed to account for up to 80% of cases and are often classified as Type I cancers. This entity, with a correlation between a hyper-estrogen status and endometrial cancer, was first noted by Bokhman 1983.[12] He described a group of endometrial cancer patients with overweight, hyperlipidemia, diabetes mellitus and signs of hyper-estrogenism:

hyperplasia of the background endometrium, low grade tumours and sensitivity to progestogens. These patients had an over-all good prognosis. In contrast, the tumours classified as Type II cancers, were more often undifferentiated (high grade), deeply invading the myometrium, metastatic at time of diagnosis and developing in a background of atrophic endometrium. The Type II patients had little response to progesterone therapy and poor survival. The distinction between these two types was later utterly refined by histopathological subtyping and immunohistochemical profiling: high proportion of endometrioid histological subtype, estrogen and progesterone receptors positivity in Type I cancers while Type II cancers were dominated by non-endometrioid histological subtypes including serous papillary, clear cell and undifferentiated subtypes, and loss of hormone receptors.[13, 14]

Excess estrogen relative to progesterone stimulates endometrial-cell proliferation, inhibits apoptosis and promotes angiogenesis[5,15], all processes in favour of carcinogenesis. Conditions leading to long-term estrogen overexpression relative to the expression of progesterone promote increased risk of Type I endometrial cancer: obesity, persistent anovulation, nulliparity, tamoxifen use and HRT without concomitant progestin substitution. Factors associated with relative less estrogen stimulation are associated with decreased risk: multiparity and use of oral contraceptives. In addition, smoking, high coffee intake and physical exercise is correlated with reduced risk.[5, 16-19] It has been estimated that each 5 kg/m^2 increase in body weight approximately doubles the risk of developing endometrial cancer, RR 1.95.[20] Although overweight is linked with increased incidence of endometrial cancer, the link between obesity and survival after endometrial carcinoma treatment is less clear. Overweight patients have been reported to be younger, with less advanced tumours and lower histological grade at diagnosis, factors all in favour of a better prognosis.[21, 22]

Women with breast cancer, and in particular those treated with Tamoxifen, also have increased risk of developing endometrial cancer. Although estrogen may act as a common risk factor for both cancer types, subsequent endometrial cancers following breast cancer are more often of Type II (OR 2.6).[23]

For the Type II cancers, mainly non-endometrioid subtypes, the tumour arises on basis of atrophic endometrium, patients are not prone to over-weight, and a different precursor lesion, endometrial glandular dysplasia, has been described.[24] For this group, the hypothesis of a hyper estrogenic environment leading to cancer development is not applicable, and no alternative etiologic hypothesis has been established.

Although having first grade relatives with endometrial cancer approximately doubles the cancer risk[25], the majority of endometrial carcinomas has no known genetic basis and as such occurs sporadic. A minority of endometrial cancers, 2-9%, has been linked to the Lynch syndrome; hereditary non-polyposis colorectal cancer (HNPCC) with autosomal germline mutations in genes responsible for DNA mismatch repair (MMR genes).[26, 27] Endometrial cancer is the commonest extra-colonic cancer manifestation in these women, with a lifetime risk of 40-70%. For females with germ line mutations in MMR genes, yearly screening with endometrial biopsy is recommended, although firm survival benefit is not yet documented.[28] Prophylactic hysterectomy and oophorectomy is recommended by some after completed childbearing but before natural menopause, although evidence from randomised trials are lacking.[5, 29, 30]

Screening for endometrial cancer in the general population is not recommended.

1.3 Clinical aspects and diagnostics

Abnormal vaginal bleeding is the most common incident symptom in endometrial cancer, present in more than 90% of patients. Irregular bleeding is frequent in the pre-menopausal period, mostly due to transient hormonal disturbances. In contrast, postmenopausal bleeding is an alarming symptom urging most women to seek medical care. Endometrial carcinoma will be present in nearly 10% of these patients, with increasing proportion by age and time from menopause or with recurrent bleeding episodes.[31-33]

1.3.1 Biopsy, cytology and curettage

Endometrial cancer is diagnosed by biopsy from the uterine lining; slim plastic curettage devices are feasible for out-patient diagnostics with a sensitivity of 81-99% and specificity of 98%.[5, 34, 35] If the material is sparse or otherwise unfit for histopathological diagnosis, a formal curettage procedure with specimens collected from the cervix and uterine body separately (fractionated curettage) is performed. By bimanual palpation, size of the uterus is estimated and a rough assessment of the uterine mobility performed as part of the evaluation of operability. Endometrial cytology may accurately discriminate cancer from benign lesions but supplementary biopsy is necessary for optimal histologic subtyping and grading. Hysteroscopy may also be used to retrieve biopsies and has been described to detect cancer with a sensitivity of 86% and a specificity of 99%. There is a theoretical risk of spreading cancer intra-abdominally along with flushing of fluid used by hysteroscopy, although this has not been linked to reduced prognosis.[36] Thus hysteroscopy will often be applied for those patients where other methods have been inconclusive.

The histopathological diagnosis of cancer in the endometrial mucosal biopsy is the cornerstone in the diagnostic algorithm; this will initiate the planning of further treatment. Proper classification of histological subtype and grade is strongly recommended as part of the preoperative work up; non-endometrioid subtypes including serous papillary, clear cell and undifferentiated subtypes as well as carcinosarcomas are aggressive, with high frequency of extra-uterine spread.[37] Likewise high grade endometrioid tumours dominated by nuclear atypia and solid growth are correlated with high FIGO stage and poor prognosis.[38] Proper identifying high-risk subtypes is important to allocate patients for appropriate surgical treatment at tertiary centres as indicated.[39, 40] The correlation between preoperative assessment based on biopsy/curettage and postoperative evaluation of the hysterectomy specimens varies. Wang and co-workers[41] described accuracy as low as 37% for detecting correct grade preoperatively compared to final grade based on hysterectomy specimen. Although accuracy increased with higher grade, overall 50% were upgraded. Using frozen section increased the concordance to 69%. Corresponding

figures from other studies indicate an accuracy of 64-71% for grade and 58-85% for frozen section.[42-44]

1.3.2 Sonography

Sonography by an experienced gynaecologist is a good diagnostic tool to evaluate the possibility of cancer as the cause of a postmenopausal bleeding. A thin, regular endometrial lining ≤4 mm measuring the double endometrial thickness[33], corresponds to a probability of 1 % for having cancer, reduced to 0.7% if a cut-of of ≤3 mm is applied.[35] The evaluation of premenopausal patients is more challenging due to cyclical changes in endometrial thickness.

Cervical or myometrial tumour infiltration can be assessed preoperatively by ultrasound with an accuracy of 92-98% and 77-84% respectively.[45-48] If cervical infiltration is detected, this opts for extended surgical treatment with radical hysterectomy including excision of paracervical and parametrial structures, similar to the surgical treatment of primary cervical cancer. Tumour infiltrating deeply in the myometrium (>50% of the wall thickness) significantly increases the risk of lymph node metastasis.[49, 50] This is one key factor evaluated in many algorithms for decision-making regarding lymphadenectomy in connection with the primary surgical treatment.[51-53]

1.3.3 Radiological diagnostics

Chest X-ray or CT (computer tomography) is recommended preoperatively to detect distant spread including lung metastasis. Pelvic- MRI (Magnetic Resonance Imaging) is superior to CT in evaluating extent of pelvic disease.[46, 54] At present neither is considered a good predictor of lymph node metastasis.

The accuracy of MRI evaluation of cervical or myometrial tumour infiltration is in line with what can be achieved by Sonography. For both modalities the prediction of cervical infiltration is better than for myometrial infiltration.[46, 48]

1.3.4 Serological analyses

Serological analyses are not used routinely in the diagnostic work-up of endometrial cancer. Still there are some reports supporting CA-125 as a predictor of patients with high risk features, including lymph node metastases. In one study of 124 patients, a preoperative level of CA-125 >40 U/ml correlated significantly with advanced stage, deep myometrial infiltration and lymph node metastasis, predicting metastatic lymph nodes with a sensitivity of 71% and specificity of 81%.[55] A more recent study found that CA-125 with a cut-off >23.3 U/ml similarly predicted lymph node metastasis with a sensitivity of 65%, specificity of 64% and accuracy of 65% analysing only patients with endometrioid tumours.[56]

1.4 Treatment

1.4.1 Surgery, including lymphadenectomy

Surgery is the main component in primary treatment of endometrial cancer. Hysterectomy with bilateral salpingo-oophorectomy is performed if the patients' general co-morbidity does not preclude this. Peritoneal washing for cytological testing is recommended. Extended radical hysterectomy including excision of paracervical and parametrial structures is performed if tumour tissue invades the cervix, in line with the surgical approach to treatment of cervical cancer. With a histologic diagnosis of clear cell or serous papillary subtypes, resection of the omentum is also recommended due to high frequency of intra-abdominal spread of these subgroups.[40, 57-59]

Traditionally, primary surgery has been performed by laparotomy, but may also be performed by laparoscopy, laparoscopic assisted vaginal hysterectomy or robotic assisted laparoscopic technique, according to the patient status and surgeons' skills, preferences and availability of equipment (robotics). Laparoscopic procedures

generally have longer operation time but less blood loss and shorter hospital stay.[60, 61] Long-term outcome comparisons are sparse and awaiting.

According to the FIGO recommendations[62-64], pelvic and para-aortic lymph node sampling should be performed as part of complete surgical staging. The proportion of patients where this is actually performed varies considerable. Even in centres strongly advocating this procedure it is still not performed for 34-49% of patients.[65, 66] A survey among tertiary cancer centres in USA reported that 45% of centres routinely performed lymphadenectomy[67], with corresponding numbers reported from European centres of 24%.[68] One of the latest reports from the large SEER database (USA)[9], describes an increasing rate of lymphadenectomy performed from 25% in 1988 to 44% in 2001 with mean number of nodes harvested of 6. In Norway the national guidelines from 2002 recommended sampling if suspicious enlarged nodes were encountered during surgery.[57] In the revised guidelines from 2009 a risk stratification based on endometrial subtype and grade and myometrial infiltration recommends pelvic lymph node sampling for medium-risk patients, and pelvic and para-aortal lymphadenectomy for high-risk patients[40], see table 1.

Table 1 Risk of recurrence, stratification in relation to histological subtype and grade and myometrial infiltration.
Adapted from the Norwegian Gynaecological Society's Guidelines; http://www.legeforeningen.no/ngf

	Myometrial infiltration <50%	Myometrial infiltration ≥50%
Endometrioid Grade 1-2	Low-risk	Medium-risk
Endometrioid Grade 3	Medium-risk	High-risk
Clear cell, serous papillary, undifferentiated, carcinosarcoma	High-risk	High-risk

It is well documented that metastatic lymph nodes, corresponding to FIGO stage IIIC is a strong predictor of poor prognosis with 57% 5-years survival compared to 91 % for FIGO IA/IB, 85% for IC, 83%for IIA and 74% for FIGO stage IIB.[49]

Factors known to correlate with lymph node metastases are high grade endometrioid tumours and non-endometrioid histological subtypes.[50, 69] Also, cancers infiltrating deeply in the myometrium are more prone to lymph node spread.[49, 50] The reported rate of metastatic lymph nodes is 6% for <50% myometrial infiltration compared to 12 % for >50% infiltration and 40% for the subgroup of histological grade 3 tumours with deep infiltration. The risk of para-aortic metastasis without concomitant pelvic lymph node positivity was generally low: 0.3-2.9%, highest for grade 3 tumours suggesting that systematic sampling of para-aortic lymph nodes will improve prognostication for this subgroup of patients.[49]

Although detecting the presence of metastatic lymph nodes identifies patients with poor prognosis, it is still a matter of debate whether removing lymph nodes (performing lymphadenectomy) improves outcome. There are cohort studies showing better survival for patients where lymph nodes have been removed [70, 71] but most cohort studies do not find any significant survival differences comparing FIGO stage I patients with or without lymph node sampling performed.[51, 70, 72-77] For summary see table 2.

Two large randomised trials of lymphadenectomy have been performed, neither finding any survival benefit for lymph node sampled patients.[78, 79] Also investigation of the pooled data showed no significant survival differences with HR = 1.07 (95% CI 0.81-1.43) and HR = 1.23 (95% CI 0.96 -1.58) for overall and recurrence-free survival respectively.[80] Lymphadenectomy has, however, been linked to higher complication rates: for the sampled group of patients a significantly higher risk for surgically related systemic complications and lymph oedema or lymph cysts formation has been reported with RR = 3.72 (95% CI: 1.04-13.27) and RR = 8.39 (95% CI: 4.06-17.33) respectively.

Table 2 Studies comparing outcome of lymph node sampling (LNS+) versus no lymph node sampling (LNS-) in treatment of endometrial carcinoma.

1. Author/ Year	Inclusion	Study/ Periode	Outcome measure	LNS- n/%	LNS+ n/%	Survival Log-rank p-value	Impact Multivariate analysis	
							HR LNS+	P-value
Cusido 2011[74]	Endometrioid	Retrospective 1990-2008	DSS	n=85 93%	n=143 93%	n.s	n.a	n.a
Bassarak 2010[76]	Endometrioid	Retrospective 1990-2002	DSS FIGO I	n=63 90%	n=151 95%	0.032	0.31	0.005
			DSS all	85%	90%	0.044		
			OS FIGO I	70%	95%	0.001		
			OS all	60%	85%	0.001	0.40	0.001
			PFS FIGO I	92%	94%	0.69		
			PFS all				0.38	0.022
Kang 2009[77]	Endometrioid Grade 1-2 <50% MI (MRI)	Retrospective 2002-2004	PFS	n=58 98%	n=64 97%	0.61	n.a	
			OS	98%	98%	0.95		
Neubauer 2009[83]	Endometrioid Grade1	Retrospective 1970-2006	PFS	n=313 89%	n=268 90%	ns	0.96	0.82
			OS	93%	92%		1.00	0.99
Kitchener 2009[79]	Clinical stage I	Prospective RCT 1998-05 Multicenter	OS	n=704 81%	n=704 80%		1.04	0.83
			RFS	79%	73%		1.25	0.14
Panici 2008[78]	Clinical stage I	Prospective RCT 1996-06 Multicenter	DSS	n=250 82%	n=264 81%	0.68	1.20	0.41
			OS	90%	86%	0.50	1.16	0.59
Zuzterzeel 2008[84]	FIGO I / II Intermedian/ High risk	Retrospective 2 centres 1983-2004	PFS	n=123 87%	n=172 84%	0.46	LNS n.s.	
Chan 2007[70]	Endometrioid	Prospective Multicenter 1988-2001		n=27 063	n=12 333			
			DSS FIGO I	97%	96%	>0.05	0.75	<0.001
			DSS FIGO II	82%	90%	<0.0001		
			DSS FIGO III	63%	74%	<0.0001		
			DSS FIGO IV	27%	53%	<0.0001		
Denschlag 2007[71]	FIGO III	Retrospective 1989-2003	DSS	n=51 59%	n=60 73%	0.039	0.33	0.017
Hidaka 2007[72]	Endometrioid G1-2,<50%MI	Retrospective 1992-3003	DSS	n=60 98%	n=68 96%	0.56	n.a	
			OS	98%	99%	0.66		
Ceccaroni 2004[73]	Clinical stage I	Retrospective 1986-1994	OS	n=76 88%	n=55 93%	0.38	n.a	
Mariani 2000[51]	Endometrioid Grade 1-2 <50%MI	Retrospective 1984-1993	OS Tumour <2cm	n=141 100%	n=187 100%	1.00	n.s	
			OS Tumour >2cm	100%	93%	0.10		
			RFS Tumour>2cm	100%	91%	0.05		
Bar-Am 1998[75]	Endometrioid Clinical Stage I	Retrospective 1980-1989	OS	n=62 89%	n=183 88%	0.88	n.a	

n.a: not accounted for, n.s: not statistical significant, p-value unspecified, HR: Hazard ratio, DSS: Disease-specific survival, OS: Overall survival, PFS: Progression-free survival, RFS: Recurrence-free survival
Blue: Randomised controlled trials, Red: p-value <0.05

Still, uncertainties related to the standardisation of the procedures applied for lymph node sampling and lymphadenectomy, as well as a potential therapeutic effect for specific subgroups of patients, and for high risk groups in particular, is still debated.[81]

Alternative approaches to identify patients of high risk for lymph node metastasis by applying intra-operative investigations have been attempted. Unfortunately, the detection of deep myometrial infiltration by the surgeon based on visual inspection has been described with as low as 30% accuracy for Grade 3 tumours[82], while the pathologist based on frozen sections had 85% concordance.[44]

1.4.2 Adjuvant treatment

Radiation therapy

Radiation therapy can be delivered vaginally as brachytherapy (VB), externally to the pelvis (EBRT), or extended to include the para-aortal area or the whole abdomen. Radiation therapy is mostly used as adjuvant treatment aiming to decrease the risk of local and regional relapse. Medically inoperable patients may be considered for radical radiotherapy.

