Endometrial carcinoma: a step closer to individualized therapy?

Exploring transcriptional alterations in relation to prognostic biomarkers

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«Alt for å finne det sannes mysterium – det er den ekte forskers kriterium.»

Peer Gynt, Henrik Ibsen

The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim
Because it was grassy and wanted wear,
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I,
I took the one less traveled by,
And that has made all the difference.

(*Robert Frost 1874-1963*)

SCIENTIFIC ENVIRONMENT

"*If I have seen further it is by standing on the shoulder of giants."*
(Sir Isaac Newton, 1643-1727)

This PhD project has been performed within the *Gynecologic Cancer Research Group* directed by Professor Helga B. Salvesen and the *Tumor Biology Research Group* directed by Professor Lars A. Akslen.

Professor Helga B. Salvesen has been my main supervisor and Professor Lars A. Akslen, my co-supervisor.

Translational research in gynecological cancer in general, and endometrial cancer in particular, has for several years had a solid foundation in Bergen, led by Professor Helga B. Salvesen (principal investigator) at Department of Clinical Science, University of Bergen and Department of Obstetrics and Gynecology, Haukeland University Hospital. A systematic collection for a biobank from gynecologic malignancies, at the Department of Obstetrics and Gynecology, Haukeland University Hospital was initiated in 2001. After confirmed consent, fresh frozen tumor and blood samples from women treated for gynecologic cancers have been prospectively collected at our institution and in a multicenter setting (MoMaTEC). The overall goal for the scientific activity is to explore potential biomarkers to improve and individualize treatment for women with gynecologic cancer.

In relation to Professor Salvesen's group, at present around 20 members (research fellows, postdocs, students and technicians). Five theses have been completed, 8 post doc projects and 9 PhD projects are ongoing.

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

Department of Pathology, University Medical Center Hamburg Eppendorf, Hamburg, Germany, is involved in FISH analyses of identified candidate genes in validation series. Professor R. Thomas, Max-Planck Institute for Neurological Research, Cologne, Germany, is involved in mutation screens of oncogenes (OncoMap).

Professor Lars A. Akslen at The Gade Institute, Section for Pathology, University of Bergen (from 2013 reorganized to Department of Clinical Medicine, Section of Pathology) and Department of Pathology, Haukeland University Hospital with his Tumor Biology Research Group has been a fundamental collaborator through these years. The *Tumor Biology Research Group* at The Gade Institute led by Professor Akslen was established in 1995 and has aimed to perform translational cancer research at an international level, identifying biomarkers of aggressive cancers that can assist in prognostication and prediction of targeted treatment response. The biomarker studies have been especially related to angiogenesis and tumor-vascular interactions, and importance for the metastatic process, as well as tumor cell proliferation and cell cycle regulation. Studies have been performed across different tumor types (breast, endometrial and prostate cancer and malignant melanoma) with long-time collaboration with clinical investigators, and also including extensive national and international collaboration networks. The research group currently counts around 20 members (research fellows, postdocs, senior researchers, students and technicians). From 2013, Akslen is the director of *Centre for Cancer Biomarkers*, a Norwegian Centre of Excellence funded by the Research Council of Norway.

Professor Karl-Henning Kalland at Department of Clinical Science, University of Bergen, has been a long-term collaborator in microarray studies.

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My warmest hugs go to our kids, Tora, Sturla and Åsmund who give me their honest feelings every day, and remind me of the importance of having a balance between family life and professional life.

Last, but not at all least; my husband and best friend Brynjar! Without his support in whatever field of life, no PhD would have come through. Fruitful discussions on logic, philosophy, mathematics and other important issues in life, as well as his patience, his love and his work in our family in my absence and absent-minded presence have been immensely important in this period. Thank you!

Bergen, June 2013 Elisabeth Wik

ABBREVIATIONS

ACTB:	Actin, beta
ANGPTL4:	Angiopoetin-like 4
AKT:	v-akt murine thymoma viral oncogene homolog
APC:	Adenomatous polyposis coli
AURKA:	Aurora kinase A
BAX:	BCL2 associated X protein
BCL2:	B-cell CLL/lymphoma 2
bFGF:	Basic fibroblast growth factor
BMI-1:	BMI1 polycomb ring finger oncogene
BRCA1/2:	Breast cancer 1 and 2, early onset
BSO:	Bilateral salpingo-oophorectomy
CA125:	Cancer antigen 125
CCND1:	Cyclin D1
CCNE1:	Cyclin E1
CDH1:	Cadherin 1, type 1, E-cadherin
CDK:	Cyclin-dependent kinase
CDKN2A:	Cyclin-dependent kinase inhibitor 2A
cDNA/ cRNA:	Copy DNA/RNA (deoxy/ribo nucleic acid)
CSC:	Cancer stem cell
CT:	Computed tomography
C_T:	Cycle threshold
D&C:	Dilatation and curettage
DNA:	Deoxyribonucleic acid
ECARS:	Endometrial Carcinoma Recurrence Score
ECM:	Extracellular matrix
EEC:	Endometrioid endometrial cancer
EGFR:	Epidermal growth factor receptor
EMT:	Epithelial- mesenchymal transition
ER:	Estrogen receptor
ERBB2:	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastomaderived oncogene (avian)
ESR1:	Estrogen receptor 1
FBXW7:	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase
FDR:	False discovery rate

FFPE:	Formalin fixed, paraffin embedded
FGFR2:	Fibroblast growth factor receptor 2
FIGO:	International Federation of Gynecology and Obstetrics
FISH:	Fluorescent *in situ* hybridization
FOXC2:	Forkhead box C2
FVIII/Ki67:	Factor VIII/Ki67 co-expression
GAPDH:	Glyceraldehyd-3-phosphate dehydrogenase
GDF15:	Growth differentiation factor 15
GPER:	G protein-coupled estrogen receptor 1
GSEA:	Gene set enrichment analysis
H&E:	Hematoxylin and eosin
HIF:	Hypoxia inducible factor
HMGA2:	High mobility group AT-hook 2
HNPCC:	Hereditary non-polyposis colorectal cancer
HR:	Hazard ratio
HSP90:	Heat shock protein 90
IHC:	Immunohistochemistry
IGF-1:	Insulin-like growth factor 1
IGF1R:	Insulin-like growth factor 1 receptor
IL-11:	Interleukin 11
KLF8:	Kruppel-like factor 8
KRAS:	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
L1CAM:	L1 cell adhesion molecule
LCD:	Laser capture dissection
LSAB	Labeled StreptAvidin Biotin
LOH:	Loss of heterozygosity
LVI:	Lymph vascular invasion
MAPK:	Mitogen-activated protein kinase
MET:	Mesenchymal-epithelial transition
miRNA:	micro RNA
MLH1:	MutL homolog 1, colon cancer, nonpolyposis type 2 (E.coli)
MMMT:	Malignant mixed Mullerian tumor
MMP:	Matrix metallopeptidase
MMR:	Mismatch repair
MRI:	Magnetic resonance imaging
mRNA:	messenger RNA
MSH2:	MutL homolog 2, colon cancer, nonpolyposis type 1 (E.coli)

MSI:	Microsatellite instability
MSigDB:	Molecular signatures database
mTOR:	Mammalian target of Rapamycin
MVD:	Microvessel density
MVP:	Microvessel proliferation
MW:	Microwave
MYC:	v-myc myelocytomatosis viral oncogene homolog (avian)
NEEC:	Non-endometrioid endometrial cancer
PAI-1:	Plasminogen activator inhibitor-1
PARP1:	Poly (ADP-ribose) polymerase 1
PBS:	Phosphate buffered saline
PDGFR:	Platelet-derived growth factor receptor
PDK1:	Phosphoinositides dependent kinase 1
PET:	Positron emission tomography
PHH3:	Phosphohistone-H3
PI3K:	Phosphatidylinositid 3-kinase
PIK3CA:	Phosphatidylinositol-4,5-bisphosphate 3-kinase,catalytic subunit α
PIK3R1:	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
PR:	Progesterone receptor
pStathmin(S38):	Phospho-Stathmin(Serine38)
PTEN:	Phosphatase and tensin homolog
pStathmin:	phospho-Stathmin
qPCR:	qualitative polymerase chain reaction
RASSF1A:	Ras association (RalGDS/AF-6) domain family member 1
REMARK:	Reporting recommendations for tumor marker prognostic studies
RNA:	Ribonucleic acid
RTK:	Receptor thyrosine kinase
SAM:	Significance analysis of microarray
Ser:	Serine
SHBG:	Sex hormone binding globulin
SI:	Staining index
SNP:	Single nucleotide polymorphism
SPSS:	Statistical package for social sciences
STK11:	Serine/threonine kinase 11
TCGA:	The Cancer Genome Atlas
TGF-β:	Transforming growth factor β
TLDA:	TaqMan® low density array

TMA:	Tissue microarray
TP53:	Tumor protein 53
TSP-1:	Trombospondin-1
TVS:	Transvaginal sonography
US:	Ultrasonography
VEGF(R):	Vascular endothelial growth factor (receptor)
VI:	Vascular invasion
VPI:	Vascular proliferation index
WHO:	World Health Organization
ZEB1:	Zinc-finger E-box binding homeobox 1

ABSTRACT

Background:

Endometrial cancer is diagnosed early and has in general a good prognosis. The more important it is to diagnose and treat the poor-prognosis cases. Therapy of endometrial carcinoma patients per today is to a large extent empirically based. Improvements on therapeutic strategies with more personalized focus are needed. Preparing the ground for later clinical studies, by combining clinico-pathologic and molecular data from preclinical studies and cancer patients, is an important step to individualize therapy in cancer.

The incidence of endometrial cancer is increasing and the prognostic categorization used in clinical practices today is suboptimal for separating patients according to survival. Despite a focus on translational research in endometrial cancer for some decades, it has proven difficult to bring new biomarkers to the clinic to improve prognostication and prediction of therapy response in this cancer type. Endometrial cancer is behind other cancer types as breast, lung and colorectal cancer in clinical application of molecular classification of tumors to select patients for targeted therapy. Better tumor biological understanding of subgroups, applicability of prognostic markers in a routine clinical setting, and targets for therapy including markers predicting response to such, is important to improve personalized treatment strategies to benefit the endometrial carcinoma patients.

Main objectives: The main objective was to study biomarkers potentially associated with endometrial carcinoma progression, to assess their potential as prognostic markers and explore on targets for therapy associated with pathologic expression of these markers. By this, we aimed to provide a rationale for further testing of candidate markers as prognostic and predictive markers in clinical trials. Also, we wanted to focus on biomarker implementation through an important step in the stair-case from research to clinical use; *biomarker validation* in independent patient series and in a routine clinical setting.

Materials and methods: Overall, subsets of one retrospective and one prospective patient cohort were analyzed (**Paper I-IV**), in addition to an external gene expression microarray (**Paper II**) and endometrial cancer data from *The Cancer Genome Atlas* (TCGA, **Paper IV**), with comprehensive clinico-pathologic and follow-up annotations for all series. DNA oligonucleotide microarrays were analyzed (**Paper I-IV**). ERα and pStathmin(S38) immunostaining was performed (**Paper II and III,** respectively). Also, previously published data were included (*e.g.* EMT markers, data on vascular invasion, proliferation markers, PIK3CA sequencing data, Stathmin and SNP array data). RNA sequencing for gene expression levels were retrieved from 333 endometrial carcinoma samples in The Cancer Genome Atlas (TCGA).

Results: DNA aneuploidy was associated to higher age at diagnosis, non-endometrioid histology and high histologic grade in both series studied, and with independent association with reduced survival in multivariate analyses. We found the research and routine diagnostic series to be comparable, with no significant differences in distribution in standard clinico-pathological variables (**Paper I**).

ERα-low tumors were associated with aggressive endometrial cancer and reduced survival in 4 independent patient series. Transcriptional differences based on ERα status revealed pathways, single genes and transcription factors linked to epithelial-mesenchymal transition (EMT) enriched in ERα negative tumors, also validated in an external gene expression data set and validated by mRNA and immunohistochemistry in two independent patient series. ERα-low tumor status was also significantly correlated to various markers for PI3Kinase pathway alterations. Furthermore, the gene expression signatures of PI3K/mTOR inhibitors were correlated to ERα-low gene signatures in two independent patient series (**Paper II**).

High pStathmin(S38) immunostaining associated with an aggressive clinico-pathologic phenotype and reduced survival, in both the investigation and validation cohorts. Gene expression patterns related to cell cycle progression were enriched in pStathmin(S38)-

high cases. pStathmin(S38) also correlated with a panel of established markers for tumor cell proliferation: Ki67, mitotic count and S-phase fraction. Gene expression signatures representing effect of PI3K/mTOR and HSP90 inhibitors associated with a pStathmin(S38)-high gene expression signature. High pStathmin(S38) correlated significantly with several potential markers for PI3K activation (**Paper III**).

The 29-gene signature score validated to identify patients with increased risk of recurrence, also in patient subgroups with presumed favorable outcome. The 29-gene endometrial carcinoma recurrence score (ECARS) also associated with clinico-pathologic data of aggressive endometrial cancer. ECARS validated to predict overall survival in 332 cases from The Cancer Genome Atlas (TCGA) database. High ECARS associated with vascular invasion and measures for EMT and potential measures for PI3K pathway activation. Assessing ECARS and an EMT signature in metastatic lesions demonstrated an increase of theses signatures from primary to metastatic tumors (**Paper IV**).

Conclusions: DNA aneuploidy identifies aggressive endometrial carcinoma and predicts poor outcome, also in a routine clinical setting (**Paper I**).

Low ERα in endometrial carcinoma is associated with epithelial-mesenchymal transition, vascular invasion and PI3K alterations (**Paper II**).

High pStathmin(S38) associates with high tumor cell proliferation and measures for PI3Kinase activation in endometrial carcinomas (**Paper III**).

The endometrial carcinoma recurrence score (ECARS) validates to identify endometrial carcinomas with shorter recurrence free survival. ECARS increases from primary to metastatic lesions and is associated with measures for PI3Kinase activation and epithelial-mesenchymal transition (**Paper IV**).

Low ERα, high pStathmin(S38) and high ECARS predict aggressive endometrial carcinomas and reduced survival, and may suggest treatment with PI3K/mTOR and or EMT inhibitors in clinical trials (**Papers II, III and IV**).

LIST OF PUBLICATIONS

I. **Wik E**, Trovik J, Iversen OE, Engelsen IB, Stefansson IM, Vestrheim LC, Haugland HK, Akslen LA, Salvesen HB. Deoxyribonucleic acid ploidy in endometrial carcinoma: a reproducible and valid prognostic marker in a routine diagnostic setting. *Am J Obstet Gynecol. 2009;201:603.e1-7*

II. **Wik E**, Ræder MB, Krakstad C, Trovik J, Birkeland E, Hoivik EA, Mjos S, Werner HMJ, Mannelqvist M, Stefansson IM, Oyan AM, Kalland KH, Akslen LA, Salvesen HB. Lack of Estrogen receptor α is associated with epithelial-mesenchymal transition and PI3K alterations in endometrial carcinoma. *Clin Cancer Res. 2013;19:1094-105.*

III. **Wik E**, Birkeland E, Trovik J, Werner HM, Hoivik EA, Mjos S, Krakstad C, Kusonmano K, Mauland KK, Stefansson IM, Holst F, Petersen K, Oyan AM, Simon R, Kalland KH, Ricketts W, Akslen LA, Salvesen HB. High Phospho-Stathmin(Serine38) expression identifies aggressive endometrial cancer and suggests an association with PI3K inhibition. *Clin Cancer Res. 2013;19:2331-41.*

IV. **Wik E**, Trovik J, Kusonmano K, Birkeland E, Raeder MB, Pashtan I, Hoivik EA, Krakstad C, Werner HJM, Holst F, Mjøs S, Halle MK, Mannelqvist M, Mauland KK, Oyan AM, Stefansson IM, Petersen K, Simon R, Cherniack AD, Meyerson M, Kalland KH, Akslen LA, Salvesen HB. Endometrial Carcinoma Recurrence Score (ECARS) validates prospectively to identify aggressive disease and associates with markers of epithelial-mesenchymal transition and PI3K alterations. *Manuscript submitted*

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1. INTRODUCTION

Cancer - a disease of unregulated cell growth and with a potential of cancer cell invasion into neighboring organs as well as spread to distant organs. Cancer may develop in any tissue originating from embryonic mesoderm, ectoderm or endoderm, and is of many today regarded as a "genetic disease at the cellular level",[1] as genetic alterations in somatic cells are thought to be required for initiation of the carcinogenic process in the development from normal to cancerous tissue. There is a large range of clinical presentations in cancers, depending amongst other on the cancer type, localization and stage of the disease.

Endometrial carcinoma is an epithelial cancer, developing in the epithelial lining of the uterine cavity, with a potential to invade into the myometrium and cervical stroma and spread to distant body sites.

1.1 Epidemiology

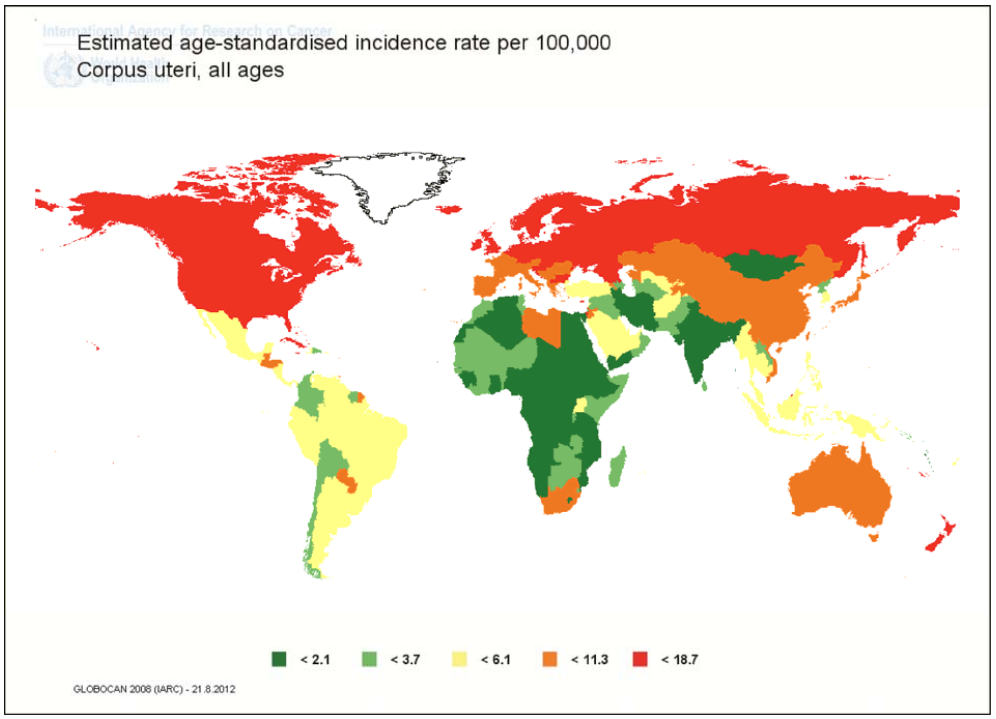
Incidence

Worldwide, cancer has a major role in the burden of diseases, being the number one cause of death in developed countries and the second most frequent cause of death in developing countries. Almost 13 million new cases and 7.6 million cancer deaths were estimated in 2008.[2] Endometrial cancer is one of the most common cancer types in women and the 4th most frequent cancer type in women of developed countries after breast, colorectal and lung cancer,[2] affecting more than 140 000 new women per year. Endometrial cancer is also the most frequent pelvic gynecologic cancer type in the Western world.[3,4]

The endometrial carcinoma incidence is reported to be increasing in Europe.[5] The age adjusted incidence rate (new cases per 100 000 person years) in Norway is 16.5/100 000

person-years[4] (**Figure 1**), and has increased from 7.0 in 1955-59 through 12.2 in 1980-85 to 16.4 in 2005-2009 (**Figure 2**).[4]

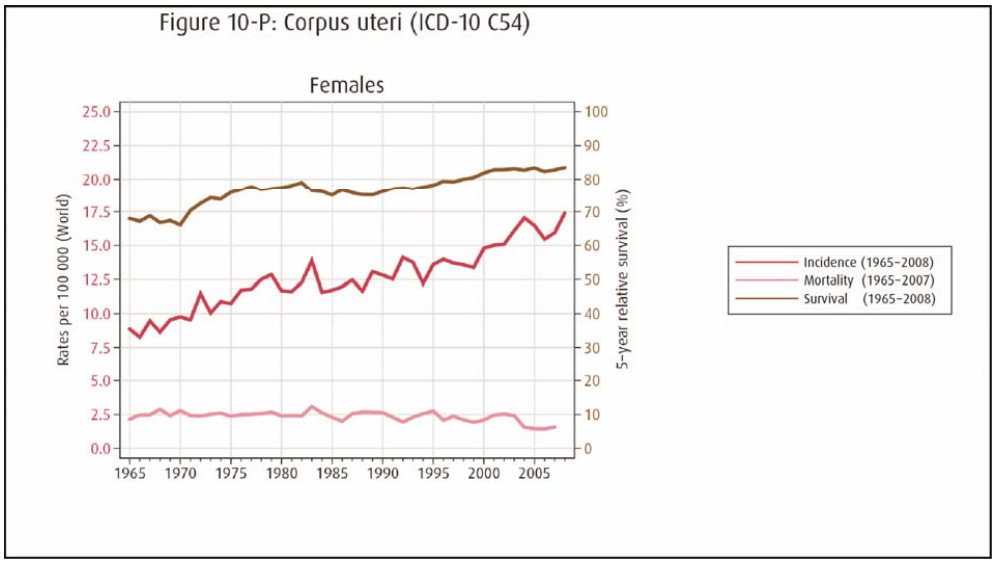
Figure 1. Estimated age-standardized incidence rate of corpus uteri cancer per 100 000 person-years (adapted from *http://globocan.iarc.fr*)



In cancer and death registries, endometrial carcinomas are recorded within the group of uterine cancer, indicating that the crude numbers of endometrial carcinomas are lower than the reported overall numbers for uterine cancers, the latter also including uterine sarcomas in the statistics.[4] Uterine sarcomas are demonstrated to comprise 3-9% (depending on histological definition) of all uterine cancers,[6,7] and to occur with a relatively stable incidence of 0.3-0.4 per 100 000/year in the Nordic countries,[8]

supporting that the observed increase in the numbers of uterine cancer over the same period mainly reflects the increased incidence of endometrial carcinoma.