A Cochrane review including four randomised controlled trials of patients with FIGO stage I disease treated with external pelvic radiation versus no adjuvant treatment showed a significant reduction of vaginal relapse for the treatment group (RR=0.28; 95% CI 0.17-0.44) but no overall improvement of survival. Patients in the treatment arm with multiple high risk factors as deep myometrial infiltration and grade 3 tumour, had a trend towards improved survival compared to the controls.[85] Based on this, radiation therapy is no longer recommended for low-risk patients with FIGO stage I endometrioid grade 1 disease with less than 50% myometrial infiltration. The PORTEC-2 trial demonstrated that vaginal brachytherapy was as efficient as external radiation in reducing local recurrences but with fewer side effects for high-intermediate risk patients defined as FIGO stage I grade 1-2 with deep infiltration, grade 3 with <50% infiltration or FIGO stage II with superficial infiltration of grade

1-2.[86] A trend for shift in treatment regimen in favour of vaginal brachytherapy rather than external radiation therapy has been documented.[87] For the time being only high risk groups within FIGO stage I defined as grade 3 endometrioid tumours with deep myometrial infiltration or non-endometrioid histological subtypes are considered for external radiation therapy[88], in line with the revised Norwegian National guidelines[40] In line with the international trend, there has clearly been a reduction in the use of adjuvant radiotherapy in Norway from routinely applied in the vast majority of endometrial carcinoma patients through a period with administration of radiotherapy to intermediate- and high risk groups until current practice.[89] (Trovik, **Paper V**)

Systemic therapy

Anti-hormonal therapy is no longer advocated as adjuvant treatment for endometrial cancer. Two Cochrane reviews have addressed this; Kokka and co-workers reported 6 randomised controlled trials with different regimens in adjuvant or recurrent setting and found insufficient evidence for any survival benefit[90], but in five out of these six trials hormone receptor status was not incorporated. Martin-Hirch and colleagues reported seven randomised trials of gestagen therapy in a pooled meta analysis finding no survival benefit from adjuvant treatment with gestagens[91]. It should be noted, however, that none of these randomised trials were based on hormone receptor status in the tumours or metastatic lesions, so anti-hormonal treatment might have a yet unexplored potential for subgroups of receptor positive endometrial carcinoma patients.

Chemotherapy for high-risk endometrial patients is increasingly recommended. A recent Cochrane review of 9 randomised controlled trials of adjuvant chemotherapy, mostly platinum based, describes significant survival improvement for patients treated with chemotherapy, both alone or in addition to radiation therapy. Overall survival HR was 0.74 (95% CI 0.64-0.89) and progression-free survival 0.75 (95% CI 0.64-0.89) in favour of chemotherapy.[92] The risk of recurrence outside the pelvis was significantly reduced with chemotherapy while pelvic recurrence tended to be better prevented by radiation therapy although not statistical significant (RR=1.28; 95%CI

0.97-1.68). The reported proportion of patients receiving adjuvant chemotherapy has also increased, from 2 to 13% from 1996 to 2001.[49]

Despite the introduction of more extensive primary surgery and adjuvant chemotherapy for high risk groups, treatment of patients with advanced or recurrent disease generally have an unfavourable outcome; with 5-year survival of 36-57% for FIGO stage III and 20-21% for FIGO stage IV.[93]

The exception is an isolated vaginal recurrence where radiation therapy in former radiation naïve patients has a similar survival as if radiation is administered as a primary adjuvant treatment.[94]

One study (GOG 19) regarding patients with measurable recurrent or advanced endometrial cancer tested continuously administered tamoxifen citrate and progestin intermittently found a significant longer survival for patients with pre-treatment tumour biopsies positive for estrogen receptor with HR 0.47 (95% CI 0.24-0.92), median overall survival of 8 months versus 19 months with negative ER.[95]

The searching for better treatment regimens has prompted investigation of underlying molecular alterations important in endometrial carcinogenesis.[96] Exploration of tumour biology, uncovering dysregulation in several cellular pathways, is one way of identifying novel treatment strategies. Several of such molecular approaches to new therapy are now being explored in clinical phase I and II trials. Of studies recently completed evaluating therapies in recurrent or metastatic setting, the partial response rate range from 5-12.5 % and stable disease from 10-49%.[97] Ongoing studies are mostly exploring EGFR, VEGF or mTOR/PI3Kinase as shown in table 3.

Table 3 Ongoing Phase I/II trials of targeted therapies for endometrial cancer.

Clinical trial ID	Target	Biological Agent	Additional treatment	n	Restricted to biomarker
NCT00920257	AKT	GSK2141795		70*	
NCT01476137	AKT&MEK	GSK1120212, GSK2110183		335*	
NCT01138085	AKT&MEK	GSK1120212, GSK2141795		40*	
NCT01273168	ER	Endoxifen		72*	ER/PRstatus
NCT00003669	ER	Arzoxifene		n.a.	
NCT00006903	ER	Fulvestrant		95	ERstatus
NCT01244438	FGFR	FP-1039		10	FGFR2mutation
NCT01454479	HER2	Lapatinib	Ixabepilone	24	HER2pos
NCT00650572	HER2/EGFR	ARRY-380		50*	HER2 pos
NCT00087685	mTORC1	Everolimus		35	
NCT00703807	mTORC1	Everolimus	Topotecan	36	
NCT01068249	mTORC1/ER	Everolimus, Letrozole		42	
NCT00770185	mTORC1	Ridaforolimus		30	
NCT00739830	mTORC1/ER-PR	Ridaforolimus, MP/M	C/P,PLD,D,To	130	
NCT01256268	mTORC1	Ridaforolimus	C/P	28*	
NCT00408655	mTORC1	Temsirolimus		39*	
NCT00729586	mTORC1/ER-PR	Temsirolimus, M,T		84	
NCT01460979	mTORC1	Temsirolimus		86*	
NCT00703625	mTORC1	Temsirolimus	Docetaxel	25*	
NCT00982631	mTORC1	Temsirolimus	PLD	30*	
NCT00703170	mTORC1	Temsirolimus	PLD	25*	
NCT01155258	mTORC1	Temsirolimus	VD	20*	
NCT01198184	mTORC1/ Notch	Temsirolimus, RO4929097		30*	
NCT00698243	mTORC1/2	OSI-027		110*	
NCT00687687	PARP	BSI-201	C/P	45	
NCT01237067	PARP	Olaparp	Carboplatin	66*	
NCT01289041	PI3K	BKM120		140	PI3K activation
NCT01068483	PI3K	BKM120		86*	
NCT00876109	PI3K	GDC-0941		99*	
NCT01458067	PI3K	GSK2636771		150*	PTEN loss
NCT01312753	PI3K	MK-2206		90	PI3CA mutation
NCT01307631	PI3K	MK-2206		90	PI3CA mutation
NCT00756847	PI3K	XL 147	C/P	74*	
NCT01364844	PI3K/mTOR	DS-7423		66*	
NCT01455493	PI3K/mTOR	GDC-0980		50	
NCT00940498	PI3K/mTOR	PKI-587		85*	
NCT00485719	PI3K/mTOR	XL765		75*	
NCT00462826	VEGF	Aflibercept		43	
NCT00879359	VEGF	Bevacizumab	C/P	31	
NCT00545792	VEGF	Bevacizumab	Radiation therapy	20*	
NCT00513786	VEGF	Bevacizumab	C/P	38	
NCT01379534	VEGF	Dovitinib		80	FGFR2mutation
NCT00977574	VEGF/mTOR	Bevacizumab, Temsirolimus	C/P/Ixabepilone	330	
NCT01010126	VEGF/mTOR	Bevacizumab, Temsirolimus		275*	
NCT00723255	VEGF/mTOR	Bevacizumab, Temsirolimus		43	
NCT01065662	VEGF/mTOR	Temsirolimus, Cediranib		50*	
NCT01225887	VEGFR/FGFR2	Intedanib		55	
NCT00888173	VEGFR2	Brivanib		43	
NCT01132820	VEGFR2	Cediranib		54	
NCT01111461	VEGFR2	E7080		130	
NCT00478426	VEGFR2	Sunitinib		30	

ID= ClinicalTrial.gov identifier, n=number of patients to be included, n.a=not accounted for

AKT=v-akt murine thymoma viral oncogene homolog, FGFR= fibroblast growth factor receptor, ER=estrogen receptor, EGFR=epidermal growth factor receptor, HER2=human epidermal growth factor receptor 2, MAP2K1=mitogen-activated protein kinase kinase 1, mTORC=mamillian target of rapamycin complex, PARP=poly (ADP-ribose) polymerase, PI3CA=phosphoinositide-3-kinase catalytic alpha polypeptide, PI3K=phosphoinositide-3-kinase, PR=progesterone receptor, PTEN=phosphatase and tensin homolog, VEGF= vascular endothelial growth factor

C/P=Carboplatin/Paclitaxel, D=Doxorubicin, M=Megestrol, MP=Medroxyprogesteron, PLD= Pegesylated Doxorubicin, T=Tamoxifen, To=Topotecan, VD= Vinorelbine ditartrate, *Including other tumour types

1.5 Tumour biology

Normal cells have a highly controlled behaviour, following strictly regulated steps. External signal molecules (ligands) trigger receptors leading to propagation of signals through intracellular circuits (pathways) finally resulting in changes in gene expression in the cell nucleus followed by altered protein production. These cell-signalling pathways are tightly controlled via several feedback loops. When cancer develops, the cell escapes these strict regulatory mechanisms due to multistep changes in the cell's DNA, as pinpointed in the phrase; "Cancer is a genetic disease at the cellular level".[98] Still, tumour cells will be influenced by the local tissue environment, micro milieu, favouring different cell clones to thrive in different tissues or remain dormant.[99] Six specific traits or hallmark characteristics acquired during the multistep cancer development have been described: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis and tissue invasion and metastasis.[100] Recently the ability to evade immunological destruction and reprogramming of energy metabolism to support neoplastic proliferation have been suggested as emerging additional hallmarks of cancer. Also, two enabling characteristics, tumour-promoting inflammation and genome instability and mutation have been introduced to the model of Hanahan and Weinberg.[101] Targeted therapies (developed against specific molecular targets) can be categorized according to their hallmark capabilities as well as the main signalling pathway it attacks.

An illustration simplifying the principles of a signalling pathways and effects of oncogenes and tumour suppressors is seen in figure 5.

Carcinogens may result in genetic changes through gene mutations, deletions, translocations, amplifications or change of gene transcription through epigenetic alterations. Three different categories of genes are typically affected during the carcinogenetic process: oncogenes, tumour suppressor genes and care-taker genes.[99]

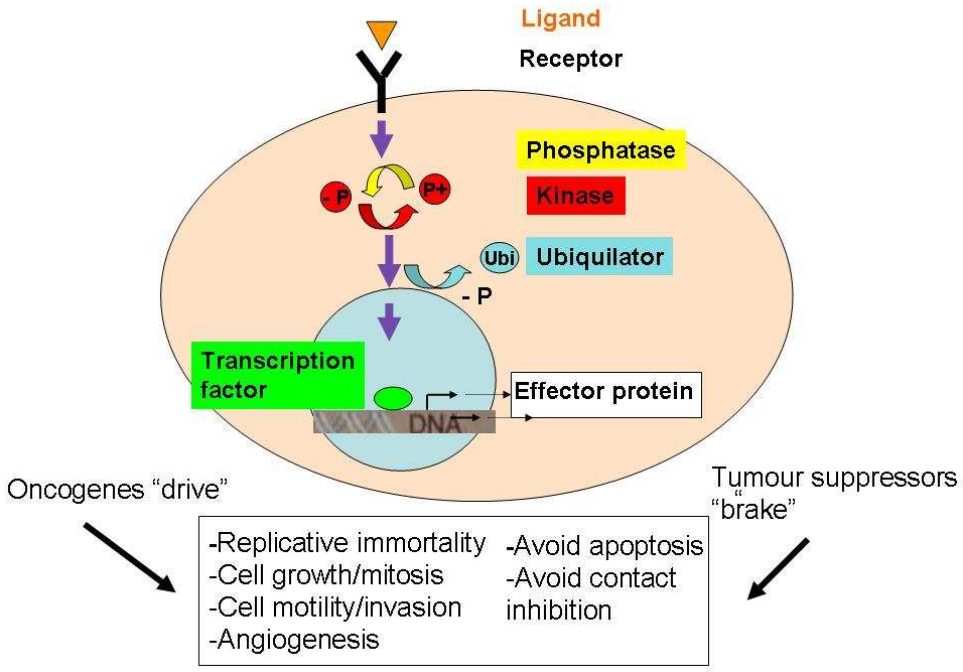

Figure 5 Signalling pathway: Extracellular signal substance (ligand), connecting to receptor, activating intracellular effectors (phosphatases, kinases or ubiquilators) and nuclear transcription factors linked to protein production.

1.5.1 Oncogenes

Normal genes (proto-oncogenes) may be changed to oncogenes by mutation, deletion, translocation or amplification. Activation of one allele of a gene pair is sufficient to alter the affected gene's transcription, and thus lead to changed protein production (oncoproteins); either as uninhibited increased quantity or with increased or

unregulated activity due to structural changes.[99] Oncoproteins often mimics normal growth signals and make cancer cell growth independent of exogenous signals. The altered protein may be a growth factor receptor (e.g. HER2/neu, FGFR2), intracellular signal mediator (e.g. K-ras, AKT, PI3Kinase) or act as a transcription factor (e.g. Myc, ER, PR). Altering of intracellular signalling pathways (e.g. the SOS-Ras-Raf-MAPK cascade and the PI3Kinase signalling pathway) enables a constitutive mitogenic signal even without sustained stimulation[100], exemplified in figure 6.

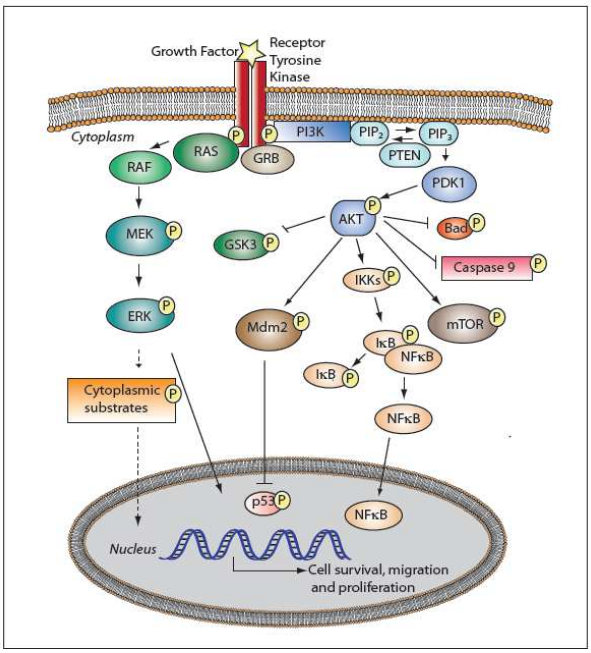

Figure 6 Illustration of growth signalling pathway; activated receptor tyrosine kinase signalling via Ras-Raf-MEK-ERK and via PI3K/AKT.
PDK1: Phosphoinositide-dependent kinase 1, PIP3:phosphatidylinositol-3,4,5 triphosphate, PIP2: phosphoinositide-4,5 bisphosphate.
Adapted from Krakstad and Chekeneya 2010.[102]

Genetic alterations in several oncogenes involved in the PI3Kinase signalling pathway have been described in endometrial cancer:

HER2/neu

ERBB2/HER2/neu; Human Epidermal growth factor receptor 2, is an oncogenic truncated receptor firing without any ligand binding thus promoting cell growth, proliferation as well as hindering apoptosis via the PI3Kinase signalling pathway. This oncogene is known to be amplified and the receptor overexpressed in 20-25 % of breast cancers. The HER2 targeting monoclonal antibody trastuzumab has successfully been implemented in the clinic in adjuvant therapy and treatment of advanced or recurrent HER2 positive breast cancer.[103, 104] In endometrial cancer this gene has been found to be amplified in 1 % of Type I endometrial cancer and 17% of Type II[105], while overexpression of the protein as detected by immunohistochemistry is reported from 3-10% in Type I and 30-40% in Type II cancers.[106, 107]

K-ras

K-ras is a G-protein (GTP-ase), controlling cell growth and differentiation. Although the Ras-pathway mostly is described to act via the Raf-MAPK cascade, Ras may also activate PI3Kinase and thus participate in the PI3Kinase signalling pathway.[99] As an oncogene Ras may be activated by amplifications or point mutations, the latter has been identified in 10-30% of endometrial carcinomas.[97, 108]

PI3Kinase

PI3Kinase is a lipid kinase that phosphorylates PIP_2 to PIP_3 which activates AKT and promotes proliferation, differentiation, motility, angiogenesis and avoidance of apoptosis.[99, 109, 110] The PI3Kinase signalling pathway is the most frequently altered pathway in endometrial cancer.[97] In human malignancies *PI3K* gain-of-function mutations are a frequent oncogenic event.[111] In endometrial cancer, mutations in the *PI3CA*, coding for the catalytic subunit; PI3CA, is found in 30% of Type I cancers and 20 % of Type II, while *PI3CA* amplifications are seen in 2-14 % and 46% of Type I and Type II respectively.[96, 97] However mutation of the regulatory unit *PI3R1* has recently been described in 43% of Type I and 12% of Type II. This mutation also

promotes phosphorylation of AKT, and thus activates the PI3Kinase signalling pathway.[112]

Stathmin

Stathmin or oncoprotein-18 is a protein destabilizing microtubules, acting as an oncoprotein by promoting cell proliferation, mobility, metastasis and resistance to antimicrotubule chemotherapy.[113, 114] Stathmin is inactivated by phosphorylation but the amount of protein is foremost regulated at the transcriptional level.[115] A point mutation in the coding region of *STMN1* has been described in oesophagus cancer cells.[116, 117] The corresponding protein thus experimentally expressed showed decreased phosphorylation. Stathmin protein expression has been associated with PI3Kinase activity in breast and endometrial cancers.[96, 118] The latter found STMN1 overexpressed in 15% of Type I cancers compared to 64% for Type II.[96]

Other oncogenes have also been reported as altered in endometrial cancer; Drug sensitive mutations in the fibroblast growth factor receptor 2 (*FGFR2*) tyrosine kinase gene have been reported in 12% of endometrial carcinomas.[119] Nuclear accumulation of β-catenin (CTNNB1), only partially explained by mutations, is detected more frequently in Type I compared to Type II cancers.[120]

Growth differentiation factor-15

Growth differentiation factor-15, formerly named as macrophage inhibitory cytokine-1, placental bone morphogenetic protein, placental transforming growth factor-A, prostate derived factor and non steroidal anti-inflammatory drug (NSAID)-activated gene-1. GDF-15 is a distant member of the transforming growth factor-β superfamily. Gene expression is stimulated by tumour necrosis factor-α, interleukins and macrophage colony-stimulating factor and regulates cell cycle, proliferation, proliferation, differentiation and apoptosis.[121, 122] Overexpression have been found in ovarian cancer[123], prostate cancer, colorectal cancer and breast cancer[124], but to our knowledge, has not earlier been described in endometrial cancer.

1.5.2 Tumour suppressor genes

Tumour suppressor genes code for proteins inhibiting growth and tumour formation, acting as gatekeepers against external growth promoting signals (e.g. Retinoblastoma protein) as well as maintainers of the internal well-being of the cell (e.g. TP53). With inactivation of one allele (by mutation, deletion or promoter region methylation), normal protein production is sustained. Inactivation of both alleles is needed to lose the protective function leading to uncontrolled cell growth. Thus for tumour suppressor genes there is a recessive trait; two "hits" are needed to trigger carcinogenesis by affecting a tumour suppressor in accordance with Knudson's two-hit hypothesis.[99]

PTEN

PTEN encodes for the Phosphatase Tensin homologue phosphatase counteracting PI3Kinase by dephosphorylating PIP_3 to PIP_2. Inactivating PTEN will promote the PI3Kinase signalling pathway and thus promote cell proliferation, cell survival and angiogenesis, and hinder apoptosis.[29, 99, 109]

In endometrial cancer PTEN loss is mostly associated with Type I tumours (50-83%) and only 5-10% of Type II cancer, thus mostly related with a less aggressive phenotype and good prognosis.[97, 125, 126]

TP53

The tumour suppressor gene *TP53* encodes for a transcription factor responsible for guarding the genomic integrity. If errors in DNA replication are detected, TP53 initiate cell cycle arrest, DNA repair or apoptosis. This is the most frequently mutated gene in human cancers, and is mutated in over 90% of Type II endometrial cancers compared to 20% of Type I.[97, 108] TP53 is inactivated by ubiquitilation by MDM2, thus overexpression of MDM2 can also lead to reduced TP53 function. Normal TP53 is rapidly degraded while non-functional mutant TP53 resists degradation. A

concordance of 76% between mutation and protein overexpression is reported in endometrial cancer.[127]

Other tumour suppressors of importance in endometrial cancer are p16 and E-cadherin, loss of these are encountered in 83% and 5-50% of Type I cancers respectively and in 5 and 62-87% of Type II cancers.[97, 128]

1.5.3 Care-taker genes

When DNA replications proceed incorrectly or other DNA damages occur, repair mechanisms are activated. The mismatch repair genes (MMR genes) initiate repair of DNA replication errors, inhibit recombination between un-identical DNA sequences and respond to DNA damage to hinder genetic instability.[99] Autosomal germline mutations of MMR genes such as *MLH1, MSH2, MSH6* and *PMS2* are found in Lynch syndrome; hereditary non-polyposis colorectal cancer (HNPCC), where endometrial cancer is the commonest extra-colonic cancer in females characterized by microsattelite instable (MSI) tumours.[26] In sporadic endometrial cancer methylation of MMR genes may lead to replication errors of microsatellites (simple repetitive DNA sequences) and microsatellite instability. MSI is prevalent in Type I cancers, 20-45%, but rare in Type II (0-5%).[97, 129]

Another caretaker gene set is *BRCA1* and *BRCA2*, responsible for repairing double strand DNA breaks. Germline mutations are seen in hereditary breast and ovarian cancers, but are seldom a cause of endometrial cancer.[130, 131]

DNA ploidy as a tumour marker

Normal cells contain 46 chromosomes and are termed diploid. With either fewer or more than these 23 chromosome pairs, the cell is referred to as aneuploid. Carcinogenesis is often characterised by aberrant mitosis, chromosomal instability

and loss of the protective TP53, thus aneuploidy is common in cancer cells.[99] In endometrial cancer aneuploidy is present in around 20% of patients and associated with Type II cancers.[132, 133]

1.5.4 Tumour markers more specific related to endometrial cancer

Hormone receptors

The endometrium is highly hormone sensitive and express both estrogen and progesterone receptors. Normally the endogen ovarian produced estrogen induces endometrial proliferation. When progesterone is added in the second half of the menstrual cycle, the estrogen driven proliferation is antagonized and the epithelium differentiates from the proliferative to the secretory phase.[134] Although both estrogen and progesterone receptor status is known to correlate with clinical features and prognosis in both endometrial and breast cancer, targeting the ER pathway yields partly different results for these cancers.[135]

ER

Hormone activated estrogen receptor, mostly the isoform ERα, is known to be important in endometrial carcinogenesis. Acting as a transcription factor, ER bound to the estrogen response element (ERE) of target genes, can induce expression of growth factors (EGF, TGF-α) and growth factor receptors (ERBB1 and ERBB2, IGF-I and IGF-II) known to stimulate both the PI3Kinase- and Ras/Raf pathways and thus stimulate mitogenic activity.[15] ERα phosphorylated by AKT can lead to ligand independent response without estrogen coupled to the receptor, increased transcription of growth factors and stimulation of mitosis.[15, 136] An alternative estrogen receptor GPER/GPR30 has been described and has been demonstrated to mediate estrogen stimulation and PI3Kinase activation in nuclear ER negative cells.[137] Loss of ER is highly linked to type II endometrial cancer.[138]

PR

PR is considered essential to inhibit estrogen induced hyperproliferation, and thus to counteract carcinogenesis. Both PR-A and PR-B are expressed in endometrial tissue[134], but in endometrial cancer mostly PR-A is investigated and generally referred to as PR. Similarly to ER, PR loss is correlated with Type II cancers.[138] Progestin effects are mainly mediated through PR, and in ER positive tumours progestin may yield an anti-estrogen effect.[134] This treatment option is mainly considered as a possibility for young women with endometrioid low grade tumours where removal of the uterus is avoided to retain the possibility of child-bearing.

In table 4, genetic abnormalities are investigated separately. In reality, tumours will exhibit multiple defects. Specific "clustering" of aberrations may affect different aspects of tumorigenesis.[101] Upregulation of certain genes combined with downregulation of other sets of genes has been reported to be characteristic for activation of specific oncogenic pathways.[147] The PI3Kinase signalling pathway is one such major pathway found to be upregulated in cancer in general, and in aggressive subtypes in particular.[109, 110] Activation of this pathway may affect sets of genes (expression signature) that may be more robust in determining the involvement of the signalling pathway compared to the regulation of a single gene. One such expression signature of activated PI3Kinase has been reported by Gustafson[148], another by Catasus.[149] Integrated analyses of genome-wide expression and copy-number data suggest that activation of this PI3Kinase signature is associated with amplification of *PIK3CA* region, aggressive phenotype and poor survival for endometrial carcinomas.[96] Other genes active in regulation of this pathway are *K-ras*, *HER2/neu*, *ER* and *PTEN*.

Table 4 A summary of genetic alterations and their relevance for Type I versus Type II endometrial cancers.

Target	Function	Alteration	Type I (%)	Type II (%)
KRAS [97, 108]	Oncogene	Mutation	11-26	0-10
ERBB2/HER-2/neu [105-107]	Oncogene	Amplification, expression	1	17-80
PIK3CA[139]	Oncogene	Mutation	21-46	46
PIK3CA[96, 97]	Oncogene	Amplification	2-14	46
Stathmin [96]	Oncoprotein	Overexpression	15	64
FGFR2[119]	Oncogene	Mutation	12	12
PTEN[97, 125, 140]	Tumour suppressor	Mutation, deletion, methylation	35-83	0-11
TP53[97, 108, 141]	Tumour suppressor	Mutation	5-20	80-93
P16[97, 142, 143]	Tumour suppressor	Mutation, methylation, expression	8	5-45
MSI[129, 144]	DNA repair	Methylation, mutation	20-45	0-5
ER, PR[138]	Transcription factors	Expression	70-73	19-24
Beta-catenin [120, 145]	Oncogene	Mutation, expression	18-47	0-5
E-cadherin[146]	Tumour suppressor	Mutation, methylation	5-50	57-87

1.6 Biomarkers predicting prognosis in endometrial cancer

A biomarker is a ”*Characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*” as defined by the National Institute of Health Biomarkers Definitions Working Group.[150] Thus, traditional clinical as well as histopathological features can be regarded as biomarkers. Regarding endometrial cancer, both age and nulliparity are noted as independent factors for survival.[5, 151]

1.6.1 Clinicopathological characteristics as biomarkers

FIGO (Federation of Gynaecology and Obstetrics) stage (table 5) is the strongest prognostic factors in endometrial cancer, with significantly reduction in disease-specific survival with higher stages. 96% 5-year survival is reported for patients with localised tumour, 67% with regional- and 17% with distant metastasis.[152] Corresponding figures using the recent revised FIGO 2009 classification is 90 and 78% respectively if tumour infiltrates inner- or outer half of the uterine wall (FIGO stage I), 74% if cervical stroma is involved (FIGO II), 56, 36, 57 and 49% respectively with adnexal, vaginal, pelvic lymph node or para-aortic lymph nodal involvement (FIGO III), and 22 and 21% respectively with bladder/rectal versus distant metastases.[93]

The impact of deep myometrial invasion on prognosis is illustrated by the significant difference in survival between FIGO IA (<50%) and FIGO IB (>50%), but is also a risk factor for advanced stage including lymph node metastasis and identified as an independent risk factor for poor survival even in high stage disease.[153-155]
Metastatic lymph nodes, either pelvic (FIGO IIIC1) or para-aortic (FIGO IIIC2) are an independent predictor of poor survival. Even when adjusted for uterine risk factors as high histologic grade, cervical stromal invasion or myometrial infiltration >50%, a large study of nearly 27 000 lymph node sampled patients from the American SEER registry, found patients with metastatic lymph nodes to have significantly lower disease-specific and overall survival than patients with negative lymph nodes.[153]

Table 5 Surgical tumour classification according to the International Federation of Obstetrics and Gynaecology (FIGO) originally from 1988, revised 2009.

Stage	FIGO 1988[62]	FIGO 2009[64]
I	IA Tumour limited to endometrium	IA Tumour with no or less than half myometrial invasion
	IB Invasion to <1/2 myometrium	
	IC Invasion >1/2 myometrium	IB Invasion equal to or more than half of the myometrium
II	IIA Endocervical glandular involvement only	II Tumour invades cervical stroma but does not extend beyond the uterus
	IIB Cervical stromal invasion	
III	IIIA Tumour invades serosa and/or adnexae and/or positive peritoneal cytology	IIIA Tumour invades the serosa of the corpus uteri and/or adnexae
	IIIB Vaginal metastases	IIIB Vaginal and/or parametrial involvement
	IIIC Metastases to pelvic and/or para-aortic lymph nodes	IIIC1 Metastases to pelvic nodes
		IIIC2 Metastases to para-aortic lymph nodes with or without positive pelvic lymph nodes
IV	IVA Tumour invasion of bladder and/or bowel mucosa	IVA Tumour invasion of bladder and/or bowel mucosa
	IVB Distant metastases including intra-abdominal metastases and/or inguinal lymph nodes	IVB Distant metastases including intra-abdominal metastases and/or inguinal lymph nodes

[62]Creasman: Gynecol Oncol;35:125-7 [64]Pecorelli: Int J Gynecol Obstet 2009;105(2):103-4

Using patients with metastatic nodes but no uterine risk factors as reference category, patients without metastatic nodes had a hazard ratio of 0.09 (95% CI 0.07-0.11) for disease-specific death and 92% 5-year survival if no uterine risk factors were present compared to a hazard ratio of 0.64 (95%CI 0.52-0.79) and 69% 5-year survival with $\geq$ 2 uterine risk factors. Within the group of lymph node positive patients, having any additional uterine risk factors increased the risk with HR 1.24 (95% CI 1.02-1.52) and

with a 58% 5-year survival compared to 68% for lymph node positive without additional uterine risk factors. Although detection of lymph node metastases is a well established prognostic factor, randomised controlled trials have not shown any definitive survival benefit from the performance of lymphadenectomy in patients with presumed FIGO stage I disease.[80] It is an ongoing challenge to properly identify patients with high risk of lymph node metastasis that may benefit from adequate lymph node sampling and to avoid potential side effect from lymphadenectomy for those who most likely have no benefit from the procedure.

Histological type and grade are also strong predictors of outcome: Non-endometrioid subtypes including serous papillary, clear cell and undifferentiated carcinomas as well as carcinosarcoma have significantly poorer outcome than endometrioid tumours. The non-endometrioid tumours are all classified as high-grade tumours. Within the endometrioid subgroup, low-grade, well differentiated tumours have significantly better survival than undifferentiated high grade cancers.[49, 70] Most tumours are endometrioid but the proportion of non-endometrioid tumours increase with increased stage; from 8% of Stage I-II to 21% of stage III-IV patients. Within Stage I disease, patients with endometrioid histology had a 5-year overall survival of 90-93%, while corresponding figures for non-endometrioid tumours were 78-85%. Also the proportion of high-grade endometrioid tumours is increased with higher stages. Within Stage I disease, Grade 2 tumours had reduced survival with HR 1.4 (95% CI 1.1-1.7) and Grade 3 HR 2.8 (95% CI 2.2-3.6) compared to Grade 1 tumours.[49]

These two distinct different prognostic groups; endometrioid estrogen driven versus non-endometrioid, have also been categorised as Type I and Type II respectively.[12, 14] Although there is considerable overlap, they also show different molecular characteristics as summarised in table 4.

Several studies have demonstrated that involvement of blood vessels[156, 157] or lymphovascular space is linked to poor outcomes, emphasizing the importance of

reporting this information in the routine histopathological reports for endometrial carcinomas.[158, 159]

1.6.2 Molecular biomarkers

Molecular biomarkers comprises evaluation by genomic or proteomic techniques. Proteomics includes investigations of proteins; often by specific protein targeted antibodies in immunohistochemical investigations.

There is a large range of genomic approaches for genetic investigations: abnormal number of chromosomes can be detected by DNA ploidy analyses, specific mutations can be identified by sequencing and gene copy number alterations by SNP array. Gene expression can be evaluated by microarray or quantitative PCR. These methods are illustrated in figure 7 in relation to assessment of alterations on DNA, mRNA or protein levels.

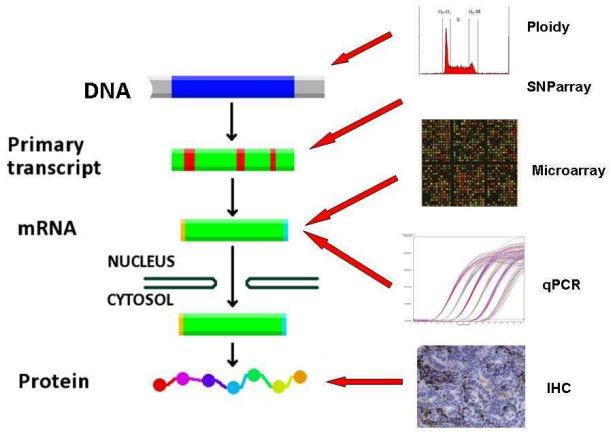

Figure 7 Illustration of different methods identifying genetic aberrations at the DNA, mRNA and protein levels.

Immunohistochemical (IHC) analyses in addition to Haematoxylin-Eosin stainings are performed in most pathology laboratories. In some diagnostic work-ups this is

performed routinely, such as HER2 testing in breast cancer.[160] IHC analyses are mainly less costly and simpler to perform than genetic analyses. Due to the applicability in routinely available formalin fixed paraffin embedded tissue, a biomarker with good correlation assessed by IHC will often be preferred to genomic approaches, if similar prognostic and predictive information can be derived.