Figure 2. Incidence, mortality and survival rates from uterine cancers in Norway 1965-2008.[4]



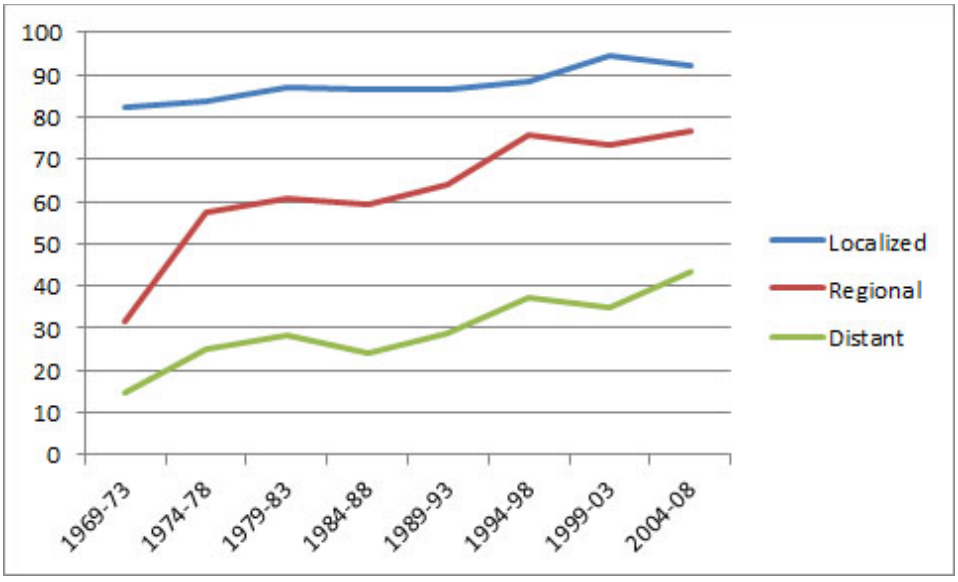
Survival

The ultimate goal when treating cancer patients is to extend life expectancy with as good life quality as achievable, for a period as long as possible. It is important to discuss which end-points are best suited when reporting on cancer prognosis, and thereby as indirect measures for effects of cancer therapy. In general, *disease/cancer specific survival* is described by time from diagnosis to death from cancer. *Overall survival time*, the time from diagnosis to death of any cause, is suggested to better reflect the overall efficacy of therapeutic interventions, also accounting for potential therapeutic side effects with survival impact. Effects on survival after recurrences also impacts overall survival. As

such, *progression free survival* (for patients with advanced cancers) and *recurrence free survival* (for patients with localized cancers), are considered to be surrogate measures for overall survival, used as secondary end-points in clinical trials, where overall survival is the primary end-point when evaluating effects from interventions.

In endometrial cancer, overall survival has increased over the last 40 years, from 72.8% to 83.5% (for all stages).[4] The increase in overall survival is seen within each of the various stages of the disease (**Figure 3**).

Figure 3. Five-year relative survival (%) from uterine cancer in Norway (1969-2008). Survival rates stratified according to disease stage (based on numbers from *Cancer Norway2008*).



1.2 Etiology and risk factors

"Correlation does not imply causation" (*RA Fisher 1890-1962*)

It is generally accepted that cancer as well as other diseases, are "caused" by certain influences. Various presumed "causes" have directed approaches to both diagnostics and treatment throughout the medical history.[9] "Etiology" is in medicine regarded as the cause of origin of a disease, and the studies of such. Classification of etiologic factors into *genetic* and *acquired* is commonly applied.[9] For only a few diseases, the simple link "one agent leading to one disease" is valid, exemplified by some infections and diseases caused by changes in one single gene. Many diseases, including cancer, are today regarded to have multifactorial etiology.[9]

Genetic factors

Cancer is considered "a genetic disease at the cellular level",[1,10] and was only a few years ago considered to be of monoclonal origin.[11] For many cancer types there is now strong evidence for cancer being a polyclonal disease.[12] Endometrial carcinoma is a heterogeneous neoplasia, associated with a variety of genetic alterations.

Genetic causes for cancer can be divided into two groups: hereditary (covered in this chapter) and somatic (covered in chapter 1.3). Overall, the life-time risk for developing endometrial carcinoma is approximately 1.6%.[2] The majority of cases are sporadic, but a subgroup of approximately 5% of endometrial carcinomas develop due to a genetic predisposition caused by inherited mutations (*i.e.* in germ cells) of cancer-related genes.[13] Patients with the inherited diseases Lynch syndrome, Cowden syndrome or Peutz-Jeghers syndrome are all at increased risk of developing endometrial cancer.

Lynch syndrome

Hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch syndrome, is an autosomal dominant disease caused by pathogenic germ line mutations in DNA mismatch repair (MMR) genes.[14] The prevalence of such mutations is 1:500-1:1000.[14] The first description of the original HNPCC family took place 100 years ago.[15] The disease was recognized as a syndrome and further defined in the 1960-70s. Patients with Lynch syndrome are at increased risk of several cancers; colorectal, endometrial, gastric and ovarian cancer, with highest lifetime risk for women (40-60%, 40-60%, 13% and 6-12%, respectively).[16,17] The prognosis for endometrial cancer related to HNPCC is similar to sporadic endometrial cancer.[18] A proposed screening program for women with Lynch syndrome includes colonoscopy once every 1-2 years, endometrial sampling once a year, urine cytology once a 1-2 year, and general history and examination yearly, starting from 21-30 years. Hysterectomy and ovarectomy are considered when the woman does not want (more) children.[19] Yearly screening with endometrial biopsy for women with known germline mutations in MMR genes is recommended by some.[20] However, in general the effectiveness by diagnosing these early stages of cancer is debated.[21]

Cowden syndrome and Peutz-Jeghers syndrome

Cowden syndrome is autosomal dominant inherited and characterized by multiple hamartomas occurring in different tissues. The incidence of Cowden syndrome is estimated to 1:200 000-250 000.[22] Cowden syndrome patients have increased risk of malignancies, in particular breast, thyroid and endometrial cancer,[23] and have an endometrial cancer lifetime risk of 5-10%.[24] The majority of Cowden syndrome patients have germ line *PTEN* mutations (~80%),[22] potentially explaining the increased cancer risk.

Multiple hamartomatous polyps in the gastrointestinal tract and mucocutaneous pigmentation characterize the Peutz-Jeghers syndrome,[23] in addition to risk of developing gastrointestinal and other cancer types. The incidence of this syndrome is 1:50 000-250

000. Autosomal dominant inherited *STK11* mutation is seen in ~90% of the patients.[25] The lifetime risk of developing endometrial carcinoma is 9% in this patient group.[26]

Screening for endometrial cancer in both Cowden and Peutz-Jeghers syndromes are at present being discussed, but per December 2012, no screening guidelines are implemented.[23]

Acquired factors

90-95% of endometrial carcinomas are sporadic occurring cancers.[14] Bokhman suggested in a clinical, descriptive study of 366 patients, two distinct pathogenetic types of endometrial carcinoma.[27] Diabetes, hypercholesterolemia, hypertension and overweight were frequently observed in a large fraction of the patient series (60-80%). These patients more often developed endometrial carcinoma with concurrent endometrial hyperplasia, and were associated with low histologic grade, superficial myometrial infiltration and response to progestagens. The tumor was considered cancer of "the first type", later named Type I endometrial carcinoma.[28,29] In the "second type", metabolic disturbances amongst the patients were absent, tumors were more often of high histologic grade with deep myometrial infiltration, and more metastatic spread occurred. Also, a different association with survival was noted between the two patient groups; the "second type cancer" was associated with reduced survival. The categorization into type I and II is still the major classification of endometrial carcinoma in clinical use today, although several molecular characteristics associated with the type I versus type II distinction have been reported.[30]

One of the early published reports on co-occurring prolonged exogen estrogen stimulation and development of endometrial cancer in patients is from 1946.[31] The study is a case-report of a 45 year old woman receiving unopposed estrogen therapy over 8 years, developing endometrial cancer by end of therapy. The case-report led to the hypothesis that endometrial cancer develops because of unopposed estrogen therapy. The surgeon G.