For endometrial cancer, biomarkers have been most extensively studied in hysterectomy specimens, but for some markers also in preoperatively collected curettage samples. A summary of the most common IHC markers and their respective impact on survival is summarised in table 6. Based on a convincing number of sufficiently powered studies with robust outcome data, DNA ploidy, ER, PR and TP53 status seem to contribute independent prognostic information adjusted for standard histopathological data[132, 138, 161], although the additional prognostic impact of biomarkers in patient series subjected to lymphadenectomy has to date been less studied.

Biomarkers applied on preoperative biopsies or curettage specimens give the additional possibility to improve the identification of patients with aggressive disease and poor prognosis prior to surgery. Even when adjusting for FIGO stage and histological type and grade, several investigations have demonstrated independent prognostic value of hormone receptors, TP53 and p16 while neither HER2 nor ploidy had independent significance when estimated in preoperative endometrial biopsies.

Preoperatively biomarkers in the assessment of risk for lymph node metastasis have been addressed by Mariani in 2005 through a retrospective case-control analysis of 82 patients. They found TP53 tested in curettage specimen as an independent predictor of metastatic lymph nodes adjusted for histologic subtype.[65]

Table 6 Biomarkers and their reported reduction (%) in survival when tested in hysterectomy or preoperative specimens.

Biomarker	**Hysterectomy specimens**	**Preoperative biopsy**
Loss of ER-PR expression		
Orescovic-04[162]	Not specified	35% n.s Cox
Engelsen-08[138]	31%	30%
High expression of TP53		
Salvesen-99[161]	30%	
Mariani-00[163]		33%
Silvermann-00[164]		21% Cox n.a.
Engelsen-06[143]		33%
Oreskovic[162]	Not specified	30%
Loss of P16 expression		
Salvesen-00[142]	34%	
Engelsen-06[143]		35%
High proliferation rate (Ki-67)		
Salvesen-99[161]	26%	
Engelsen-08[106]	16%	13% n.s Cox
High expression of Stathmin		
Salvesen-09[96]	18%	
Overexpression of Her2NEU		
Morrison -06 [107]	42%	
Mariani-00[163]		18% n.s.Cox
Engelsen-08[106]	20% n.s Cox	20% n.s Cox
Aneuploidy		
Mariani-00[163]		12% n.s Cox
Silverman-00[164]		27% n.s. Cox
Wik-09[132]	19%	

n.s: not an independent marker in multivariate Cox analyses when adjusted for FIGO stage, histologic type or grade n.a: not accounted for

CA-125 measurements have been suggested as a predictor for lymph node metastasis but neither optimal cut-off nor which supplementary analyses to use in combination in a prediction algorithm have been defined.[53, 56] Thus, there is no established serologic analyses in general clinical use to predict lymph node metastasis or poor outcome in endometrial cancer. [165]

1.6.3 Prognostic value from specific genomic analyses

Most studies of genetic aberrations in endometrial cancers have focused on the distinction between Type I and Type II cancers. Also, an approach to further molecularly classify tumours based on histological subtypes has often been applied. Many of these studies have been selective and too small for adequate survival analyses, sometimes generating conflicting data regarding prognosis. Of the studies referred to in Table 4, neither has proven any independent survival effect for the investigated genomic marker. In Morrison's investigation of *HER-2* amplification and expression these were not independent predictors of poor survival unless both factors were pathologic, with a HR of 2.30, 95% CI 1.25-5.32. The *PI3CA* amplification in Salvesen's series of 74 endometrial cancers was highly significantly associated with poor outcome in univariate analysis of recurrence-free survival, but not in multivariate analyses. However, the protein expression of Stathmin as a marker of the PI3Kinase pathway, had a HR of 2.14, 95% CI 1.28-3.59 in multivariate analysis when investigated in a larger patient series of 313 endometrial cancers.[96]

1.6.4 Predictive markers for therapy response

Hormone receptor status in tumours has been linked to the effect of antihormonal therapy. In one study of patients with metastatic tumours treated continuously with tamoxifen and intermittently with weekly medroxyprogesterone acetate (GOG 119 Study), patients with metastasis staining positive for ER showed significantly better response and overall survival. No correlation with PR and clinical response was

noted.[95] A systematic review from 2007 found no randomized trails based on hormone receptors status but five cohort studies evaluating different regimens; hydroxyprogesterone caproate, megestrol acetate, letrozole and arzoxifene. All studies showed higher response rates in ER+ and PR+ tumours compared to tumours with receptor loss.[166]

Contrasting breast, lung and colorectal cancers, none of the new targeted therapeutics has reached the clinic yet for endometrial cancers.[97] In clinical trials for endometrial cancer, drugs targeting the PI3Kinase/AKT/mTOR pathway, EGFR/HER2 or VEGFR are being evaluated as summarised in Table 3. Since PTEN mutations and loss of function is common in endometrial cancer, and promotes the PI3Kinase/mTOR signalling pathway activation, PTEN staining by immunohistochemistry has been tested as predictor for response to mTOR inhibitors, but has not so far been shown to be applicable as predictive marker for response.[97] Immunohistochemical staining for Stathmin, a surrogate marker for PI3Kinase activation, has also been suggested as an alternative marker to explore for prediction of response to PI3Kinase/AKT/mTOR inhibition.[96] Vascular proliferation and microvessel density associates with outcome in endometrial cancer[167], and vascular proliferation correlates with VEGF-A expression. Thus, such markers for activated angiogenesis have been suggested as potential markers for response to angiogenesis inhibitors, but are yet to be evaluated.

HER2 amplification is less frequent in endometrial compared to breast cancer and has not been as successful in predicting effect from trastuzumab therapy as for breast cancer. The molecularly based trial targeting both EGFR and HER-2 (NCT00748709) based on HER2 and/or EGFR amplification or mutation status, was one of the first target-specified patient inclusion phase II trials for endometrial cancer.[97]

2. Aims of the study

2.1 Background

Although endometrial cancer in general has a good prognosis, 15-20% recurs.[5] Surgery is the main component of treatment with lymph node sampling increasingly advocated as compulsory for complete staging. In metastatic disease there has been limited effect from conventional chemotherapy or antihormonal treatment, and no novel targeted therapies are yet available for this patient group. Thus, improved identification of high-risk patients and more effective and individualised therapy, surgical as well as systemic, is urgently needed. Several biomarkers including hormone receptor status, TP53 and Stathmin expression have been found to be of prognostic importance in retrospective studies. The PI3Kinase signalling pathway is one of the major upregulated oncogenic pathways and has been documented to be over-expressed in aggressive endometrial carcinomas with poor prognosis.[96] PI3kinase inhibitors as cancer treatment are now entering clinical trials.

2.2 General aims

The general aim was to evaluate if biomarkers tested preoperatively could identify aggressive endometrial carcinomas; patients with advanced disease at primary treatment, especially lymph node metastasis, and patients with poor survival. A second aim has been to focus on potentially targetable biomarkers for hormone receptor status and PI3kinase activation, to identify markers potentially applicable as predictive markers for selecting patients for treatment with antihormonal therapy and PI3Kinase-inhibitors. Also, we wanted to elucidate changes in treatment strategy and survival for endometrial carcinoma patients during a 30 year period in a population based setting.

2.3 Specific aims

1. To explore potential markers for PI3kinase activation: Investigate the immunohistochemical markers AKT, Phospho-AKT and Stathmin in relation to clinico-pathological variables and patient prognosis; to examine whether these markers reflects the level of PI3Kinase activation based on mRNA expression levels (**Paper I**).

2. To validate the prognostic value of Stathmin immunohistochemical expression in a large prospective multicenter setting; to test if Stathmin expression in preoperative curettage samples can predict lymph node metastasis (**Paper II**).

3. To investigate and validate in two independent patient cohorts if the expression level of growth differentiation factor-15 (GDF-15) in preoperative blood plasma samples correlated to clinico-pathological patient characteristics and prognosis and whether GDF-15 could predict lymph node metastasis (**Paper III**).

4. To validate the prognostic value of the immunohistochemical expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and TP53 in preoperative curettage specimen and to investigate if these markers could improve prediction of lymph node metastasis in a large prospective multicenter setting (**Paper IV**).

5. To investigate if changes in clinico-pathological patient characteristics and therapeutic strategies including increased frequency of lymph node sampling are reflected in survival changes through careful characterization of a 30-years population based cohort (**Paper V**).

3. Materials

3.1 Patient series

This study has used several patient series in the various papers, summarised in figure 8.

3.1.1 The Hordaland cohort:

To evaluate changes over time, both in underlying tumour and patient characteristic as well as treatment strategies (**Paper V**), a stringent population based cohort of patients from Hordaland treated during 30 years at Haukeland University hospital has been selected.

Haukeland University Hospital is a tertiary cancer centre responsible for treatment of women with gynaecological cancer primarily from Hordaland County but is also a referral centre for Rogaland and Sogn og Fjordane Counties. To avoid over-representation of high-risk cases, aiming for a representative population based cohort from our region in Norway, we have collected only endometrial carcinoma patients with verified permanent address in Hordaland County at time of primary treatment.

The first decade (1981-1990), 286 patients, have been extensively studied and described in several earlier publications.[120, 142, 143, 168, 169] Cross-checking with data from the mandatory Cancer Registry of Norway has confirmed that 286 of 303 patients (94%) from Hordaland with histological verified endometrial carcinoma had been correctly identified and included in our study cohort through the hospital diagnosis registries at the Departments of Pathology as well as Gynaecology and Obstetrics. The middle decade (1991-2000) was retrospectively collected by a similar approach, containing 307 patients, whiles the last decade (2001-2010) 484 patients, was prospectively included as part of the MoMaTEC trial (http://www.clinicaltrials.gov/ct2/show/NCT00598845).

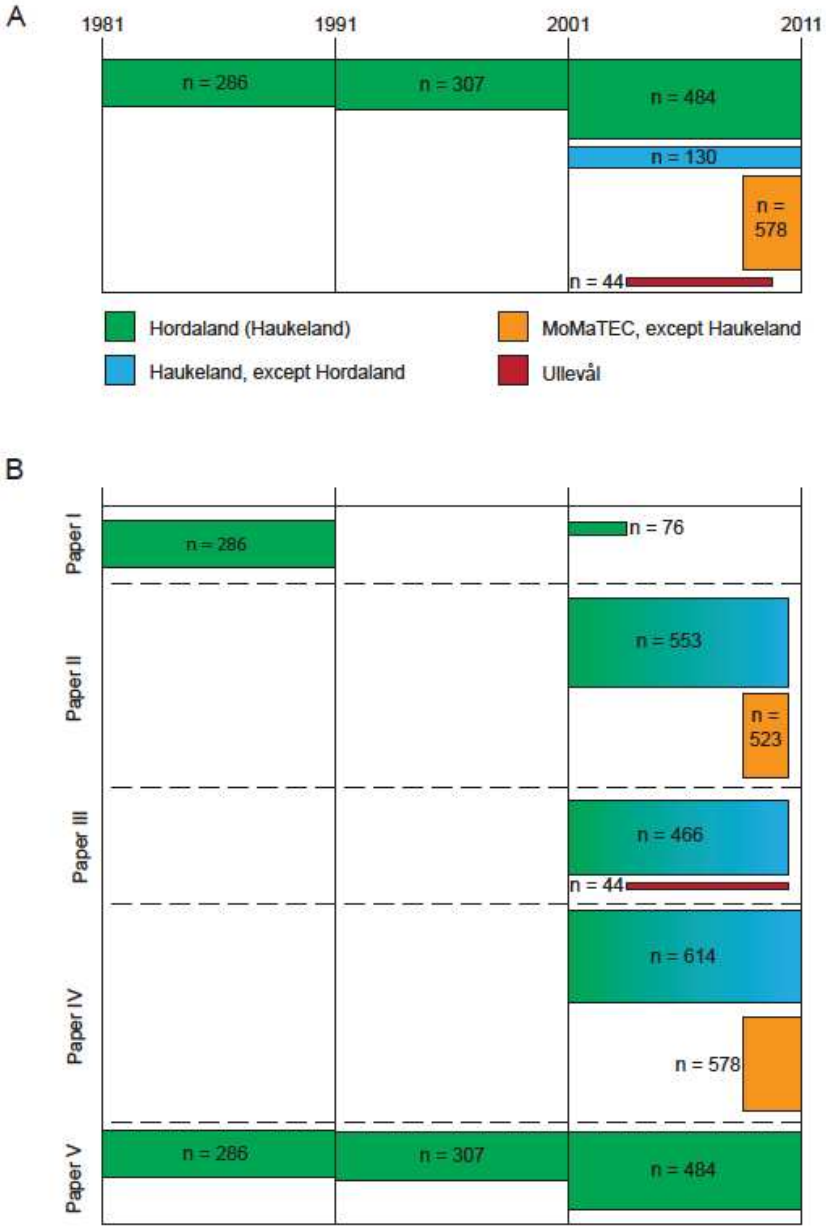

Figure 8 Patient cohorts which have been used in this thesis. **A** Number of patients in each series related to year of primary treatment and inclusion area. **B** Number of patients and cohorts used for each of the papers (**Paper I-V**) in this thesis.

Clinical data regarding age at primary operation, parity, menopausal status, height and body weight, date and type of primary and adjuvant treatment, histopathological type and grade, FIGO stage, lymph node sampling status and the course of disease were collected from patients' medical records. Supplementary information was collected from primary physicians and private gynaecologists responsible for out-patient follow-up. Data from the two last decades were, similarly to the first cohort, cross-checked for permanent address and diagnosis confirmation through the mandatory Norwegian Cancer Registry and the dates and causes of deaths were authenticated through the Norwegian Death Registry.

Hordaland County is a region comprising 9.8 % of the total 4 920 000 Norwegian population being demographically well defined.[170] The age-adjusted incidence rate of cancer in the corpus uteri (ICD-10 code C54) is similar in Hordaland as in total of Norway and has risen in parallel with a national increase since the initiation of national cancer registration in 1952.[2] The Hordaland cohort collected during our study thus is assumed to be representative of a general Norwegian endometrial cancer population.

The whole 30-years Hordaland cohort is investigated in **Paper V**, the 1981-90 decennium and the first 76 consecutive patients enrolled in the 2001-10 cohort comprise the study population in **Paper I.**

Table 7 Comparison of distribution of patient characteristics between the large retrospective 1981-90 cohort and smaller prospective series from 2001 onwards from Hordaland studied in **Paper I** support that there are no significant differences between the cohorts.

Variable	**Hordaland 1981-1990 Retrospective n=286**	**Hordaland 2001-2002 Prospective n=76**	**P-value[1]**
Mean age years (Range)	65 (33-92)	66 (39-91)	0.6
FIGO 1988[2]			0.5
I-II	230 (78)	64 (22)	
III-IV	55 (82)	12 (18)	
Histological type			0.5
Endometrioid	257 (80)	66 (20)	
Non-endometrioid	29 (74)	10 (26)	
Histological differentiation			0.3
Grade 1-2	227 (80)	56 (20)	
Grade 3	59 (75)	20 (25)	
Myometrial infiltration[3]			0.1
<50%	146 (80)	37 (20)	
≥50%	90 (71)	36 (29)	
BMI(kg/m^2) [4]			0.2
<25	103 (88)	14 (12)	
≥25	112 (82)	25 (18)	

[1]t-test used for comparing age, all other correlations tested by Chi-Square test
[2] FIGO missing for 1 case in retrospective series
[3] Myometrial infiltration available for 215 women
[4]BMI available for 215 cases in retrospective and 39 in prospective series

3.1.2 Patients treated at Haukeland University Hospital not in the Hordaland cohort

This patient series consists of 130 women treated at Haukeland University Hospital with permanent address registered being outside Hordaland County or with a histological diagnosis of carcinosarcoma. In addition to the Hordaland 2001-2010 cohort these women were studied in the complete hospital based cohort treated at Haukeland University Hospital during the 2001-2010 decade.

The reason for not including carcinosarcomas in the analyses of the population based Hordaland cohort from 1981-2010 was that this histologic subtype was not available for the first part of the period studied. When the 1981-90 Hordaland cohort was collected, carcinosarcoma was not considered an entity classified as endometrial carcinomas but as sarcomas and thus not included in the population based cohort. During later years these tumours have increasingly been treated and classified as endometrial carcinomas[171], in line with national guidelines.[40]

Identification of cases and registration of clinical data was otherwise similar for the Haukeland University Hospital and the Hordaland Cohorts.

3.1.3 MoMaTEC trial

Molecular Markers in Treatment of Endometrial Cancer http://www.clinicaltrials.gov/ct2/show/NCT00598845 is a prospective study initiated at Haukeland University Hospital in 2001 including the 614 patients (Hordaland/Haukeland University Hospital) as described above, and from 2007 expanded with 9 centres actively recruiting additional 578 women; seven Norwegian hospitals (n=330) one Swedish- (n=109) and one Belgian comprehensive cancer centre (n=139). Patients primarily treated for verified endometrial carcinoma, including carcinosarcomas, were eligible for inclusion. In addition to the same clinico-pathological data collected for the Hordaland cohort, the histopathologic diagnosis from the routine preoperative biopsy or curettage histology reports available for 1166 patients, were classified as low risk versus high risk, the latter including all endometrioid grade 3, serous papillary, clear cell, carcinosarcoma and undifferentiated subtypes. This was in line with the study objective to evaluate prospectively a panel of biomarkers in relation to the standard pre- and postoperative clinicopathologic variables derived in a routine setting representing daily clinical practice. See Appendix for copy of inclusion and follow-up forms. Initially the FIGO 1988 classification was used. When this was changed in 2009 the registration forms were updated and data reclassified accordingly.