Beatson (1848-1933) is, however, recognized as the first to present the hypothesis that ovarian hormones play an important role in the cause of cancer, based on observational studies in breast cancer patients.[32] The description of how oophorectomy in patients with advanced breast cancer improved the performance with respect to cancer dissemination came even before the concept of hormones was set forth. The functional roles of estrogen in cancer development have since then been extensively studied in epidemiological, clinical and experimental studies.[33-36] It is now generally accepted that excess estrogen relative to progesterone plays an important role for development of endometrial cancer.[37]

In the reproductive age, estrogens are synthetized and secreted primarily by the ovaries, with contributions also from adipose tissue and adrenal glands. In the postmenopausal phase, the majority of circulating estrogen is synthesized by peripheral aromatization of adrenal androgens. Intratumoral estrogen synthesis is also suggested being important in endometrial cancer.[35] Surplus estrogen, relative to progesterone, promote angiogenesis[38] and endometrial cell proliferation,[39] and inhibits apoptosis.[40,41] Progesterone is considered to counteract the proliferative effects of estrogen, contributes to cellular differentiation[42] and inhibition of cancer cell invasion.[43] A relative estrogen/progesterone excess may appear as a consequence of excess estrogen and/or progesterone deficiency. Overweight, unbalanced hormonal replacement therapy only replacing estrogen, but not progesterone, persistent anovulation, and nulliparity are conditions that lead to increased circulating estrogen, and are associated with increased endometrial cancer risk.[44-50]

Strong associations between obesity and occurrence of endometrial cancer are demonstrated,[44,51] and increasing obesity is viewed as a major contributor to the increasing incidence of endometrial carcinoma in the Western world.[28] Various mechanisms underlying this observation are suggested, conveying through the excess of estrogen relative to progesterone: Increased aromatase activity in fatty tissue together with reduced levels of Sex hormone-binding globulin (SHBG) leads to increased bioavailable estrogen,[48] and increased ovarian androgen synthesis leads to chronic anovulation and decreased levels of progesterone.[48] Obesity is also associated with

increased levels of insulin and Insulin-like growth factor-1 (IGF-1); both being ligands of receptors potentially activating the PI3K signaling pathway and downstream key processes in the carcinogenesis, such as proliferation and cell survival.[45,52] Occurrence of endometrial cancer is also associated with hypertension and diabetes mellitus,[53] but their role as causal factors is not clear.[45]

Breast cancer patients treated with the estrogen receptor antagonist Tamoxifen are associated with a two-fold increased risk of endometrial cancer, also dependent on duration of therapy.[54-56]

Late age at menarche, early age at first birth, last pregnancy at relatively high age, high number of children, longer (accumulative) period of breastfeeding, and use of combination oral contraceptives are associated with decreased risk of endometrial cancer.[37] Habitual factors such as smoking and physical activity are also associated with reduced risk of endometrial cancer.[57-60] The antiestrogenic effects of smoking through altered hormonal metabolism, weight loss and earlier menopause, are suggested mechanisms for this observation. Combined oral contraceptives are associated with reduced risk of endometrial carcinoma (ever use associated with 30% lower risk).[61,62]

1.3 Tumor biomarkers in endometrial carcinomas

Cancer has been characterized by eight hallmarks contributing to the "cancer phenotype": Self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evasion from apoptosis, sustained angiogenesis, tissue invasion and metastasis.[10] Re-programming of energy metabolism and evasion from immune destruction are two major characteristics more recently added to the list of cancer hallmarks. Underlying these hallmarks are a broad range of genetic and epigenetic aberrations as well as dysregulated communication between cancer cells themselves and between cancer cells and cells in the surrounding tumor microenvironment.[10] Endometrial

cancer is a heterogeneous disease, reflected in a variety of molecular alterations described for this cancer type.[63,64] In the following, a presentation of some of the mentioned hallmarks and underlying alterations is given and related to the endometrial carcinoma tumor biology.

Enabling characteristics in endometrial carcinomas

Genomic instability

Mutations

The human genome is dynamic, and millions of DNA damaging events and replication errors occur daily.[65] A highly efficient genome maintenance system, including detection and repair mechanisms, functions such that mutations are not frequently found in each cell generation in normal tissues.[10] Chromosomal instability is demonstrated in cancer, where the chromosomal structure and content changes over time, with high rate compared to normal cells.[66] A cancer "mutator phenotype" is suggested, where mutations in genes crucial to maintain a normal genotype occur, such as the DNA repair genes, and thereby contribute to the large amount of mutations present in cancer.[65]

Short, repetitive DNA sequences throughout the genome are called microsatellites. These sequences are susceptible to replication errors, and if loss of function of the DNA repair genes occurs, there is high risk of mutations in these satellites, denoted microsatellite instability (MSI)[67,68] and regarded as a form of genomic instability.[66] MSI occur in 11-45% of endometrioid endometrial carcinomas.[69] HNPCC families carry germ line mutations in the DNA mismatch repair genes *MLH1* and *MSH2*, potentially contributing to the increased risk of both colorectal and endometrial cancer.[63] In sporadic endometrial cancer, some of the DNA mismatch repair genes are demonstrated to be silenced by promoter methylation.[63] MSI is more frequent in Type I endometrioid carcinomas (20-45%), compared with 0-5% in type II carcinomas.[64]

Depending on the location of a mutation and its amino acid effect, various mutations of a gene exhibit different effects. Mutations in the promoter region of a gene may alter how the gene is transcribed (more/less expressed, and to other times or in other locations compared to the non-mutated state). Missense and nonsense mutations in coding regions of the genome, coding for different amino acids and stop codon, respectively, may affect the protein structure and thus alter protein function.[70] Silent mutations code for similar amino acids or amino acids that do not imply functional protein alterations. Growth signaling may be activated by mutations in oncogenes or tumor suppressors involved in regulating such signaling pathways.[66] A wide range of mutations are described in endometrial cancer (*http://cancer.sanger.ac.uk/cosmic/browse/tissue?sn=endometrium*). *PIK3CA*, *KRAS*, *FGFR2*, *TP53* and *PTEN* mutations are identified with varying frequency in type I and type II tumors (**Table 1**).[64,71] Several *PIK3CA* mutations are suggested as activating mutations with regard to PI3K signaling,[72] and a mutation in *PIK3CA* exon 20 (H1047R) has recently been suggested as a predictive marker for response to PI3K inhibitors in endometrial carcinoma.[73] Recent whole exome sequencing of serous endometrial carcinoma have identified *TP53*, *PIK3CA* mutations (82% and 24%, respectively) together with mutations in chromatin remodeling genes and ubiquitin ligase complex genes.[74,75] Dysfunctional chromatin remodeling and ubiquitin ligase is regarded to have important roles in carcinogenesis and cancer progression.[76,77]

The functional implications from various mutations are to some extent studied in experimental models and provide a basis for the understanding of effects from similar mutations in human. Still, many of the mutations found in human cancer are not functionally described in experimental models and are only associated with cancer and cancer phenotypic measures. As the whole exome sequencing is emerging within cancer research and more cancer associated mutations are identified, the functional implications and relevance for drug response of these mutations are important objects for future studies.

Somatic copy number alterations

Somatic copy number variations are common in cancer and may contribute to drive the development of cancer.[78] Salvesen et al characterized 57 endometrial carcinomas by gene expression arrays and 76 tumors by copy number profiling, and found oncogenes such as *PIK3CA*, *EGFR*, *MYC*, *KRAS*, *ERBB2* and *AURKA* significantly amplified across the samples studied.[79] Also, a tumor suppressor (*FBXW7)* was identified deleted in this population.[79] Two other studies assessed genome wide copy number alterations in serous and endometrioid subtypes and confirmed *PIK3CA* and *KRAS* amplifications and deletion of *FBXW7* in endometrial cancer.[74,80] In a study by Murayama-Hosokawa et al, the extent of the copy number alterations was categorized: Tumors were presented as chromosomal instability (CIN) negative, *i.e.* no copy number alterations, CIN-intermediate and CIN-extensive (1-4 and ≥ 5 loci of copy alterations, respectively). Patients with CIN-extensive tumors experienced significantly poorer survival as compared to those with CIN negative or intermediate tumors. In addition to standard copy number gain and loss, this study also described presence of copy number neutral (CNN) LOH, denoting loss of one allele and gain of the opposite allele, in *CDKN2A*, *PTEN* and *TP53*.[80]

Epigenetic alterations; methylation

"Epigenetic alterations" denotes inheritable changes in gene expression and phenotype not due to altered nucleotide sequence.[81] Methylation - attachment of methyl groups to cytosine in CpG sequences located in promoter regions and within gene exons and introns, is one of the epigenetic alterations associated with cancer, and both loss and gain of methylation may contribute to increased and decreased/silenced transcription, respectively.[81] Methylation is suggested to be as important as mutations for reduced transcription of tumor suppressor genes.[1]

In a study of the methylation pattern of 24 tumor suppressor genes in the carcinogenic process in endometrial cancer, promoter methylation increased from normal endometrium to simple and complex hyperplasia.[82] Hypermethylation of the DNA mismatch repair

gene *MLH1* is associated with MSI in endometrial cancer, particularly the endometrioid subtype, and is suggested to be a cause of *MLH1* silencing, as demonstrated in cancer cell lines.[83,84] Methylation of tumor suppressor genes such as *PTEN*, *CDKN2A*, *RASSF1A* and *APC* have been reported in endometrial carcinomas with frequencies in the range of 11-85%.[83] Also, low expression of the cell adhesion marker E-cadherin (*CDH1*) is suggested to occur by promoter hypermethylation.[83] Silencing of these genes by methylation is more frequent in type I than type II cancers, and it is suggested that hypermethylation has a stronger impact on the carcinogenesis in type I than type II endometrial carcinomas.[83]

Non-coding RNA:

Over the last years, the importance of non-protein coding RNA to biological processes has been recognized. Micro-RNA (miRNA) is known to post-transcriptionally repress gene expression at the level of translation by binding to mRNA, and is thus able to regulate multiple gene targets.[85] A global down-regulation of miRNA expression is demonstrated in cancer,[86] and specific miRNA alterations are associated with various cancer types and may function as oncogenes and tumor suppressors.[85] MiRNAs alterations in cancer are suggested to affect genomic instability and DNA repair mechanisms, contributing to the overall genomic instability seen in cancer.[86] MiRNAs are also suggested as metastatic activators.[87] The miR-200 family is presented as tumor suppressors and down-regulation of these is further linked to epithelial-mesenchymal transition (EMT) and the invasive process.[88] In line with this, the miR-200 family is demonstrated to be down-regulated in the mesenchymal part of carcinosarcomas,[89] and studies of endometrial cancer cell lines have demonstrated a link between low expression of miR-200 family members, increased ZEB1 expression and further low expression of E-cadherin.[90]

Dysregulated pathways

Several of the above mentioned enabling characteristics have the potential to activate specific signaling pathways that may promote cancer initiating processes and alterations

linked to cancer progression. By binding of extracellular growth factors to corresponding receptors and the following intracellular signal transduction of the growth signals to the nucleus for gene expression regulation, growth factors may exert multiple effects inside the cell.[70]

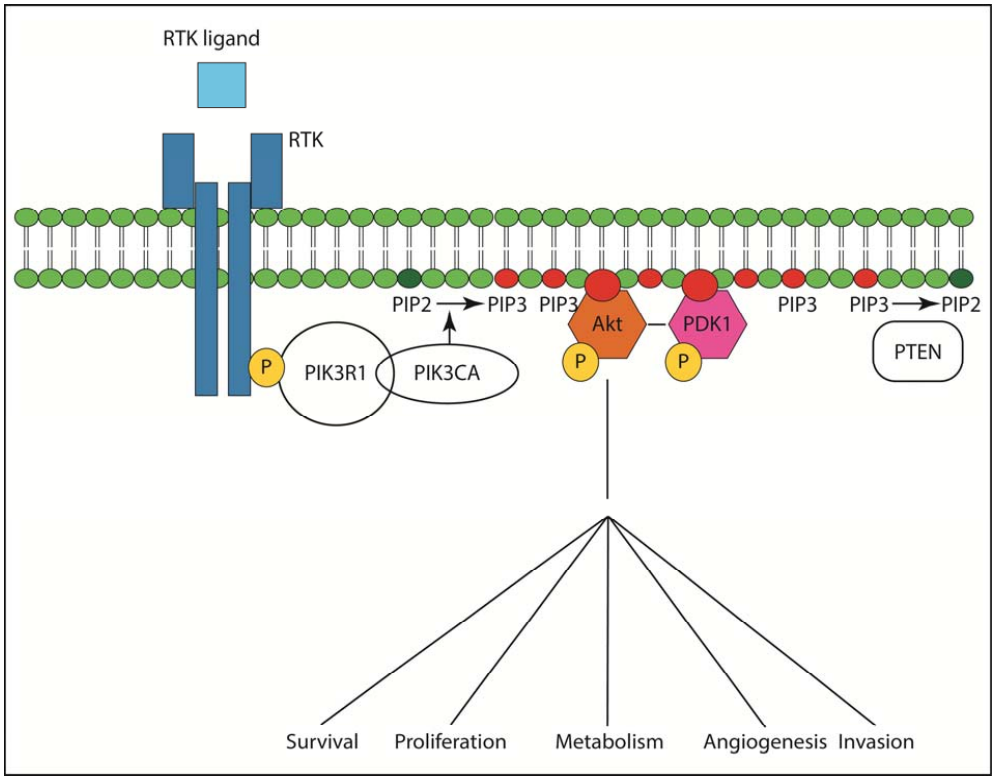
The papers included in this PhD project involve data especially on PI3K, TGF-β and ERα signaling and a brief introduction to these pathways in cancer is given.

PI3K signaling

The phosphatidyl-inositide-3-kinases (PI3Ks) are lipid kinases catalyzing phosphorylation of phosphatidylinositol, leading to activation of signaling pathways regulating a diverse panel of functions such as metabolism, vesicle trafficking, cell survival, and migration.[91,92]

Three classes of PI3Ks (I-III) are identified, with different isoforms within each class. The Class I PI3Ks, the class most studied in relation to cancer, is further subdivided into Class IA and IB. A catalytic (PIK3CA) and a regulatory subunit (PIK3R1) compose the heterodimer Class IA PI3Ks, and are coupled to and activated by receptor tyrosine kinases (RTKs).[92] The PI3K signaling pathway can be activated by binding of ligands to receptor tyrosine kinases such as *EGFR*, *HER2*, *VEGFR*, *FGFR2*, *IGF1R* and *PDGFR*, promoting tyrosine phosphorylation of an intracellular receptor domain, leading to increased affinity for specific intracellular proteins, such as *PIK3R1*. By binding of *PIK3R1* to phospho-tyrosine residues at the activated growth receptors, *PIK3R1* activates *PIK3CA* to catalyze the conversion of the membrane bound phosphatidylinositol-2-phosphate (PIP2) to PIP3. *AKT* and the phosphoinositides dependent kinase 1 (*PDK1*) are attracted to and directly bind to PIP3.[93] *PDK1* phosphorylates the serine-threonine kinase *AKT* that thereby is activated to phosphorylate downstream signaling transducers and effectors,[93] eventually contributing to biologic processes important to cancer initiation and progression, such as cell survival, proliferation, angiogenesis and metabolism (**Figure 4**).[94,95]

Figure 4. Schematic overview of PI3K signaling



Similar effects may be seen from specific somatic mutations of receptor tyrosine kinases and other PI3K pathway components that render the pathway constitutively active.[96,97] PIK3CA mutations are found both in endometrioid and non-endometrioid carcinomas,[72,98] exon 20 mutations more frequent in the endometrioid cases and exon 9 mutations mainly seen in non-endometrioid tumors.[64,99] The activating PIK3CA mutations as well as PIK3R1 and PIK3R2 mutations[100] and PIK3CA amplifications[79,101] demonstrated in endometrial carcinomas may contribute to sustained proliferation in this cancer type.

PTEN (Phosphatase and tensin homolog) contribute to PI3K pathway deactivation by dephosphorylating PIP3 to PIP2.[102] *PTEN* is frequently mutated in sporadic cancer, up to 80% reported for endometrioid endometrial carcinoma.[64] Low protein expression, promoter hypermethylation, mutations and miRNA-21 overexpression are identified in endometrial cancer[103-105] and are demonstrated to regulate *PTEN* expression and function, and potentially affecting to what extent *PTEN* further regulates PI3K signaling activation.[102,106]

The PI3K pathway is suggested as a key target for therapy in endometrial carcinomas.[30,64] Molecular alterations potentially participating to PI3K pathway activation are demonstrated with varying frequency distribution between type I and II cancer (**Table 1**).

Table 1. Frequency distribution of molecular alterations in Type I and Type II endometrial carcinoma; 1A) PI3K related alterations. 1B) Other selected molecular alterations.

1A: PI3K pathways related alterations

Target	Characteristic	Type I (%)	Type II (%)
ERBB2/Her2[107,108]	Amplification	1	17
	Overexpression	3-10	32
EGFR[108]	Overexpression	46	34
FGFR2[109-111]	Mutation	10-16	1
PTEN loss of function[103,104]	Mut/Methyl/Low expr	83	5
PDGFR[112]	Positive expression	91	73
	Phosphorylation	46	40
AKT[113]	Mutation	3	0
KRAS[71,109,114,115]	Mutation	11-26	2-4
	Amplification	2	10
PIK3CA[64,72,74,79,116]	Mutation	~30	~20
	Amplification	2-14	46
PIK3R1[96]	Mutation	20	0
PIK3R2[96]	Mutation	5	25

Table 1B: Other selected molecular alterations in endometrial carcinoma

TP53[99]	Mutation	~20	~90
TP16 loss of function[117]	Loss of expression	10	45
E-cadherin[118-120]	Low expression	5-50	62-87
Microsatelite instability[121,122]	Present	20-45	0-5
ERα, PR[123]	Low expression	31-44	56-69
VEGF-A[124]	Overexpression	16	36

TGF-β

The cytokine transforming growth factor-β (TGF-β) contributes to maintaining tissue homeostasis with regard to cellular proliferation and survival, differentiation and cell adhesion. The bioactive TGF-β dimer brings together two receptor serine /threonine kinases, TGF-β receptor I and II (TGFβRI, TGFβRII), and binding to TGFβRII is followed by phosphorylation of TGFβRI. The activated TGFβRI transmits the signal by phosphorylation of Smad transcription factors. In the nucleus, RSmad and Smad4 form a complex and recruits DNA-binding cofactors, and specific genes are targeted by each Smad4-RSmad-cofactor combination. TGF-β may in this way regulate the expression of multiple target genes at once.[125]

In cancer, the cancer cells and various cell types in the tumor microenvironment may be the source of TGF-β. In pathologic TGF-β signaling, the tumor suppressor properties from TGF-β is lost and tumor growth, evasion of the immune system, invasion and metastasis are demonstrated.[125] Pathologic effects from TGF-β may occur by inactivating components crucial to the overall TGF-β signaling, such as inactivating mutations in one of the TGF-β receptors, or by alterations in downstream pathways members that inhibit only the tumor suppressor activity from TGF-β, rendering the other TGF-β functions active and potentially supporting the carcinogenic and tumor progressive processes.[125,126]

TGF-β also exerts effects on the immune system and pro-tumorigenic effects may occur if the immunosuppressive effects are stronger than the tumor suppressive anti-inflammatory

effects.[125] TGF-β is also an inducer of epithelial-mesenchymal transition (EMT),[127] demonstrated in various cancer types, including endometrial carcinoma.[128-130]

A few studies have examined TGF-β pathway related alterations in endometrial carcinomas. Higher TGFβRII protein expression, Smad4 cytoplasmic protein and higher Smad2 and Smad 4 mRNA levels are associated with myometrial infiltration in the endometrioid subtype.[131] Also TGFβRII mutations are demonstrated to be associated with MSI and MLH1 promoter methylation and the TGFβRII alterations are suggested being due to mismatch repair deficiency.[132] Based on gene expression alterations related to high risk of recurrence, TGF-β signaling has been indicated as important for aggressive endometrial carcinoma,[130] including vascular invasion in this cancer type.[133] PAI-1, a suggested marker for TGF-β signaling,[134] is associated with clinico-pathologic features of aggressive endometrial carcinomas and reduced survival in several studies.[135-138]

Sex hormones and hormonal receptors

Estradiol signals through the nuclear estrogen receptors (ER) α and β and G-protein coupled estrogen receptor, GPER. Upon binding of the bioactive form of estrogen to ERα, the activated receptor act as a transcription factor with binding to estrogen responsive elements (ERE) for transcription of various genes. The activity of ligand bound ERα is regulated by co-factors (suppressors and activators) and receptor phosphorylation.[139] ERα is also membrane bound and may signal in a ligand-independent manner, due to phosphorylation of the receptor.[139]

ERα was the first ER discovered and is per today the ER most studied in relation to estrogen response both in normal and cancer tissue, in comparison to ERβ and GPER.[140,141] ERα and ERβ show different tissue distribution, also in the endometrium, where ERα is more abundant than ERβ.[139] The proliferative effect of ERα in the endometrium is counteracted by ERβ.[142]

In breast cancer, interactions between PI3K/AKT signaling and ERα is suggested; membrane bound ERα is phosphorylated by activated AKT and may contribute to ligand-

independent transcriptional ERα activity. ERα is subsequently found binding to PIK3R1, the regulatory subunit of PI3K, participating in PI3K/AKT pathway activation.[143] In endometrial cancer cell line studies, regulation of proliferation by ERα and GPER is demonstrated to act through Notch and PI3K/AKT signaling, respectively.[144] Also, estradiol is demonstrated to activate PI3K/AKT signaling in ERα-dependent and independent manners in ERα positive and negative cell lines, respectively.[145] In the Ishikawa endometrial cancer cell line expressing ERα, wild type *TP53* is shown to repress ERα transcriptional activity and some *TP53* mutations are linked to lack of such repression.[146]