Compared with the other secondary Norwegian centres, the tertiary centres (Sahlgrenska, Leuven and Haukeland) enrolled significantly more high-risk patients with non-endometrioid histological subtypes, grade 3 differentiation, and advanced FIGO-stage as shown in Table 8. This is in line with the referral policies in the region studied.

In **Paper II** all patients enrolled in the MoMaTEC study until end of 2009 was investigated and **Paper IV** contains all patients enrolled through 2010.

Table 8 Characteristics for 1192 endometrial cancer MoMaTEC* trial patients at time of primary treatment related to including centre classified into three groups. P-values are based on Pearson's chi-square test.

Characteristics	**Haukeland n (%)**	**Norwegian Other n (%)**	**European Other n (%)**	**P-value**
Age				0.017
<66 years	314 (51)	166 (50)	101 (41)	
≥66years	300 (49)	164 (50)	147 (59)	
Menopausal status				0.064
Pre-/perimenopausal	72 (12)	32 (10)	16 (7)	
Postmenopausal	542 (88)	298 (90)	232 (94)	
FIGO 2009 classification				0.024
Stage I-II	507 (83)	293 (89)	203 (82)	
Stage III-IV	107 (17)	37 (11)	45 (18)	
Histological subtype				0.010
Endometrioid	489 (79)	286 (87)	195 (79)	
Non-endometrioid	128 (21)	44 (13)	53 (21)	
Histological differentiation[1]				0.002
Grade 1-2	395 (65)	250 (76)	167 (67)	
Grade 3	213 (35)	78 (24)	81 (33)	

[1]Missing information about grade in 10 cases

3.1.4 The Oslo cohort

This is a sample of women treated for primary endometrial cancer at Oslo University Hospital Ullevaal during 2004-2009 with available preoperative plasma samples. This cohort was used as a primary investigation cohort evaluating the serological marker GDF-15 in the study applying plasma from 466 women with endometrial carcinomas

recruited from 2001-2010 at Haukeland University Hospital as validation cohort. (**Paper III)**. The Ullevaal cohort had similar age and stage as those from Haukeland but with more differentiated tumours (p=0.03).

3.2. Treatment and follow-up

The main treatment component in endometrial cancer is surgical therapy including hysterectomy and bilateral salpingo-oophorectomy. If tumour infiltrates the cervix (FIGO stage II) radical hysterectomy with more extensive resection of parametrial/paracervical structures is performed (Wertheim-Meigs procedure).

Lymph node sampling along pelvic vessels has increasingly been recommended as part of complete staging and lately even para-aortic sampling for high-risk groups has become customary in many centres. During 1981-1990 systematic lymph node sampling was not routinely done in Norway, and only suspiciously enlarged nodes were biopsied. From 1997 pelvic lymph node sampling was formally recommended by the Norwegian gynaecological oncology guidelines.[89] Patients not suitable for surgical treatment have been staged by clinical examination, curettage and x-ray/CT-scan results and offered external radiotherapy and/or uterine brachytherapy.

Adjuvant radiation therapy was routinely recommended during 1981-2000; intravaginally if myometrial infiltration less than 50%, external pelvic radiation (45-50GY) if deeper infiltrating or poorly differentiated tumours. From 2001 vaginal radiation (brachytherapy) was no longer routinely offered as adjuvant treatment, and external pelvic radiation was restricted to deeply infiltrating poorly differentiated tumours, aneuploid or non-endometrioid tumours. During this last period, chemotherapy has been increasingly offered as alternative to radiation therapy. Patients from Sweden or Belgium were offered significantly more adjuvant radiation therapy for stage I and II, compared to Norwegian patients as shown in table 9.

Patients without residual tumour have been followed by gynaecologists either at the hospital responsible for primary treatment or by gynaecologists in private practice. The standard follow-up protocol has been 3-4 visits first year, two the second and yearly thereafter for five years. In addition to gynaecological examination, cytological smear from the vaginal vault was performed in patient not treated with radiotherapy, and for most yearly chest x-rays have been performed.

Table 9 Treatment given to 1192 endometrial cancer patients included in the MoMaTEC trial related to centre for primary treatment classified as three groups. P-values are based on Pearson's chi-square test.

Characteristics	**Haukeland n (%)**	**Norwegian Other n (%)**	**European Other n (%)**	**P-value**
Lymph node sampling				<0.001
Performed	474 (77)	257 (78)	125 (50)	
Not performed	140 (23)	73 (22)	123 (50)	
Lymph nodes removed				<0.001
< 10	130 (28)	85 (33)	12 (10)	
≥ 10	343 (73)	171 (67)	111 (90)	
Adjuvant primary treatment				
FIGO Ia				<0.001
no	317 (94)	177 (93)	103 (72)	
any[1]	20 (6)	14 (7)	41 (29)	
FIGO Ib/II				0.002
no	100 (67)	59 (59)	23 (39)	
any[2]	52 (34)	41 (41)	36 (61)	
FIGO III/IV				0.574
no	13 (15)	5 (14)	3 (8)	
any	76 (85)	31 (86)	35 (92)	

[1]Radiotherapy for 1/34 patients in Norwegian and 33/41 in non-Norwegian centres
[2]Radiotherapy for 49/93 in Norwegian and 35/36 in non-Norwegian centres

Patients were urged to take contact in between scheduled appointments if symptoms of recurrence (vaginal bleeding) occurred. Individual follow-up regimens were constructed for patients not radically treated.

Date of last follow-up was June 15th 2011 for the Hordaland Cohorts with a mean follow-up time for survivors of 17.5, 9.7 and 3.5 years for the three respective

decades studied and a mean for all patients 7.5 years (range 0.1-23.2). 191 patients died from endometrial cancer during the follow-up period.

For the MoMaTEC patients, the date of last follow-up was February 15th 2011, with mean follow-up time for survivors 32 months, range 0-96. 119 patients died from endometrial cancer during follow-up period.

3.3 Clinical and histopathological variables

Clinical data such as patient age, parity, menopausal status, height and weight at time of admission for primary treatment, as well as date and type of primary therapy was recorded. Also treatment of recurrent or progressive disease, location and time of recurrence and date and status of last follow-up including cause of death if deceased, were retrieved from patient records. Histopathological diagnosis and myometrial infiltration were retrieved from the routine pathological reports for final hysterectomy specimens, for the 1991-2010 Hordaland cohort and all MoMaTEC patients' preoperative biopsy/curettage report were also collected. During the 1981-1990 period, routine histology at Haukeland did not consistently include differentiation nor subtypes for endometrial carcinomas, and for these patients the histopathological features have earlier been revised according to the WHO criteria.[172] Vascular invasion was classified as present if invasion in more than one vessel or vascular space was seen. Upper quartile of mitotic count pr high power field (x400) hot spot area was classified as high, corresponding to $\geq$17 mitosis.

Myometrial infiltration was measured as the distance tumour invaded the uterine wall and recorded as either less than or equal or more than half of the myometrial thickness.

Ploidy analyses comprising the ratio of aneuploid DNA content to the amount of normal diploid cells were measured in primary tumours from hysterectomy specimens and were available for 566 patients from the Haukeland series.

Patients were originally staged according to FIGO 1988 criteria[62], and this was used in **Paper I-III** and **Paper V**. In 2009 the FIGO classification was officially changed.[63, 64] In the revised classification former IIA (tumour spread in cervical mucosa/glands) was classified as stage I according to depth of myometrial infiltration: IA if less than 50%, IB if equal or deeper than 50%. Positive abdominal cytology without any other metastatic lesions was no longer classified as IIIA. In addition IIIC (former: metastatic pelvic lymph nodes) were expanded as IIIC1: pelvic lymph nodes and IIIC2 para-aortic lymph nodes, summarised in table 5. For the ongoing MoMaTEC trial patients have been reclassified according to the FIGO 2009 criteria and used in **Paper IV**. For comparison see table 10.

Table 10 Comparison of 5-year survival for FIGO stages according to 1988 and 2009 criteria in the MoMaTEC cohort of 1192 patients.

Variable	FIGO 1988 n (%)	5-years survival	FIGO 2009 n (%)	5-years survival
FIGO I	876 (74)	93%	919 (77)	93%
FIGO II	112 (9)	85%	84 (7)	80%
FIGO III	147 (12)	58%	133 (11)	55%
FIGO IV	57[1] (5)	19%	56 (5)	19%

[1]One case with enlarged para-aortic metastasis originally classified as FIGO IVB changed to IIIC2

4. Methods

Formalin fixed paraffin embedded (FFPE) tumour tissue from both preoperative curettage and hysterectomy specimens have been collected and applied for immunohistochemical studies. Freshly frozen primary tumours from hysterectomy specimens were applied in the mRNA microarray investigations. Plasma samples were applied in immunoassay analyses. An overview of specimens applied and biomarkers studied in the different papers is given in figure 9 and table 11.

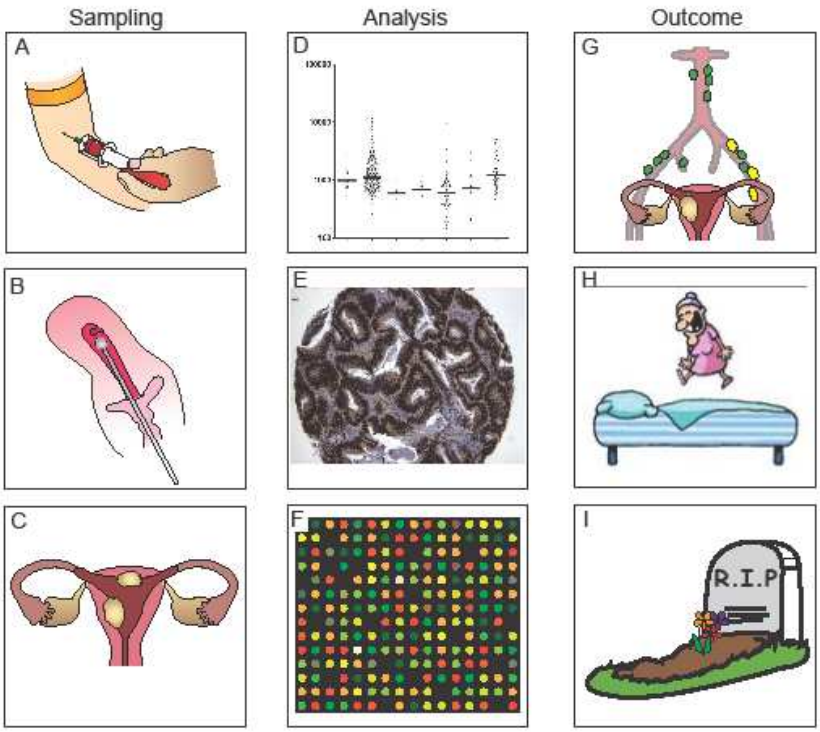

Figure 9 Overview of types tissues sampled, analyses performed and outcome studied.

A Blood samples **B** Curettage/preoperative biopsy **C** Hysterectomy tissue from operation **D** Plasma protein analysis **E** Immunohistochemistry **F** mRNA microarray **G** Clinicopathological features including lymph node metastases **H** Survival **I** Death

Table 11 Overview of the various patient series, specimen types, methods applied and biomarkers investigated in the different papers.

Article	Patient cohort n=patients	Tissue specimens	Tissue preparation	Biomarker	Method	Number specimen with evaluable data
Paper I	Hordaland 1981-1990 n=286	Hysterectomy	FFPE/ TMA[1]	Stathmin	IHC[2]	241
				AKT		245
				pAKT		228
		Curettage		Stathmin		175
	Hordaland 2001-2002 n=76	Hysterectomy		Stathmin	IHC	70
				AKT		69
				pAKT		70
			Freshly frozen	PI3Kinase-Score	mRNA MicroArray	76
Paper II	MoMaTEC 2001-2009 n=1076	Hysterectomy	FFPE/ TMA	Stathmin	IHC	477
		Curettage		Stathmin		818
Paper III	Haukeland 2001-2009 n=564	Blood plasma	Freshly frozen	GDF-15	Immuno-Assay	466
	Ullevål	Blood plasma		GDF-15		44
Paper IV	MoMaTEC 2001-2010 n=1192	Curettage	FFPE/ TMA	ER, PR, TP53	IHC	841
Paper V	Hordaland 1981-2010 n=1077	Clinico-pathological data only	-	-	-	-

[1]FFPE: Formalin Fixed Embedded Paraffin, TMA: Tissue Micro Array [2]IHC: Immunohistochemistry

4.1 Tissue MicroArray (TMA)

To gain high profile output and efficient investigation of the large number of samples derived from these patient cohorts, three 0.6 mm cylinder biopsies from each individual tumour specimen were transferred from the donor to a recipient block. A representative area of tumour of highest grade based on evaluation of haematoxylin and eosin stained slides were selected and punched out by using a custom made

precision instrument (Beecher Instrument, Silver Spring, MD, USA). These biopsies where mounted in recipient paraffin blocks and 5 µm sections prepared for subsequent immunohistochemical stainings. This method of producing tissue microarrays (TMA) have been described and validated in several studies.[138, 173, 174]

While curettage samples were collected from all centres participating in the MoMaTEC trial, TMAs from hysterectomy specimens were only generated in parallel for Haukeland patients. As shown in table 12, patients with inadequate tissue samples in the TMAs prepared from the curettage specimens, more often had non-endometrioid histological type and high histological grade, otherwise patient characteristics, including survival, were similar.

Table 12 Comparison of patient- and histopathologic characteristics according to availability of tumour tissue in TMAs for the 1192 MoMaTEC trial patients. Cases with representative tumour tissue from curettage available in TMAs were further studied by immunohistochemistry for potential biomarkers.

Characteristics	**IHC not performed n (%)**	**IHC performed n (%)**	**P-value**
Age ≥66years	152 (52)	459 (51)	0.8
Para ≥1[1]	239 (84)	744 (84)	1.0
Postmenopausal	261 (89)	811 (90)	0.6
BMI ≥25.0(kg/m^2)[2]	119 (62)	253 (64)	0.7
Myometrial infiltration ≥50%[3]	78 (29)	270 (34)	0.1
FIGO 2009 stage III/IV	47 (16)	142 (16)	0.9
Curettage histology non-endometrioid[4]	78 (28)	128 (14)	<0.001
Curettage differentiation grade 3[4]	102 (36)	211 (24)	<0.001
Hysterectomy histology non-endometrioid	78 (27)	147 (16)	<0.001
Hysterectomy differentiation grade 3[5]	112 (39)	260 (29)	0.002
Primary surgery curettage or debulking	15 (5)	30 (3)	0.1
Lymph node sampling not performed	90 (31)	246 (27)	0.3
Metastatic lymph nodes	22 (11)	83 (13)	0.5

Missing data [1]21 for parity, [2]602 for BMI, [3]136 for myometrial infiltration, [4]26 for curettage histology and differentiation, [5]8 for hysterectomy differentiation

4.2 Immunohistochemistry

Thin slides (5 µm) cut from TMA blocs made as described above were dewaxed with xylene/ethanol before antigen retrieval by microwaves and after peroxidase block (S2032 Dako Cytomation) incubated with antibodies according to optimized protocols as summarised in table 13. Tissue samples known to express the individual antibody were added as positive controls while omitting the primary antibody and using diluent only was used as negative control. The staining procedures were either done manually (AKT, Phospho-AKT and Stathmin in **Paper I**), or by using the DAKO Autostainer (no. 3400-9567) automated slide-processing equipment as described by the manufacturer (for Stathmin in **Paper II** and ER, PR and TP53 in **Paper IV**). The detection system used was EnVision+(K4006) DacoCytomation, with the enzyme labelled polymer of secondary antibody and DAB+ chromogen. Slides were finally counterstained with Haematoxylin (Dako S2020). For ER, PR and TP53 the protocols published earlier[138, 143], have been utterly refined and Stathmin staining was tested out and optimised under guidance and collaboration with the technician (G.L.H) and the senior pathologist (L.A.A).

Table 13 Survey of antibodies and immunohistochemical staining protocols used in this study.