Progesterone signals through two different isomers of the progesterone receptor (PR), PR-A and PR-B. PR-A is the one most studied and plays the major role in the endometrium where the ligand bound receptor down-regulates the actions exerted from ligand-bound ERα.[141] Expression of ERα and PR in endometrial carcinoma is regarded a sign of differentiation. In endometrioid histological grade 3 tumors and in non-endometrioid endometrial carcinoma, low ERα and PR expression is common.[63,147,148] A recent study suggests intact progesterone signaling as important for preserved immunosurveillance and to inhibit an invasive phenotype.[149]

Endometrial cancer biology in light of the "cancer hallmarks"

Sustained proliferative signal and evasion from growth suppression

For cell reproduction, where DNA is replicated and eventually split into two daughter cells, the cell passes through 4 stages (G1, S phase, G2 and M phase), each phase with specific progress in the reproductive cycle. In brief, G1 is a growth phase with protein synthesis required in particular for the DNA replication that takes place in the following S phase. In G2, the protein synthesis is again increased, related to large extent to microtubule formation, preparing for the coming mitosis in M phase. Cells that are not actively proliferating are quiescent and stably localized in G0.[150] For cells to be activated

from G0 and enter the cell cycle, mitogens or growth factor stimulation are needed. Cyclins and their corresponding cyclin dependent kinases (*CDK*) regulate the passage through various stages of the cell cycle. Some of the cyclins are targets for the downstream signal transduction from growth factors binding to their corresponding receptors. Potential DNA damage during cell cycle transitions induces cellular responses evoked by cell cycle checkpoint signaling in G1, G2 and M checkpoints, culminating in cell cycle arrest if replication errors are identified. Dysfunctional checkpoint signaling may lead to chromosomal aberrations potentially evoking carcinogenesis.[150]

Four sub-phases of the M (mitotic) phase exist (**Figure 5**), in addition to cytokinesis that parallels the telophase: In prophase, the chromatin is condensed and the centrosomes (consisting of centrioles and associated microtubules) are generated. The chromosomes are aligned in the equatorial plane in metaphase, while in anaphase the chromosomes are split and the microtubules are shortened. In telophase, the nuclear membranes are generated and chromosomes are decondensed. Cytokinesis contributes to completion of the cell division.

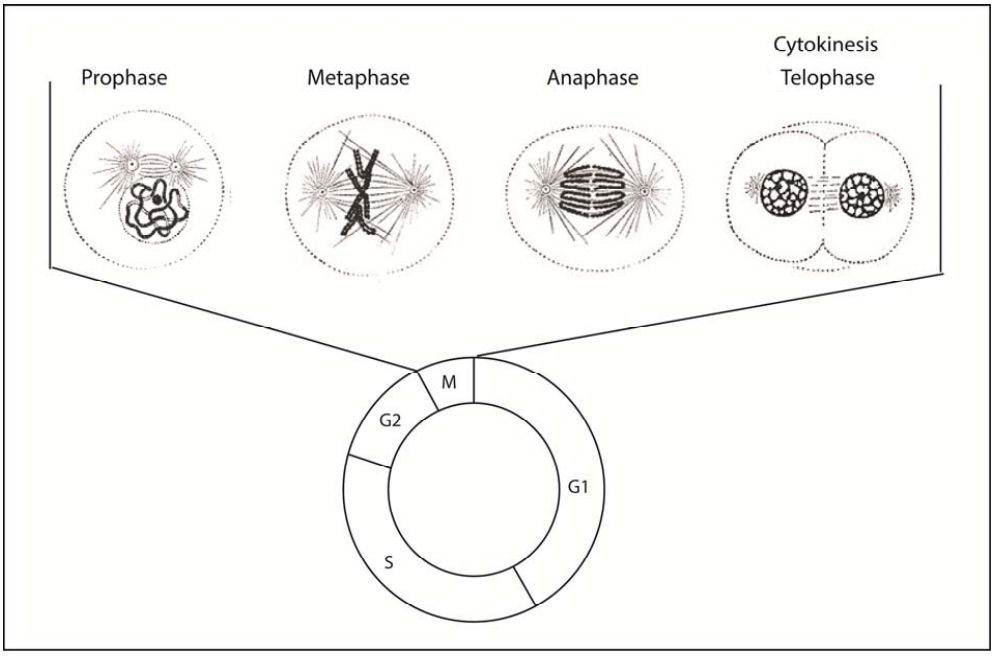
Microtubules, the cytoskeletal tubulin polymers, are involved in a plethora of cellular functions and also play an important role in mitosis.[151] The microtubules are dynamically shifted between states of elongation and shortening.[152] Stathmin is demonstrated to destabilize microtubules potentially through two different routes: By preventing the assembly of tubulin and by stimulation of the microtubule catastrophe.[152] The E2F family of transcription factors and *TP53* are transcriptional regulators of Stathmin.[153] Post-translational inactivation by phosphorylation of four Stathmin phospho-sites takes place. This stabilizes the microtubules in the cell cycle stages before de-phosphorylation and the eventual shortening of microtubules in anaphase.[151,153]

Mitogen signaling in cancer cells may occur through various routes:[10] 1. Autocrine proliferative signaling, where the cells produce growth factors themselves, for signaling through the corresponding cell surface receptors; 2. The cancer cells stimulate cells in the

tumor microenvironment to produce growth factors; 3. Altered levels or conformational changes of growth factor receptors may modify signaling in response to low levels of ligands or in a ligand-independent manner, respectively; 4. Constitutive activation of pathway members downstream of the growth receptor also promote ligand-independent signaling.

Several markers of cell proliferation are present in endometrial cancer. High mitotic count, high expression of the tumor proliferation marker PHH3, and elevated levels of Ki67 expression and S-phase fraction are regarded markers for tumor cell proliferation and are all associated with aggressive features and reduced survival in endometrial carcinomas.[154-157]

Figure 5. Overview of the cell cycle stages and details from mitosis (*Adapted from Gray's anatomy of the Human Body, 1918*)



High expression of Cyclin D1, with a regulatory role in G1, is associated with aggressive endometrial carcinomas. *CCND1* (encoding Cyclin D1) amplifications are more frequent in non-endometrioid endometrial carcinomas[158] and a *CCDN1* mutation interfering with the degradation of Cyclin D1 has been demonstrated in the endometrioid subtype.[159] In a recent study on genome wide assessment of 23 serous endometrial carcinomas, 57% had either a mutation in the ubiquitin protein ligase gene *FBXW7* or *CCNE1* amplification (encoding cyclin E),[74] both potentially contributing to increased proliferation. Cyclin E is an important regulator of the cell cycle progression, and is frequently up-regulated in cancer.[76] The amount of Cyclin E is regulated by *FBXW7*.[76] In line with these findings, high Cyclin E expression is associated with the proliferation marker Ki67 and features of aggressive endometrioid endometrial carcinomas.[160]

Activating mutations of PI3K pathway members (*e.g. PIK3CA*, *AKT*) and other PI3K pathway activating alterations are proven tumorigenic and able to induce sustained proliferation despite low levels of other mitogenic stimuli.[10,161] Also, activating *KRAS* mutations, as seen in endometrial carcinomas,[71,162] are regarded important to sustained tumor cell proliferation.[163] As mentioned, PI3K alterations with potentially increased PI3K pathway activity are frequently reported in endometrial carcinomas.[64,72,79,98]

TP53, a major tumor suppressor in many cancer types, supports evasion from growth suppression when loss of function occur.[164] *TP53* is frequently altered in endometrial carcinomas, more often in type II carcinomas,[64] and is associated with aggressive cancer and reduced survival.[115,165] *CDKNA2* (encoding p16) is regarded a tumor suppressor gene and plays a major regulatory role in G1/G1-S transition. *CDKNA2* is frequently altered in endometrial carcinoma; lower p16 protein expression, promoter hypermethylation and deletion of *CDKN2A* are demonstrated,[166] more frequent in non-endometrioid than endometrioid cancers, also reflected in reduced survival.[117,167]

Resisting cell death

Programmed cell death by apoptosis is limiting cancer development.[10] The regulators of apoptosis are divided into the *extrinsic program* (processing extracellular signals) and the *intrinsic program* (originating intracellularly) that both activate proteases that further participate in finalizing the apoptotic process, where eventually the cell debris is ingested by surrounding cells.[10] In homeostasis, pro- and anti-apoptotic proteins counterbalance the apoptotic signals. A low expression of the anti-apoptotic marker *Bcl-2* is reported in normal and hyperplastic endometrium and early stages of endometrial carcinoma, but with a higher expression in advanced cancer.[168,169] An opposite expression pattern, from higher expression in normal endometrium to lower expression in hyperplasia and cancer, has been demonstrated for of the pro-apoptotic marker *Bax*, with highest expression in early stage cancer.[168] The pattern of lower levels of apoptosis markers seen in advanced endometrial carcinoma may be due to the overall loss of cell homeostasis control in these cases.[168] The anti-apoptosis marker survivin is demonstrated to be stronger expressed in cancer as compared to normal endometrium.[170] The PI3K pathway, through *AKT*, is suggested to play an important role in the resistance to apoptosis.[171] Recent endometrial cancer cell line studies have supported such a link through modulation of *AKT* expression and drugs targeting the PI3K/AKT/mTOR pathway.[172-174]

Sustained tumor angiogenesis:

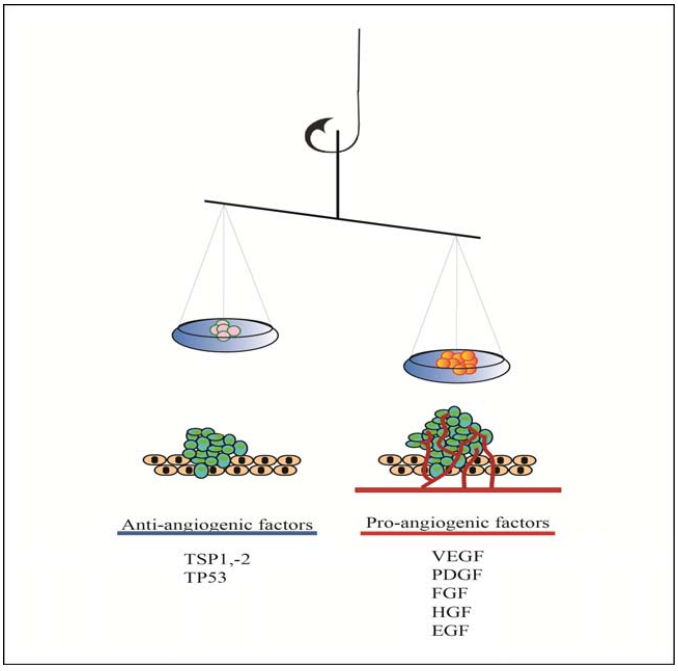
Algire and colleagues proposed in 1945 that rapid tumor growth is dependent on development of a vascular supply.[175] Judah Folkman, regarded an innovative pioneer in the field of angiogenesis research,[176] further explored on the observations by Algire and laid a foundation for angiogenesis research.[177-179] Folkman was the first to suggest that anti-angiogenesis treatment could be used in cancer therapy.[178]

Like normal tissues, tumors need nutrients, oxygen, and to deposit metabolic waste. Vascularization is needed for a tumor to exceed the size of 1-2 mm.[180] Formation of new vessels through vasculogenesis (generation of new endothelial cells into vascular tubes)

and angiogenesis (sprouting of new vessels from existing vasculature) aids to this. Angiogenesis is regarded to be balanced by pro- and anti-angiogenic factors (**Figure 6**), and an "angiogenic switch" to the angiogenic state, where the neovascularization is "turned on" is seen in development, wound healing and cancer.[10]

The molecules that stimulate or inhibit angiogenesis may be ligands of endothelial receptors exerting the pro/anti-angiogenic effects. Vascular endothelial growth factor-A (VEGF-A) and trombospondin-1 (TSP-1) are examples of important pro- and anti-angiogenic regulators, respectively. The ligand VEGF-A signals through three receptor tyrosine kinases (VEGFR1-3), and transcription of VEGF-A is stimulated by hypoxia and oncogenic signaling.[181]

Figure 6. Factors contributing to the "angiogenic switch".