Biomarker	Protein targeted	Antigen retrieval: Microwave	Primary antibody	Provider	Dilution	Incubation	Detection
Cell cycle regulator	Stathmin	10 min 750W 15 min 350W Citrate pH 6	#3352	Cell Signalling Technology MA, USA	1:50	60min RT[1]	EnVision+ Rabbit
Intracellular signal mediator	AKT	10min750W 15 min 350W Citrate pH 6	#4685	Cell Signalling	1:50	Overnight 4grC	EnVision FLEX+ Rabbit
Intracellular signal mediator	pAKT	10min750W 30 min 350W TrisEDTA pH 9	Ser437 #971	Cell Signalling	1:10	Overnight 4grC	EnVision+ Rabbit
Steroid receptor	ER	10min750W 15 min 350W TrisEDTA pH 9	ER50 M7047	Dako, Copenhagen Denmark	1:50	30 min RT	EnVision+ Mouse
Steroid receptor	PR	10min750W 15 min 350W TrisEDTA pH 9	PR 636 M3569	Dako	1:150	30min RT	EnVision+ Mouse
Cell cycle regulator	TP53	10 min 750W 15 min 350W TrisEDTA pH 9	TP53 M7001	Dako	1:1000	60min RT	EnVision+ Mouse

[1]RT : Room temperature

4.3 Evaluation of staining

Slides were evaluated blinded for patient characteristics and outcome by two of the authors (J.T and H.B.S) using a standard light microscope. A semiquantitative and subjective grading system described in several earlier publications[138,143,175], calculating a staining index (range 0-9) consisting of the product of staining intensity (0-3) and area of tumour with positive staining (0= no staining, 1= <10%, 2 = 10-50% and 3 >50%). ER, PR and TP53 staining were mainly expressed in nuclei, Stathmin and AKT in the cytoplasm, while Phospho-AKT was expressed both in nuclei and cytoplasm. In line with earlier studies[138], lower quartile was defined as the cut-off for loss of ER and PR, corresponding to staining index $\leq$3 and 0 respectively (**Paper IV)**. For TP53 (index $\geq$4, **Paper IV**) and Stathmin, AKT and Phospho-AKT (all indexes $\geq$6, **Paper I**) upper quartile was used as cut-off, similar to earlier publications.[96, 143] In **Paper II,** the overall staining intensity in hysterectomy specimens were stronger,

corresponding to staining index = 9 for the upper quartile, while for curettage the cut-off remained as ≥6. The staining intensity was not related to differences in fixation time neither for curettage nor hysterectomy specimens recorded for 126 random patients (p = 0.43 and p = 0.40 respectively, Pearson's Chi-square test).

Inter-observer reproducibility have been evaluated for ER, PR, TP53 (curettage) and Stathmin (curettage and hysterectomy specimens) by rescoring random slides blinded for previous scoring for 97, 104, 76, 168 and 88 cases yielding Kappa-values of 0.91, 0.88, 0.86, 0.72 and 0.86 respectively.

4.4 mRNA analysis - gene signature

Biopsies from representative part of primary tumours sampled at primary surgery from the hysterectomy specimens were snap-frozen and subsequent ground to powder in a mortar under liquid nitrogen. Tumour content was evaluated in adjacent haematoxylin stained frozen sections and confirmed to have tumour purity of more than 50% and for the majority > 80%. Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer protocol. Quality and yield of total RNA was assessed by agarose electrophoresis, Agilent Bioanalyser 2100 and spectrophotometry. Cy-3 labelled cRNA was hybridized on Agilent G4112F Whole Human Genome (4x44k). Oligo Microarrays with SurePrint Technology (www.agilent.com) and hybridization data were extracted and formatted as published.[176]

A multigene signature representing activation of the PI3K pathway was obtained from previously published expression data comparing cell lines transfected with PIK3CA (p119α) with GFP controls. The PI3Kinase signature included 495 genes that varied significantly in expression (Bonferroni-corrected 2-sided t-test P-value of 0.05) between control cells and the cells with PI3K pathway activity.[96] Expression data of these genes were in our data set normalised for each gene to a common mean and scaled to the same standard deviation. The PI3Kinase activation score was calculated,

subtracting the sum of expression values of genes significantly down-regulated in cells with *PIK3CA* activation (relative to cells with GFP controls) from the sum of expression values of genes up-regulated in these cells.[96]

4.5 Plasma analyses

As part of the MoMaTEC trial blood samples have been collected before primary treatment of participating women. EDTA-blood vials were centrifuged 2000 rounds per minute for 10 minutes and plasma stored at -80^{o} until analysed. Patients where blood samples were available (n=466) differed somewhat from the unsampled women by having higher parity, BMI, histological differentiation, and more radical surgical treatment including lymph node sampling as shown in table 14.

Table 14 Comparison of distribution of patient and histopathologic characteristics from 564 Haukeland patients with blood analysed for GDF-15(n=466) or no analyses performed (n=98).

Characteristics	**GDF-15 not performed n (%)**	**GDF-15 performed n (%)**	**P-value**
Age ≥66years	55 (56)	216 (46)	0.08
Para ≥1[1]	72(74)	397 (85)	<0.01
Postmenopausal	89 (91)	408 (88)	0.36
BMI ≥25.0(kg/m^2)[2]	43 (51)	258 (65)	0.02
Myometrial infiltration ≥50%[3]	23 (29)	169 (37)	0.17
FIGO 2009 stage III/IV	24 (25)	75 (16)	0.05
Curettage histology non-endometrioid[4]	23 (25)	79 (17)	0.10
Curettage differentiation grade 3[4]	33 (35)	112 (24)	0.03
Hysterectomy histology non-endometrioid	26 (27)	87 (19)	0.08
Hysterectomy differentiation grade 3[5]	43 (44)	153 (33)	0.04
Primary surgery curettage or debulking	20 (20)	11 (2)	<0.001
Lymph node sampling not performed	38 (39)	92 (20)	<0.001
Metastatic lymph nodes	4 (7)	45 (12)	0.22

Missing data [1]2 for parity, [2]83 for BMI, [3]29 for myometrial infiltration, [4]10 for curettage histology and differentiation, [5]5 for hysterectomy differentiation

GDF-15 analyses were performed by an immunoradiometric sandwich assay using a polyclonal, affinity chromatography-purified goat anti-human GDF-15 IgG antibody (R&D Systems, Minneapolis, MN). Analyses were performed in duplicate, blinded for clinicopathological data, in the laboratory where the assay have been developed at the Department of Cardiology and Angiology, Hannover Medical School, Hannover, Germany. This assay has a detection limit of 20 ng/l, an intra-assay imprecision of $\leq$ 10.6% and an inter-assay imprecision of $\leq$ 12.2%. The method is further described by Kempf et al.[177] All endometrial cancer samples were analysed in same run, reducing inter-assay variation.

4.6 Statistical methods

Statistical analyses have been performed by the statistical program PASW 18 (Predictive analytics SoftWare Statistics version 18.0, IBM, New York, USA). Categorical associations were assessed by Pearson's chi-square test, substituted by Fischer's exact test if estimated expected counts less than 5. Linear-categorical comparisons have been performed by Mann-Whitney test. Receiver operating characteristics ROC-curves were constructed by plot of 1-specificity (false positive rate) against sensitivity (true positive) test values. Differences in disease-specific and overall survival, censoring patients alive at last date of follow-up, were estimated by log-rank (Mantel-Cox) test, while univariate analyses of time to death were performed by the Kaplan-Meier method. Variables with significant impact in univariate survival analyses were further evaluated by log-minus-log plot to decide if these could be incorporated in the Cox' proportional hazard regression model, in addition several categories were tested for interaction. Unadjusted and adjusted multivariate hazard ratios (HR) were thus estimated. Binary logistic regression was used to evaluate odds ratios (OR). All statistical tests were two-sided and considered statistical significant if $p<0.05$ with the exceptions of testing the one-sided hypothesis that overexpression of immunomarkers correlated with measures of PI3Kinase-score (**Paper I**) and that high

GDF-15 in the small pilot Ullevaal cohort correlated with metastatic disease and poor survival (**Paper III**). Cut-off values for the different categorizations were based on tertiles or quartiles, considering the frequency distribution for each marker, size of subgroups and number of events, merging groups with similar survival.

4.7 Approvals

This study has been approved by the Norwegian Data Inspectorate (961478-2), the Norwegian Social Science Data Services (15501) and the local Institutional Review Board (REKIII nr. 052.01). The MoMaTEC trial have been registered at the Clinical Trials Website (NCT00598845).[178] The funding source has had no influence neither regarding evaluation of results nor in writing of the papers or thesis.

5. Main results

Paper I

In this study we found the immunohistochemical Stathmin expression in tumour from hysterectomy specimens to be strongly correlated with aggressive clinicopathological characteristics such as high histological grade, deep myometrial infiltration, vascular invasion, high mitotic rate, loss of estrogen and progesterone receptors and poor recurrence-free as well as disease-specific survival. Analysing endometrioid tumours separately, those over-expressing Stathmin demonstrated a similar significant poor patient prognosis. Stathmin overexpression in curettage specimens correlated significantly with high histological grade, high mitotic rate, aneuploidy and estrogen receptors loss, but showed weaker correlation with survival. Neither for AKT nor phospho-AKT was there any clear correlation with clinicopathological tumour features nor survival. High PI3Kinase activation score was significantly associated with overexpression of Stathmin but not with phospho-AKT or AKT expression.

Paper II

Immunohistochemical overexpression of Stathmin was detected in 37% of the curettage and in 18% of the hysterectomy specimens investigated. Stathmin overexpression in curettage as well as hysterectomy specimens were highly correlated and significantly associated with non-endometrioid histology, high grade and aneuploidy. In addition high Stathmin staining in curettage specimens also correlated with lymph node metastasis and high FIGO stage. Stathmin analysis in curettage samples was an independent predictor of lymph node metastases also when adjusting for the available preoperative histological subtype and grade. High Stathmin expression both in curettage and hysterectomy specimens was associated with poor disease-specific survival. Endometrioid tumours with overexpression of Stathmin in

curettage specimens correlated significantly with poor prognosis and a similar tendency was seen when analysing the hysterectomy specimens. High Stathmin expression, in curettage as well as from hysterectomy specimens, was a significant predictor of poor survival both in univariate and in multivariate Cox analysis, adjusting for age, FIGO stage, histological subtype and grade.

Paper III

Median plasma GDF-15 concentration from the endometrial cancer patients was, similar to the ovarian cancer group, significantly higher than levels measured in healthy pre- and premenopausal women or in women with benign or borderline ovarian tumours. In the large validation cohort of endometrial carcinomas, high plasma GDF-15 was significantly associated with FIGO stage III/IV disease, non-endometrioid histology, high grade, older age, postmenopausal status and lymph node metastases. Even when adjusting for age and preoperative histological risk classification based on preoperative biopsy, plasma GDF-15 independently predicted increased risk of lymph node metastasis. High GDF-15 was also an independent predictor of poor disease-specific and recurrence-free survival.

Paper IV

Focusing on the well-established immunohistochemical prognostic biomarkers estrogen receptor, progesterone receptor and the tumour suppressor TP53 in this paper, we validated their prognostic value when tested in preoperative curettage specimen in a large prospective multicenter setting. 18% of tumours were double negative for estrogen- and progesterone receptors (ER-/ PR-) while 23% over expressed TP53. Pathologic expression of each marker correlated significantly with high age at diagnosis, high FIGO stage, non-endometrioid histology, high grade, metastatic nodes and poor prognosis. Double negative ER-PR also correlated with

deep myometrial infiltration. 72% of patients were subjected to nodal sampling, of which 12% had metastatic nodes. Double negative receptor status independently predicted lymph node metastasis adjusted for preoperative curettage histology. A combination of high-risk preoperative histology and double negative hormone receptor status was the strongest predictor of metastatic nodes. ER-PR negative status independently predicted poor survival adjusted for age, FIGO stage, histological type, grade and myometrial infiltration. Within the low-risk endometrioid grade 1-2 tumours, ER-/PR- status independently predicted recurrence. For patients not subjected to lymph node sampling, double negative ER-PR status predicted poor survival even when adjusted for age, FIGO stage, myometrial infiltration histological type and grade. Even for the lymph node negative endometrioid tumours, ER-PR negative status influenced survival independent of tumour grade.

Paper V

In line with the increased incidence in Norway, the number of patients treated from Hordaland County increased from 286 (1981-90) through 307 (1991-2000) to 484 (2001-2010). The main changes in treatment these three decades were a significant increase in routine pelvic lymphadenectomy performed from 0% through 9% to 77%, adjuvant radiotherapy declined from 75% through 48% to 12% while adjuvant chemotherapy increased from 0% through 3% to 9%. During this period body mass index increased, the proportion of nulliparous women decreased and disease stage at diagnose became significantly less advanced. Disease-specific survival as well as overall survival increased significantly during these 30 years. Adjusting for age, parity, BMI, FIGO stage, myometrial invasion and histological subtype and grade; treatment period had independent prognostic significance in multivariate analysis, but was no longer significant when adjusting for type of surgical treatment.

6. Discussion

Endometrial cancer is available for histopathological investigation by pipelle biopsy or dilatation and curettage. The information regarding histologic subtype and differentiation grade is used for risk assessment and planning of surgery including lymph node sampling. Still, the histopathological information based on curettage tissue is discordant with the final diagnosis based on investigation of the hysterectomy specimen regarding subtype in 67 to 83%[179, 180] and grade in 36 to 63%.[41, 44] In many cases, curettage tissue is insufficient for reliable determination of type and grade. The prediction of prognosis by preoperative histologic information has rarely been addressed in the present literature. Thus, reliable biomarkers applied in preoperative curettage or blood samples will potentially be helpful tools to differentiate between low- and high-risk patients for lymph node metastasis and poor survival.

6.1 Discussion of materials

6.1.1 The patient cohorts

The Hordaland cohort

The Hordaland cohorts (**Paper I and V**); the first decade 1981-90 has been verified to contain 94% of patients with endometrial cancer from Hordaland County (Hordaland comprising 10% of the total Norwegian population) by crosschecking data from the compulsory Norwegian Cancer Registry with the patients retrieved from the hospital records. The identification and follow-up of patients from the two latter decades has been performed by similarly checking hospital files and the Cancer Registry for follow-up data. The age distribution, incidence and stage distribution of endometrial carcinoma in Hordaland is paralleled in Norway in general[2], thus our cohort is considered representative for Norwegian endometrial cancer patients during the time span investigated.

The MoMaTEC cohort (**Paper II, III and IV**)

Haukeland, being a tertiary cancer centre, treat patients with significantly more aggressive subtypes and higher FIGO stage than several other Norwegian hospitals. Similarly, the Swedish and Belgian cancer centres recruited patients with similar characteristics as those from Haukeland, thus the whole MoMaTEC cohort is to some extent enriched for aggressive tumours. Still, the percentage of positive lymph nodes is 12% and within the range reported from other studies: 13.3% from the Mayo clinic[181], 10.8% in the ASTEC trial[79] and 13.4% in the Italian study by Panici and co-workers.[78] The selection criteria for sampling were uniform in seven Norwegian centres: sampling in principle all patients, but omitting those with severe co-morbidities or if hyperplasia was the preoperative diagnosis. Loeven and Ullevål omitted sampling also in small Grade 1-2 tumours (< 2cm) with <50% myometrial infiltration. Sahlgrenska sampled only intermedium risk patients defined as having only one of the following risk factors; >50% myometrial invasion, TP53 positivity, aneuploidy or Grade 3 differentiation. This is reflected in the lower percentage of patients sampled amongst the non-Norwegian centres (see table 9). Mean number of nodes sampled was also significantly different between these hospital groups; 25 for the non-Norwegian compared to 15 for the Norwegian centres, $p<0.001$, still the frequency of metastatic nodes were not significantly higher 16.8% versus 11.5%, $p=0.095$. The overall mean and median numbers of nodes sampled (16 and 14, respectively) is similar to the ASTEC trial (median 12) but lower than the Panici trial (median 26 pelvic, 30 total).[78, 79]

Our study protocol included and recorded pelvic lymph node sampling. We have no systematic information whether additional para-aortal sampling was performed, thus the studies evaluating biomarkers as predictors of metastatic lymph nodes (**Paper II, III and IV**) can not necessarily be applicable in relation to para-aortal metastasis.

For the Norwegian centres, the routines for administration of adjuvant treatments were consistent, while the non-Norwegian centres provided significantly more radiation therapy. When evaluating survival effects of adjuvant treatment in our studies, adjuvant therapy showed no independent impact. This appears to be in line with several studies not finding any survival benefit from radiation therapy for early-stage endometrial cancer.[182]

We have information about the proportion of patients included in the MoMaTEC trial for 8 of 10 participating centres recruiting 1149 of the 1192 included patients. In total 1499 women have been treated for endometrial cancer at these centres, thus 77% of all potentially eligible patients were included and clinical and follow-up information provided, ranging from 25 to 38% from the small hospitals (Tønsberg, Haugesund and Førde) while the large centres ranged from 40 to 99% (Ullevål, Trondheim, Sahlgrenska, Leuven and Haukeland).

In sum, the distribution of clinical characteristics for the patient series from the different including hospitals is relatively homogenous, supporting that results from our studies most likely will be valid be for endometrial cancers from Norway, Scandinavia and Northern Europe dominated by a Caucasian population.

Retrospective versus prospective series

The two first cohorts from the Hordaland County were retrospectively collected, while the last cohort as part of the MoMaTEC trial was prospectively recruited. Thus, results from exploratory investigations of biomarkers from the early series (**Paper I**) and earlier studies[138, 143] could now be validated in the larger MoMaTEC multicentre trial (**Paper II and IV**).

Since histologic subtyping was not routinely performed in the early periods studied, histopathological revision was performed for the Hordaland 1981-1990 series, while the diagnosis from the routine pathology reports were considered valid and applied for the prospective validation series, also aiming to investigate if biomarkers could

contribute additional information to the diagnostic tools applied in a standard everyday basis for clinical decision making.

6.1.2 Tissues/samples available

Curettage versus hysterectomy specimens

Hysterectomy specimens have only been collected from Haukeland (**Paper I and II**), while curettage/preoperative biopsy specimens have been collected from all centres. Using curettage/biopsy specimen rather than hysterectomy specimen for analyses of biomarkers has the potential advantage that they may be used for preoperative risk analyses and aid in planning and stratification of treatment. The disadvantage is that compared to final hysterectomy specimens, biopsy material is often sparse, representing more superficial parts of the tumours. Zonal differences that may be of importance, such as the invading and more deeply invasive front of the tumour, may be less accessible for investigation using curettage tissue.

Freshly frozen tissue

For **Paper I**, freshly frozen tissues from 76 patients have been used for mRNA expression analyses**.** These patients did not differ significantly from the patients from the 1981-90 Hordaland cohort, as seen in table 7. However, one should be aware of a potential enrichment for high risk cases in studies aiming for >80% of tumour purity in the inclusion criteria. Analysing a larger set of 273 freshly frozen tumour biopsies from the MoMaTEC series, demonstrates a significantly higher proportion of non-endometrioid, high grade tumours in samples with >80% tumour purity.[183]

Blood samples

For the preoperatively collected blood samples (**Paper II**), we see a different potential selection bias; significantly more of those without blood samples available for investigations were nulliparous, with normal body weight with high grade tumours not radically treated by surgery, while FIGO stage was not significantly different.

Thus, the study population appears to some extent to be enriched for patients with favourable risk factors.

TMA specimens

For curettage specimens, we find that tissues from patients with non-endometrioid and high grade lesions were significantly less often representatively mounted in the TMA blocs for immunohistochemical evaluation (see table12). There was no correlation between parity or type of surgical treatment and whether tissues were available for analysis in TMAs or not. A reason for this finding may be that non-endometrioid tumours are arising in an atrophic and more scant endometrium[184], yielding more scarce material for biopsies than the endometrioid tumours from an abundant hypertrophic endometrium. Similarly, high grade tumours may be more prone to necrosis also leaving less viable tissue for TMA processing. The overall availability of 841 specimens analysed is still relatively high, representing 75% of all patients, thus even the rarer non-endometrioid subtypes and high grade endometrial lesions are present in 29% and 13% of the cases respectively, permitting analyses to be valid also for these categories. Former biomarker studies of curettage specimens regarding lymph node metastasis or survival have had non-endometrioid subtypes present in 8.9% of 236 tumours analysed[143], 14.9% of 134[164], 18.4% of 136[162], 20% of 76[65] and 27% of 125.[163] Our relatively high proportion of non-endometrioid tumours may be related to the inclusion of carcinosarcomas representing 4% of our study population, often being excluded in earlier studies.[143]

6.2 Discussion of methods

6.2.1 Immunohistochemistry applied in curettage tissue

We have used antibodies that from earlier studies in our centre have shown good correlation of expression between curettage and hysterectomy specimens: TP53 ($p<0.0001$)[143], ER and PR ($p<0.001$).[138] P16, although prognostically relevant in earlier analysis, did not show a similar strong statistical significant correlation

between expression in curettage and hysterectomy specimens (p=0.06)[143], one reason for not investigating this marker in the present studies.

Due to our recent data suggesting the importance of PI3Kinase activation in aggressive endometrial cancers[96], we also wanted to investigate new, candidate biomarkers reflecting PI3Kinase activation in hysterectomy specimens from a retrospective cohort (AKT, phospho-AKT and Stathmin in **Paper I**). We found a correlation between Stathmin overexpression, PI3Kinase activation and poor prognosis, but none of the other investigated markers were significantly correlated with PI3Kinase activation or prognosis. Stathmin was further investigated in curettage specimens and found to be a marker for poor prognosis. Also, further investigations in the prospective cohort of hysterectomy as well as curettage specimens, validated the association between Stathmin expression and phenotype (**Paper II**). High Stathmin expression was significantly correlated with poor prognosis based on investigations of curettage and hysterectomy specimens, both p-values <0.001, for the retrospective series, and p=0.002 in the prospective series.

Fixation time is often different for hysterectomy versus curettage specimen, potentially influencing performance of immunohistochemical investigations. We have evaluated this for Stathmin; 126 randomly selected corresponding curettage - hysterectomy sample pairs. The fixation time was significantly shorter, median 1 day for curettage as compared to 6 days for hysterectomy specimen, but there were no correlation with fixation time and immunohistochemical expression of Stathmin for neither of the specimens. This marginal effect of fixation time on staining intensity is in line with other immunohistochemical staining studies.[185]

6.2.2 Tissue MicroArray (TMA)

We have used TMAs for all immunohistochemical investigations in our studies (**Paper I, II and IV**). TMA is a good method of high output analysis with the benefit of investigating large numbers of samples at the same time. Testing all or most of a

series investigated for the same antibody in one run reduces potential variability in the staining technique. Selecting representative tissue for TMA preparation aiming for the least differentiated and probably most aggressive component of the tumour will potentially not reflect all of the biologic diversity in the tumour.[186] To increase representativeness and reduce case drop-outs, we included three 0.6 mm cores from each tumour sample, in line with most recommendations.[174]

Storing time of the formalin fixed paraffin embedded tissue blocks has also been shown to reduce staining intensity for several antibodies.[185] Even for our prospective series (initiated 2001), the tissues have been stored variable amount of time before TMA was processed, starting in 2008. Testing for the influence of storing time using 3-year intervals showed no cumulative trend change in staining scores for any of the four investigated biomarkers ER, PR, TP53 or Stathmin ($p>0.05$ for all).

Although TMA is very useful in an investigational setting, markers explored using this technique will have to be validated in full sections before applied in a clinical setting.

6.2.3 Immunohistochemistry

Morphological characteristics of tissues are the cornerstone in histopathologic diagnostics, but immunohistochemistry (IHC) may aid in the detection of biologically relevant functional changes in tumours. Immunohistochemical staining procedures involves many steps; deparaffinization, epitope demasking through antigen retrieval, antibody dilution, incubation length and temperature as well as final antibody detection and counterstaining, each with potential pitfalls. We have used stringent protocols developed in the research laboratory over several years, and for TP53, ER and PR they have been firmly established.[138, 143] For Stathmin, AKT and phospho-AKT test stainings were performed according to manufacturer's suggestions exploring different buffers during microwave antigen retrieval as well as different dilutions of antibody, temperature and length of incubation. Stainings were evaluated against tissues known to express and not express the relevant antigen, as well as

substituting the antibody with diluent only, as negative control. The final protocols are summarized in table 13.

6.2.4 Cut-points for staining indices

To evaluate the stainings, a semiquantitative score incorporating both staining intensity and area of staining was used. This scoring system was developed in our research laboratory[175], and has been used for several antibodies and in several tumour types.[187, 188] Our scorings were performed blinded for the patients' clinical and outcome data. When evaluating cut points, the frequency distribution for each marker was considered in relation to the size of subgroups and number of events in each category. Groups with similar survival were merged. In line with former studies, lower quartile for ER and PR and upper quartile of TP53 expression were considered pathologic.[138, 143] For ER, the lower quartile included staining index 0-3, while for PR, all specimens in lower quartile had no nuclear staining at all (Index=0). When determining cut-points for IHC stainings it is generally recognized that this has to be evaluated at each centre according to actual antibody used and population studied. This appears to be in line with what is applied for breast cancer, where estrogen receptor testing has been performed for several years in a routine clinical setting. The definition of receptor negative tumours is also not restricted to only staining index = 0. There are large studies using 10% of cells staining positive as upper limit for receptor negativity[189], while the joined guidelines of the American Society of Clinical Oncology and the College of American Pathologists now recommends a limit of 1% tissue with nuclear staining.[190] The latter limit increasing the proportion of ER positive tumours is chosen in order not to miss patients with ER positivity who could possibly benefit from the relatively non-toxic tamoxifen treatment.

For Stathmin, the upper quartile corresponded to index $\geq$6 in the curettage samples and index =9 in the hysterectomy specimens in the largest prospective series, while for the retrospective series the upper quartile correspond to index $\geq$6 as cut-off value for both specimen types. The explanation for this is not clear. The prospective series were stained during the same run at the same day for curettage and hysterectomy TMA slides, still the hysterectomy specimens appeared to generally have significantly

higher staining indices. As already mentioned, fixation time did not significantly influence staining indices. Since staining is higher in non-endometrioid and high grade tumours, differences in the proportion of these for the two TMA series could be another factor. However, these were not overtly different: for the curettage specimens the proportion of non-endometrioid cases and high grade endometrioid tumours were 15.7 and 13.3% while for the hysterectomy specimens these were 17.8 and 13.9% respectively. Still, the TMAs from hysterectomy specimens may contain a selection from more undifferentiated tumour parts than the curettings. These differences in cut-offs illustrate that the process from the exploration and investigation of a new antibody until an optimal staining procedure and cut-off limit for use in clinical practice is developed, is cumbersome and will need to involve many validation steps. This can be illustrated by the timeframe from the first investigations of the HER2/neu oncogene in 1984 until the American Society of Clinical Oncology and the College of American Pathologists published standardised joint guidelines for HER-2 testing in breast cancer in 2007.[160]

6.2.5 Plasma analyses

Analyses from the endometrial cancer patients (both the Oslo and the Haukeland cohort) were run in duplicate and all samples in same run. GDF-15 has been analysed from preoperative plasma samples collected from 2001 through 2009. Potentially different preanalytic storage time could influence the measurement, however, correlating year of diagnosis with proportion of high GDF-15 levels showed no cumulative trend change ($p>0.05$).

6.2.6 mRNA gene signature analysis

Microarray analysis, yielding signature scores for different signalling pathways, is a powerful tool in cancer research. It may identify potential new targets for treatment in aggressive tumours, defined by specific gene expression patterns that potentially may benefit from specific targeted therapies. Such microarray studies involve complex bioinformatics and integrated analyses, which had been performed prior to the present

study.[96] In the present thesis we have applied these data regarding activation of one such gene signature, tested in 76 freshly frozen primary tumours in relation to the protein expression of Stathmin, AKT and phospho-AKT to investigate which protein might best reflect the signature (**Paper I**).

6.3 Discussion of results

6.3.1 Improved prediction of lymph node metastasis

Lymph node metastasis is a highly significant marker for poor survival in endometrial cancer. Therefore it has been incorporated in the surgical staging as a separate entity in the FIGO classification.[64] In a recent publication comparing FIGO 88 versus FIGO 09 classification[191], patients with lymph node metastasis FIGO 88 stage IIIC had 5-year disease specific survival of 59% and according to the FIGO 09 criteria 60% if only pelvic nodes were involved (FIGO IIIC1) and 53% if aortic nodes were positive (FIGO IIIC2). Still, systematic pelvic lymph node sampling has not proved to increase survival in randomised trials.[78, 79] Sampling increases operation time and surgically related morbidity, such as postoperative ileus and deep-vein thrombosis with a RR 1.23 (95% CI 1.04-1.45) as well as the risk of lymph cysts or lymph oedema in the legs with RR 8.39 (95% CI 4.06-17.33).[80] When only 12% of sampled patients have positive nodes as in our study, the sampling of the vast majority (88%) may contribute to increased morbidity with no benefit for these patients.

Depth of myometrial infiltration, histological subtype as well as histological grade has all been found as independent predictors of poor prognosis.[49, 70, 153, 155] These factors can be combined in different ways for risk stratification. The Mayo clinic defines patients as low-risk if endometrioid Grade 1-2 histology and <50% of the thickness of the myometrium is invaded by tumour cells.[51] This is in line with risk stratification presently used in Norway[40] as well as by the ASTEC-group.[182] A retrospective cohort study by Convery and co-workers confirmed that for this low-risk group, the prevalence of metastatic nodes was very low, 2% of those with sampling performed,

and neither recurrence- nor progression-free survival were significantly influenced by lymphadenectomy.[192]

The results from the studies of Mariani and Convery are based on intraoperative frozen section assessment of histopathology including depth of myometrial infiltration. Many centres do not have such pathology service as readily available, and thus will have to rely more on gross inspection of infiltration depth and preoperative assessment of histopathology. Applying preoperative curettage histology, the low-risk group defined as endometrioid histology grade 1-2, identified a patient subset with 8% risk of lymph node metastasis in our MoMaTEC population. Taking into account the information regarding ER-PR status does not alter this 8% risk for lymph node metastasis, even when only ER-PR positive endometrioid grade 1-2 tumours were defined as a low-risk group. However, for the high-risk group, preoperative diagnosis of non-endometrioid or endometrioid grade 3 histology, ER-PR negative tumours had a significantly higher frequency of metastatic lymph nodes: 29% as compared to 18% for ER-PR positive high risk histology patients. In comparison, high Stathmin expression in combination with high-risk preoperative curettage had a 26% risk of metastatic lymph nodes.

We have not found TP53 to be of independent value in predicting lymph node metastasis. This is in contrast to the study from Mariani and colleagues where TP53 was an independent predictor when adjusted for histological subtype or grade as well as the apoptosis marker BCL-2.[65]. Their study is smaller, using a case-control design of 82 patients as compared to our 605 patients with available data from immunohistochemical staining and lymph node sampling. Contrasting our study, they did not include hormone receptor status in their investigation, which also may have contributed to the difference. We found ER-PR to be a stronger predictor than TP53, thus TP53 was not of independent predictive significance for lymph node metastasis. The same applies to our series if we simultaneously add Stathmin and ER-PR status to the logistic regression; Stathmin looses its independent predictive value for lymph node metastasis contrasting double negative ER-PR with the significant OR of 2.32

95% (CI 1.28-4.21) adjusting for curettage risk groups. Thus, based on our results, aiming to improve prediction of metastatic nodes in a routine setting, we would recommend testing for ER-PR in preoperative specimens and opting for lymph node sampling if preoperative histology is non-endometrioid or endometrioid grade 3 or double negative for hormone receptor status.

Hormone receptor status has been routinely applied in the breast cancer diagnostics for several years. Thus, pathology laboratories have thorough knowledge and experience with these stainings shown to be robust and have high reproducibility. These have now been validated in a prospective multicentre setting regarding endometrial carcinoma (**Paper IV**).

6.3.2 Immunomarkers predicting survival

Several immunomarkers tested in hysterectomy specimens have been reported to predict survival. Those most consistently reported with independently prognostic value are ER, PR and TP53.[138, 161, 163] These markers have shown to be of importance in many other cancer types as well, notably breast and ovarian cancers.[190, 193, 194]

Based on the potential targetable nature of the PI3Kinase pathway often elevated in aggressive endometrial cancers, we explored a panel of immunohistochemical markers for their possible applicability to detect PI3Kinase signalling pathway activation. We have investigated AKT, phospho-AKT (pAKT) and Stathmin. The role of phospho-AKT staining as a prognostic marker has been explored in other cancer types, such as ovarian[195, 196], breast[197, 198] and cervical cancer[199], with conflicting results: only two of these studies, those of Guo and co-workers and Wu and collaborators, showed an independent value in relation to survival.

Previous studies of endometrial cancers have found the immunohistochemical

expression of phospho-AKT to vary considerably, from 40%[200] to 100%.[201] Likewise, the relation to clinico-pathologic phenotype is inconsistent, as one study reported a correlation between high phospho-AKT and deep myometrial infiltration,[202] while two others did not find any association with clinical or pathological factors.[203, 204] In our study (**Paper I**), we found no correlation of phospho-AKT neither in relation to clinico-pathological characteristics nor survival. None of those studies that have investigated phospho-AKT in endometrial cancer have demonstrated this as an independent prognostic factor, in line with our findings.

The fact that we find that phospho-AKT staining is not related to activation of the PI3Kinase signalling level at the mRNA level (**Paper I**), supports that phospho-AKT may be a reliable pathway marker under highly controlled situations, as found in cell culture studies, but that the available reagents do not work well in routinely collected clinical specimens.[118]

For AKT immunohistochemical staining, no correlations with clinico-pathological factors and survival have been reported from several studies investigating breast-, gastric- and bronchial cancers.[205-207] Our study of endometrial carcinomas adds to this general impression that AKT is not valuable as a cancer prognosticator. Also, we find that AKT staining is not related to activation of the PI3Kinase signalling level at the mRNA level (**Paper I).**

Stathmin has been found to correlate with aggressive clinico-pathological features and poor prognosis in several cancers[115] as breast-[118, 208], ovarian-[209] and cervical cancers.[210] One study supported Stathmin as a surrogate marker of PI3Kinase pathway activation, and of independent prognostic value.[96] In **Paper I** we found high expression of Stathmin in hysterectomy specimens to correlate with high PI3Kinase mRNA activation score as well as being an independent predictor of poor survival. In **Paper II** we validated high Stathmin expression in tumour samples from

hysterectomy specimens to be an independent prognosticator with HR of 1.68 (95% CI 1.13-3.35) adjusted for age, FIGO stage, histologic type and grade. Another study exploring biomarkers in paired primary tumour versus biopsy from recurrent lesions, demonstrated Stathmin expression in the primary tumour to be significantly changed (defined as $\geq$ 2 step change in staining index) in the recurrent lesion in 38% of cases, although expression status in the metastatic lesion in itself, was not correlated with survival.[211] Currently, we have not found any other studies published exploring Stathmin in endometrial cancers.