The neovasculature in cancer is characterized by aberrant vessel morphology and endothelial proliferation, distorted blood flow and altered permeability.[182,183] A pathologic tumor circulation increases the risk of tumor hypoxia that further stimulates angiogenesis. There is a complex interplay between tumor cells, the vasculature and other cells in the microenvironment surrounding the cancer cells that participate in the regulation of the tumor angiogenic process.[184] Cells from the innate immune system are also demonstrated to play important roles in the cancer associated angiogenesis.[185] Also, evidence for angiogenesis in cancer precursor lesions is demonstrated, and is suggested to be an early event in tumorigenesis.[186]

Microvessel density (MVD; number of vessels per tissue square unit) is a measure of tumor vasculature but does not necessarily indicate the degree of angiogenesis or functionality of the present vasculature.[187] MVD is therefore suggested as a measure of the tumor metabolic burden and not angiogenesis per se.[188] High MVD is associated with features of aggressive endometrial carcinomas and reduced survival.[124,189] Amongst angiogenesis related measures in endometrial carcinomas, high VEGF-A and bFGF protein expression is reported to be associated with clinico-pathologic features of aggressive disease.[124,190] VEGF-A and VEGFR1 expression is also shown to be associated with aggressive disease and reduced survival in the endometrioid subset of endometrial carcinomas.[191] Various measures of proliferating microvessels have been suggested as markers of angiogenesis. Microvessel proliferation (MVP) measured by dual immunostaining of Nestin/ Ki67 or Factor VIII/Ki67 for proliferating vessels (proliferating microvessel density; pMVD) is demonstrated to be associated with aggressive cancer and reduced survival.[192,193] Vascular proliferation index (VPI; the ratio between the number of proliferating vessels and the total number of tumor microvessels) is suggested as a better marker for cancer neovasculature, and potentially angiogenesis.[187] In endometrial carcinomas, high VPI is associated with VEGF-A expression, tumor necrosis and reduced survival.[124]

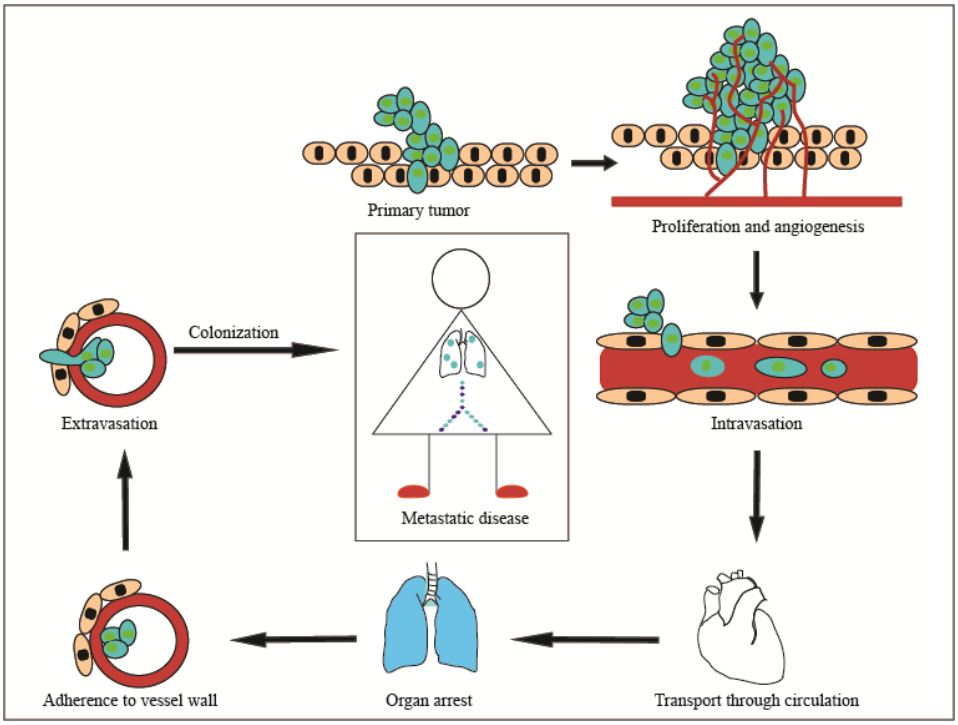
Invasion and metastasis:

The English surgeon Stephen Paget postulated "the seed and soil hypothesis" in 1889,[194] that tumor cells (denoted "seeds") have affinity for specific tissue environments in certain organs (denoted "the soil"), and laid by this the foundation for a large amount of invasion and metastasis research that has been performed for more than a century after this publication.[10]

Tumor cell invasion and metastasis are multistep processes immensely detrimental. The route to cancer dissemination is suggested by distinct steps; local infiltration, intravasation and transport of the cancer cells in the lymphatic or hematogenous systems, extravasation of cancer cells from the vessels to distant sites where micrometastases may form and grow to macroscopic lesions (**Figure 7**).[195,196] Before setting off in the invasion-metastatic cascade, it is regarded crucial that the tumor cells fulfill prerequisites such as the ability to detach and move from the original colony, with unlimited proliferative potential and a capacity to evade from destruction.[197] Genes that seem to support the metastasis to specific organs have been identified, although their exact functional mechanisms are more difficult to reveal.[198] The underlying effectors in the invasion-metastasis cascade is suggested to be classified as *metastasis initiating*, *metastasis progressing* and *metastasis virulent.*[198] Metastasis initiating genes generate a supportive environment that facilitates tumor infiltration to surrounding tissue. A tumor environment that facilitates cancer invasion is considered important to the invasive process.[10] Membrane bound proteases as MMPs and ADAMs may contribute to this, by remodeling the tissue in manners that ease the movement of cancer cells in the tumor tissue and by regulating the availability of extracellular growth factors.[199] In endometrial carcinoma, various MMPs are associated with features of aggressive disease and reduced survival.[200-202] MMP2, -3 and -9 is demonstrated associated with vascular invasion and myometrial infiltration.[133,201] Metastasis initiating genes may further promote angiogenesis, evasion from immune destruction and epithelial-mesenchymal transition (EMT), with all its implications to the cancer metastatic processes.[198]

Epithelial-mesenchymal transition: A conversion from an epithelial to a mesenchymal-like phenotype is regarded important for normal development and wound healing.[203,204] The ability of cancer cells to reversibly alter their phenotype in response to external signals is also denoted "plasticity",[205] and is regarded important for the metastatic process in general and for EMT in particular.[206,207] When pathologically activated, EMT may have significant impact on cancer invasion, apoptosis resistance, drug resistance and evasion from immune surveillance.[127] Epithelial cells that enter the EMT program lose their adherence to neighboring cells and the apicobasal cell polarity and also acquire migratory and invasive properties.[127] Reduced expression of E-cadherin, a protein with cell adhesive properties, is regarded one of the "EMT hallmarks".[10,127]

Figure 7. An overview of the invasion-metastasis cascade (*adapted from* [195]). In the patient figure, turquoise dots denote metastatic tumors (lung and lymph nodes).



Low expression of membranous E-cadherin and β-catenin, molecules implicated in cell-cell adhesion, is demonstrated in endometrial carcinoma and associated with aggressive clinico-pathologic phenotype and reduced survival.[120,208-210] N- and P-cadherin are also regarded as EMT markers,[127,211] and are together with the cell adhesion marker catenin p120 demonstrated to be altered in aggressive endometrial cancer.[120,209]

The E-cadherin repressors Snail, Slug, ZEB 1 and 2 and KLF8 are transcription factors that bind to the E-cadherin promoter and are regarded EMT inducers together with Twist, Goosecoid and FOXC2 that indirectly repress the E-cadherin expression.[10,127] A cooperation between EMT inducing transcription factors is suggested, and both spatial and temporal factors seems to be included in this complex interplay.[127] High Snail and Twist expression is associated with low E-cadherin in endometrial cancer.[212-215] One study suggested Snail upregulation linked to activation of EGFR.[216] L1CAM is associated with aggressive endometrial carcinomas and is suggested as an EMT marker in this cancer type. There is support for L1CAM upregulation being induced by TGF- β through Slug.[213] Several signaling pathways are suggested to trigger EMT, the TGF-β signaling being one potent inducer.[10,127,217] TGF-β/Smad mediated HMGA2 expression promotes transcription of Twist, Slug and Snail and subsequent E-cadherin repression.[218] HMGA2 is also found higher expressed in serous compared to endometrioid endometrial carcinomas.[219] A study of gene expression alterations related to recurrent endometrial carcinomas identified TGF-β signaling in tumors that recurred.[130] Functional cell line experiments confirmed an important role for TGF-β in the invasive phenotype. Also, TGF-β is associated with vascular invasion and EMT in endometrial cancer.[129,133] A recent and thorough exploration of EMT drivers in FIGO stage I endometrioid endometrial carcinomas revealed that E-cadherin repressors as Snail, Slug, Zeb1, Twist and HMGA2 was stronger expressed in endometrial carcinoma than normal endometrial tissue, and tumors with deep myometrial infiltration had higher expression of Slug, Zeb1 and HMGA2 compared to samples with none or superficial infiltration.[214] This study suggested the MAPK/ERK pathway as an important driver in EMT in early endometrioid endometrial carcinomas.