6.3.3 Immunohistochemical analyses in curettage specimen as prognostic markers

Markers in curettage specimens consistently reported to add prognostic information are immunohistochemical stainings for estrogen receptor (ER), progesterone receptor (PR) and TP53.[138, 143, 162, 163, 201] These are predominantly retrospective studies with fewer patients included than in the present studies. The study of Oreskovic and colleagues[162] included 136 patients and found curettage TP53 of independent prognostic value whereas ER and PR were not. Engelsen and co-workers found TP53 and hormone receptors to have independent significance in their study of 236 patients; TP53 with a HR of 2.8 (95%CI 1.5-5.6) while ER or PR each had a HR of 2.6 (95%CI 1.3-5.4) when investigated simultaneously in multivariate analysis.[138] The study by Steinbakk and co-workers[201] found TP53 combined with p21 and Survivin to be independent prognosticators of survival in their cohort comprised by FIGO stage I-II tumours; however ER or PR was not investigated.

In our larger prospective MoMaTEC trial (**Paper IV**) we find double negative ER-PR to have independent significant prognostic value and an HR 1.99 (95% CI 1.12-3.53) for disease specific death when adjusting for age, FIGO stage, myometrial infiltration, endometrial subtype and grade, while TP53 lost its independent prognostic

significance.

In **Paper II** we found that high curettage expression of Stathmin adjusted for age, FIGO stage, histologic type and grade, independently predicted poor survival with an adjusted HR of 1.68 (95% CI 1.05-2.67). This HR remains unaltered even if ER-PR is added as a factor in this multivariate analysis. Using results from Stathmin expression from hysterectomy specimens still leaves Stathmin with an HR of 2.26 (95% CI 1.22-4.21) when ER-PR is included.

Using the risk stratification incorporating subtype, grade and myometrial infiltration based on final histologic evaluation of the hysterectomy specimen, being a similar approach as applied in the ASTEC radiation trial[182], we see a significant worse survival in the intermediate risk group if either Stathmin expression is high (p=0.025) or ER-PR expression is lost (p=0.009) and similar for the high risk group with ER-PR loss (p=0.009) as illustrated in figure 10.

As shown in **Paper IV,** loss of hormone receptor expression independently predicted poor prognosis in patients not subjected to lymphadenectomy, in patients with endometrioid tumours and negative nodes, and even within the most favourable group of endometrioid grad 1-2 tumours.

Thus, both hormone receptor status as well as Stathmin expression tested in preoperative tissue specimens independently identifies patients with poor prognosis even adjusted for histological subtype and grade. This information may be available before initiating primary surgery and adjuvant treatment and clearly has a potential of tailoring treatment according to more refined risk stratification.

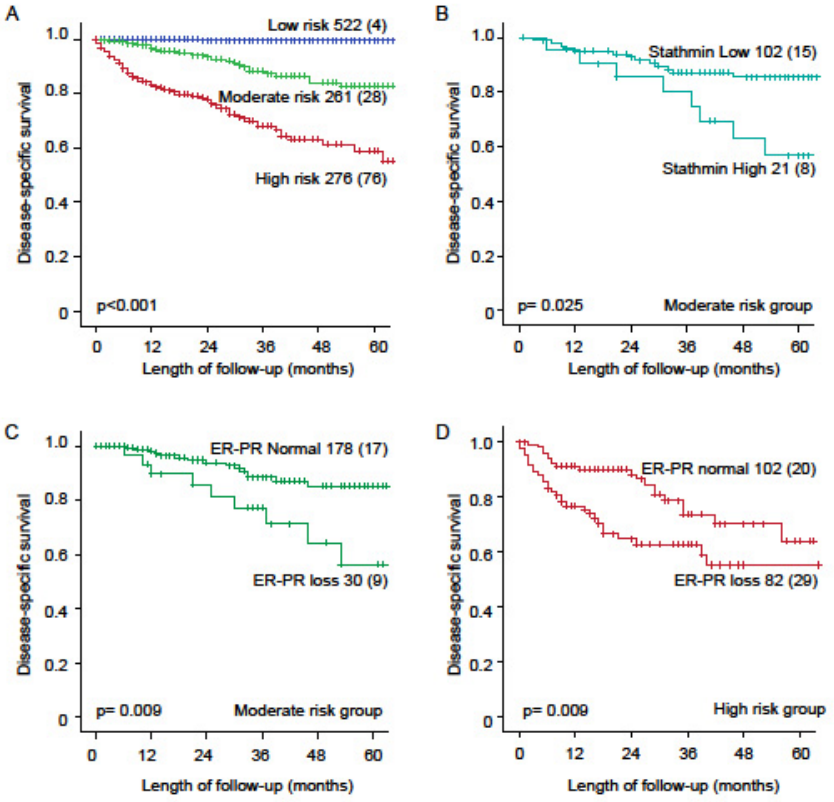

Figure 10 Disease specific survival related to risk groups and biomarker expression. A 3 Risk groups B Stathmin expression in moderate risk group C ER-PR expression in moderate risk group and C ER-PR expression in high risk group.

Low risk (blue): Grade 1-2 <50% myometrial invasion (MI)
Moderate risk (green): Grade 1-2 > 50% MI or Grade 3 > 50% MI
High risk (red): Grade 3 >50% myometrial invasion and all Non-endometrioid tumours

6.3.4 Biomarkers for treatment stratification

Stathmin known to be participating in the regulation of microtubules, could potentially be related to the effects of microtubule drugs such as Taxanes. Breast cancer cell-line studies indicate that high Stathmin expression correlate with poor effect of microtubule-acting drugs and that repressing Stathmin counteracted this and sensitise cells to Vinblastine and Paclitaxel.[114, 212] mRNA expression of Stathmin in pre-treatment biopsies from lung cancer patients demonstrated a significant shorter progression free survival in patients receiving Vinblastine.[213] In ovarian cancer pre-treatment biopsies, high mRNA expression of Stathmin likewise demonstrated worse progression-free survival in patients treated with Taxanes in combination with Platinum.[209] In contrast, another study of clear cell ovarian cancers did not find such survival difference related to protein expression of Stathmin. One recent abstract (*Werner et al, ESGO 2011, Milan, unpublished results*) reported Stathmin expression as a significant predictor of response to Taxane treatment in endometrial cancer. So far we have found no other papers describing this relation. Taxanes is presently one of the major components in chemotherapy in endometrial cancer.[214] Since we find a relation between Stathmin expression and aggressive phenotype and poor survival, this would justify the investigation of Stathmin as a predictor for response to Taxanes.

PI3Kinase inhibitors in cancer treatment are promising drugs entering clinical phase I and II trials[97,215], also illustrated in table 3. The PI3Kinase/mTOR pathway is frequently upregulated in endometrial cancer.[96, 139] In the study from Salvesen and collaborators[96], Stathmin expression was found to correlate with a PI3Kinase activation signature. Our present study (**Paper I**) validates the link between activation of the PI3Kinase signalling pathway, Stathmin overexpression and aggressive clinico-pathologic phenotype. None of the recently completed trials of targeted therapies of the PI3Kinase- or mTOR have applied biomarkers as part of their design[97], while four ongoing studies are now addressing this as shown in table 3. Further studies relating Stathmin expression and response to drugs targeting the PI3Kinase/mTOR pathway will be important to explore its potential value as a predictive marker.

Adjuvant antihormonal therapy has not demonstrated to improve survival in randomised trials and is thus not routinely recommended.[91] Still, as these studies were not biomarker restricted, such therapy may have an unexplored potential for subgroups of patients. Hormone receptor status predicts survival and response to anti-hormonal therapy in metastatic endometrial cancer. Gestagen therapy is often tried in the metastatic setting and is also an option in the primary setting for patients unfit for surgery due to high age and severe co-morbidity.[90] Still, in contrast to breast cancer treatment, hormone receptor status has not yet routinely been implemented in the clinic to improve and individualize endometrial cancer therapy. This despite the fact that estrogen and progesterone receptor status has consistently been reported as strong prognostic markers in endometrial cancer, and again verified as an independent prognosticator in our large, prospective multicenter study (**Paper IV**). Thus, incorporating hormonal receptor status in a more systematic manner for clinical trials evaluating surgical- and systemic therapies appears to be overdue for endometrial cancer.

6.3.5 The role of serological biomarkers in endometrial cancer

At present there are no serological marker of routine use in endometrial cancer although the commonly used CA-125 in ovarian cancers, has been suggested also for endometrial carcinomas.[165] GDF-15 has been investigated in other cancers, and high plasma levels correlated with reduced time to recurrence and reduced overall survival in colorectal cancer.[216] In ovarian cancer high level of GDF-15 in ascites was an independent predictor of poor survival[121], and preoperative plasma levels an independent predictor of poor survival.[123]

We have shown GDF-15 as an independent predictor of lymph node metastasis (**Paper III**). When incorporating ER-PR status in the logistic regression analysis, GDF-15 is still an independent predictor of lymph node metastasis with an OR of 2.64 (95% CI 1.23-5.65, p=0.012) together with ER-PR expression with an OR 2.46 (95%

CI 1.02-5.93, p=0.046) Thus, GDF-15 plasma level in addition to curettage histology and hormone status in tumour could improve the prediction of metastatic lymph nodes. Also, in Cox analysis regarding disease-specific survival, GDF-15 is an independent predictor of poor prognosis when adjusted for age, histological type and grade and ER-PR status with a HR of 2.60 (95% CI 1.42-4.75, p=0.002). If our findings are validated in further studies, one might consider further evaluating this marker in relation to detection of recurrence or progression.

6.3.6 Treatment strategies over time

Potentially, the knowledge of negative lymph node status in properly staged endometrial cancer patients might lead to decreased use of adjuvant treatment. In a SEER study of patients treated during 1988-2006 by Sharma and colleagues, they found no influence from lymph node sampling in the administration of external beam radiation therapy for the low-risk FIGO (88) stage IA any grade or Stage IB grade 1-2 tumours. In contrast, for higher risk groups as FIGO stage I grade 3 and stage IC any grade, they see a shift from external radiation towards vaginal radiation therapy if lymph nodes had been sampled negative.[217] Still, the overall percentage of FIGO stage I patients receiving any radiation therapy was 25% in the lymph node sampled group compared to 16% in the unsampled group. This may be explained by more lymph node sampling performed amongst patients with high risk histology or suspected deep myometrial infiltration. The accompanying editorial timely points out that the data from the SEER study also indicate that we may be over treating patients as it seems like lymphadenectomy may not be altering the decision making in terms of using any radiation or not.[81] From our MoMaTEC trial (**Paper II and IV**), we see a different pattern: only 6% of lymph node sampled FIGO (88) stage I patients received any radiation therapy in contrast to 18% of the unsampled patients. Similar numbers for the population based Hordaland cohort over three decennia, from 2001-2010 radiotherapy was given to 3% and 9% for sampled and unsampled FIGO stage I patients, respectively; this is a reduction from 69% receiving radiation therapy in 1981-1990 when no patients were sampled (**Paper V**). Thus, in Europe it seems like

lymphadenectomy has a stronger and more systematic influence on the decision making regarding the use of adjuvant treatment. Also, randomized studies reporting no survival benefit from radiotherapy may have influenced the change in treatment strategy seen over time.[182, 218]

In line with our 30-year perspective form Norway (**Paper V**), there has been an increased survival in Europe: 5-years survival has increased from 76% to 79% during 2005-2009[219]; also a similar trend was found in Britain.[220] In contrast, a SEER study has noticed an opposite trend: worse survival of their population during 1988-2001.[9] The American study noticed a significant increase in non-endometrioid subtypes, and this was mainly found in women of Afro-American ethnicity. In the British study, there was no increase in the non-endometrioid subtypes, similar to the findings from Norway. Whether the data are derived from mandatory population based registries versus non-mandatory hospital based registries may also have influenced the reported results.

The reduction of radiation therapy seen in Norway is in line with the conclusion of the randomised PORTEC-1 trial[218] and ASTEC-radiation trial[182], showing no survival benefit from adjuvant radiotherapy. Lately, there has been increased focus on systemic treatment for high risk endometrial carcinomas. A Cochrane review concluded that Platinum based therapy had an increased overall survival with HR 0.74 (95% CI 0.64-0.89) and progression free survival of HR 0.75 (95% CI 0.64-0.89).[92] The trend of adding chemotherapy for 9% of the Norwegian patients during the last decennium (**Paper IV**) and 18% of the MoMaTEC patients (**Paper II and IV**) thus seems to be in line with this recently developed knowledge.

7. Conclusions

1. Stathmin immunohistochemical staining is superior to AKT and phospho-AKT staining in detecting PI3Kinase signalling activation and aggressive endometrial carcinomas with poor outcome (**Paper I**).

2. Stathmin staining in primary tumours has been validated to identify endometrial carcinomas with aggressive clinicopathological features in a large prospective multicenter setting (**Paper II**).

3. Immunohistochemical staining of Stathmin in preoperative biopsies (curettage) independently predicts lymph node metastasis and poor survival (**Paper II**).

4. Plasma GDF-15 has been documented as elevated in two independent patient cohorts of endometrial cancer patients compared to controls. High preoperative GDF-15 was significantly associated with aggressive phenotype and a significant and independent predictor for lymph node metastasis and poor survival (**Paper III**).

5. Double negative hormone receptor status (ER and PR negative) in preoperative endometrial cancer curettage has been validated to identify patients with poor prognosis in a prospective multicenter setting. ER-PR negativity independently predicts lymph node metastasis. (**Paper IV**).

6. Reduction in adjuvant radiotherapy and increase in routine pelvic lymphadenectomy during the last 30 years (1981-2010) are associated with improved

disease-specific- and overall survival in a population-based setting of endometrial carcinoma patients with steadily increasing body mass index with time (**Paper V**).

8. Future perspectives

We have validated ER, PR and Stathmin expression in preoperative specimens as robust markers for predicting aggressive endometrial carcinomas with increased risk for lymph node metastasis and poor survival. Since routine lymphadenectomy has not been documented to increase survival, it would be of interest to evaluate if incorporating ER-PR-Stathmin status can improve definition of low- and high-risk patients for tailored surgical therapy in a prospective randomized multicentre trial.

Randomised trials enriched for high-risk patients based on conventional histopathological markers and biomarker status, can potentially improve trials evaluating effect of extensive lymph node surgery including para-aortal lymphadenectomy.

Aneuploidy has been validated to be an independent predictor of aggressive disease and poor survival in endometrial cancer.[132] Small-scale studies[163] have found that preoperative assessment of tumour ploidy may predict extra-uterine disease. It would be of interest to further explore if assessment of DNA ploidy tested in preoperatively collected specimens from our large MoMaTEC cohort, can predict lymph node metastasis and prognosis.

Molecularly based randomized trials of systemic antihormonal treatment may be important to reveal a yet unexplored potential for these drugs amongst receptor positive metastatic lesions, due to lack of incorporation of biomarkers in most earlier studies evaluating antihormonal treatment in adjuvant or recurrent settings.

Stathmin expression was found to be an independent predictor of poor survival and a surrogate marker for PI3kinase signalling, and would therefore be an interesting candidate to test as predictive marker for response to therapy in clinical phase I/II trials of mTOR/ PI3Kinase inhibitors targeting the PI3Kinase pathway.

Stathmin is known to be partaking in the microtubule dynamics, and high Stathmin has been linked to reduced effect of Taxanes in other cancers. Thus, Stathmin would be of particular interest to explore as a potential predictive marker for Taxane response in metastatic endometrial cancers as well. The role of Stathmin may be further explored in endometrial cancer cell lines and animal models of endometrial cancers. Also by evaluating Stathmin expression status in sequential biopsies from patients undergoing preoperative or palliative conventional and novel systemic treatments for locally advanced disease, may in particular offer a unique and alternative approach to study molecular alterations in tumour during treatment in relation to response, and potentially improve the clinical implementation of molecularly targeted therapy.

Regarding plasma level of GDF-15, further studies are needed to explore the applicability of GDF-15 measurements as a marker for stratification for lymph node sampling or for early detection of recurrence in routine follow-up.

Preoperative plasma GDF-15 has, in addition to endometrial cancer, been found to be elevated in ovarian cancer patients compared to women with a benign pelvic mass and other healthy controls.[123] Preoperative identification of malignant pelvic masses, with or without irregular vaginal bleeding, is a great clinical challenge for tumours originating from the uterus as well as the fallopian tubes or the ovaries. In particular it may be of interest to evaluate plasma GDF-15 as a marker for uterine sarcomas where no marker is available in the clinical setting to distinguish preoperatively between the very commonly occurring benign myoma uteri and the rare but highly malignant sarcoma of the uterus.

An expansion of data registered in the population based Norwegian Cancer Registry to include more detailed clinical-, histopathological- and therapy related data for endometrial cancer patients, may in the future be helpful to evaluate the overall quality of the treatment offered, and potential related survival effects.

Errata

Corrections in bold:

Paper I: p 645, Table 3: Foot note, line 1;" *One-sided Pearson chi-square test." should read : *One-sided **Mann-Whitney** test.

Paper III: p 4826, GDF-immunoassay section, last line"12.2% or more" should read: "12.2% or **less**".

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