Micro-RNAs (miRNAs) are promoted as important contributors to EMT in endometrial cancer.[89,129]

Angiogenesis and vascular invasion is considered to be part of the *metastasis initiating process*. Vascular invasion is associated with clinico-pathologic features of aggressive endometrial cancer.[155,220] Absent expression of CD44v6, an isoform of the cell-cell adhesion molecule CD44 is associated with deeply infiltrating endometrial carcinomas and vascular invasion.[221] A study exploring on gene expression alterations related to vascular invasion in endometrial cancer identified VEGF and TGF-β as potentially related to vascular invasion and a 18-gene expression signature associated with vascular invasion was identified.[133] ANGPTL4 was one of the genes in the signature and was more highly expressed in aggressive tumors and also associated with vascular invasion. ANGPTL4 is previously linked to TGF-β signaling and suggested to facilitate trans-endothelial passage of tumor cells.[222]

Genes supporting *metastasis progression* promotes extravasation and survival of the cancer cells outside of their original environment.[198] Cancer cells that have entered the circulation may extravasate and infiltrate distant organs. It has been demonstrated that specific vascular beds have distinct molecular expression and tumor cells expressing the corresponding receptor may become entrapped in this capillary bed, as the step before extravasation.[196] Extravasation through capillary barriers further require specific molecular tumor cell characteristics that enable the cells for this function.[198] The endothelial cells in capillaries of some organs are fenestrated, making the passage of tumor cells more likely to occur. For extravasation to other organs with a tight endothelial layer, organ specific mediators are suggested for extravasation.[198] ANGPTL4 is pointed to as a specific mediator of lung metastasis,[198] and also demonstrated higher levels in aggressive endometrial cancer.[133]

For colonization to occur, a process where the disseminated cancer cells reside in their new microenvironment and grow into macrometastases, adaptation of the tumor cells to

this environment is required.[10] To succeed in the metastatic colonization, the cancer cells need to overcome microenvironmental hostility and activate self-renewal pathways.[223] Specific cancer cell gene expression is suggested to direct organ specific tropism; One example is the expression of IL-11 that facilitates breast cancer bone metastases.[223] A "receptive" environment at the future metastatic location, set up before the colonization of tumor cells, is denoted the *pre-metastatic niche*.[224] There is evidence that cancer-specific factors released from the primary tumor promotes changes in the future metastatic microenvironment before the tumor cells arrive to this location. Also, bone marrow cells migrate to the pre-metastatic niche in response to the systemically released factors, facilitating the environment for the cancer cells to "thrive".[225,226] Conversely, the presence of "resistant niches" has recently been described.[227]

The EMT-inducers Snail, Zeb1 and Twist promote self-renewal properties in cancer cells, a characteristic that promotes the metastatic colonization.[127] The gene and protein expression of organ specific metastases is not elucidated to large extent in endometrial carcinomas. However, higher frequency of KRAS amplification and low GPER expression is demonstrated in both lymph node metastases and distant metastases compared to primary endometrial cancer.[71,228]

1.4 Clinical picture and diagnosis

Clinical aspects

Primary endometrial carcinoma is located in the endometrium of the uterus. Bleeding from tumor may pass through the vagina, and abnormal bleeding is therefore a common early symptom.[28] Women in the pre- and perimenopausal periods will relatively frequently experience irregular bleeding,[229] for the majority of women caused by hormonal imbalance.[230] In contrast, a postmenopausal bleeding is an alarming sign for most women, and those experiencing this more urgently seek immediate medical examination to diagnose the cause of bleeding. The overall risk of endometrial cancer for a woman with postmenopausal vaginal bleeding is 5-10%, the risk increasing with increasing age and other additional risk factors.[231]

The spread of cancer occurs through three routes:[9]

1. Direct spread occurs by the direct seeding of tumor cells from the tumor to nearby areas. In endometrial cancer, direct spread may occur to the peritoneal cavity by tumor extending through the myometrium and uterine serosa, to the vagina through spread downwards from the uterine cavity or cervix, or growth may extend directly into the para-cervical area.
2. Lymphatic spread happens by transport through lymphatic vessels located at the tumor margins.[232] The pattern of metastatic involvement of lymph nodes follow the natural routes of lymphatic drainage, but "skip metastases" may occur when pelvic local lymph nodes are being bypassed in the metastatic spread,[9] as also observed with sole paraaortic lymph node metastases in endometrial carcinoma.[233,234]
3. Hematogenous spread occurs by tumor cell invasion mainly to veins. Tumor cells follow the blood flow draining the area where the tumor is localized, and reside in capillary beds encountered.[9] This spread pattern therefore frequently involves the liver and lungs. In endometrial cancer, distant metastases, in addition to distant

lymph node metastases, are found most frequent in the lungs, but also in liver, bone and brain.[234]

The diagnostic measures taken when suspecting endometrial cancer are primarily used to establish an accurate diagnosis of cancer, or exclude such one. Secondarily, having demonstrated the cancer diagnosis, the next critical step is to assess the extent of the disease to tailor surgical and systemic therapies. The goal is to identify cancer patient subgroups that are at higher risk for developing recurrence and therefore in need of more extensive surgical and systemic therapy, including adjuvant treatment. Although not yet implemented in clinical practice in a standardized manner, biomarkers such as hormone receptor status, may further identify high risk patients and represent a potential target for therapy, also indicating whether a patient is more likely to respond to the alternative therapies in the adjuvant and metastatic settings.[30]

Diagnosis

Abnormal vaginal bleeding often requires examination of endometrial cytology or biopsy.[235,236] If malignancy is suspected or the diagnosis based on a cytological examination is inconclusive, an endometrium biopsy is crucial. The majority of endometrial cancer patients are diagnosed by such a biopsy; out-patient endometrium biopsies by Pipelle de Cornier curettage or a classic fractional dilatation and curettage (D&C) with sampling separately from the uterine corpus and cervix.[28,237] The biopsy is required to confirm a diagnosis of cancer and in particular to reveal preliminary histopathologic diagnosis. Both the Pipelle curettage and D&C are considered equally sensitive and specific for diagnosing endometrial cancer,[238-240] although caution should be taken if negative or inconclusive cancer diagnosis by Pipelle, as it is demonstrated that Pipelle more often than D&C provide insufficient amount of tissue for proper histopathologic diagnosis.[241,242] Correct preoperative histopathologic diagnosis is important to guide the surgical treatment planning; Preoperative high-risk cases are

recommended referred to tertiary centers and operated by gynecologic oncologists with complete pelvic and para-aortal lymphadenectomy. Other patients may be safely operated at secondary centers.[237,243] More wide-ranging surgery by radical hysterectomy may be planned if disease extends into the cervical stroma.[244,245]

Imaging

Transvaginal sonography (TVS) is a diagnostic tool recommended in the evaluation of abnormal bleeding.[235,236] Assessing the endometrium of premenopausal women might be difficult, as the cyclic hormonal changes influence the endometrial thickness. The risk of endometrial cancer is reported to be only 1-2.3% if a postmenopausal endometrium is measured by TVS to be <4-5mm, including both endometrial layers.[246,247] This cut-off has been commonly used in the clinical setting when assessing risk of endometrial cancer based on findings from TVS.[236,248] The cut-off might need to be adjusted, depending on whether irregular bleeding is present or not. The authors of a recent meta-analysis recommend a cut-off for the endometrial thickness of < 3mm to exclude endometrial cancer in postmenopausal bleeding.[248]

TVS has been considered a potential tool to determine depth of myometrial infiltration and cervical stroma infiltration pre-operatively; the sensitivity for detecting myometrial infiltration ≥ 50% range from 62-78% and specificity from 81-94%.[249-252] For detection of cervical stroma invasion, sensitivity is reported to be 77-86% and specificity 85-99%.[251,252] Three-dimensional (3D) ultrasound is also suggested as a potential measure for improved assessment of myometrial infiltration.[253]

Examination of the chest and abdomen/pelvis for potential metastatic lesions is part of the pre-operative examination of endometrial cancer. Chest X-ray or computed tomography (CT) and abdominal/pelvic CT or magnetic resonance imaging (MRI) detect metastases in the depicted areas. Contrast enhanced MRI has been the preferred imaging method in preoperative staging the last years, and has demonstrated to be superior to CT and TVS in identifying deep myometrial invasion and cervical involvement.[254,255] The ability to detect

cervical invasion by MRI exceeds that of histologic evaluation by endocervical curettage as part of the D&C.[256] PET-CT is more sensitive for detecting metastatic disease, but the clinical benefit is unsettled and thus not yet part of standard preoperative examinations for endometrial carcinomas.[30]

Other potential diagnostic tools

As specific changes may occur in biological fluids and tissues before development of cancer symptoms, such alterations can in principal be used as markers for early diagnosis.[257] No serum or urine marker is presently available for routine clinical application to handle endometrial cancer patients today. CA125,[258] GDF15,[259] prolactin[260] and HE4, alone or in combination with CA125,[261] are amongst the serum/plasma markers shown to predict prognosis in endometrial cancer. The utility for these markers in early diagnosis and to individualize therapy for endometrial cancer patients remains to be further studied in larger and prospective studies.

Per today, screening for endometrial carcinoma in the general population by ultrasound, pipelle or serum markers is not recommended.[262,263]

1.5 Histopathology

The gross appearance of endometrial carcinomas may be one or more polypoid tumors, or the growth can be seen as a more diffuse expansion in the uterine cavity. The *Pathology Report Guidelines* by the Norwegian Society of Pathology (2012) recommends reporting on histologic subtype, histologic grade, vascular invasion, depth of myometrial infiltration and presence of cervical stroma infiltration, lymph node involvement and other extrauterine disease (surgical staging) as part of the routine histopathological reports for endometrial carcinomas.[264]

The histologic subtype of endometrial carcinomas is defined by the cell type(s) that constitute the specific tumor and is regarded to be important with respect to determine the clinical course of uterine cancers.[67] By histologic examination, 85-90% of endometrial carcinomas are endometrioid adenocarcinomas with a glandular pattern similar to normal endometrial epithelium.[28,64] These cancers also comprise adenocarcinomas with squamous, secretory or ciliated differentiation. Endometrioid tumors are graded based on the amount of solid growth of the glandular component, adjusted by nuclear features (FIGO grading):[9,265]

Histologic Grade 1: Adenocarcinoma with easily recognizable glandular pattern. Less than 5% solid growth.
Histologic Grade 2: Well-formed glands with interspersed solid sheets of malignant cells. Less than 50% solid growth.
Histologic Grade 3: Solid sheets of cells with barely recognizable glands. More nuclear atypia and mitotic activity. Solid growth > 50%.
Severe nuclear atypia raises the grade by one.

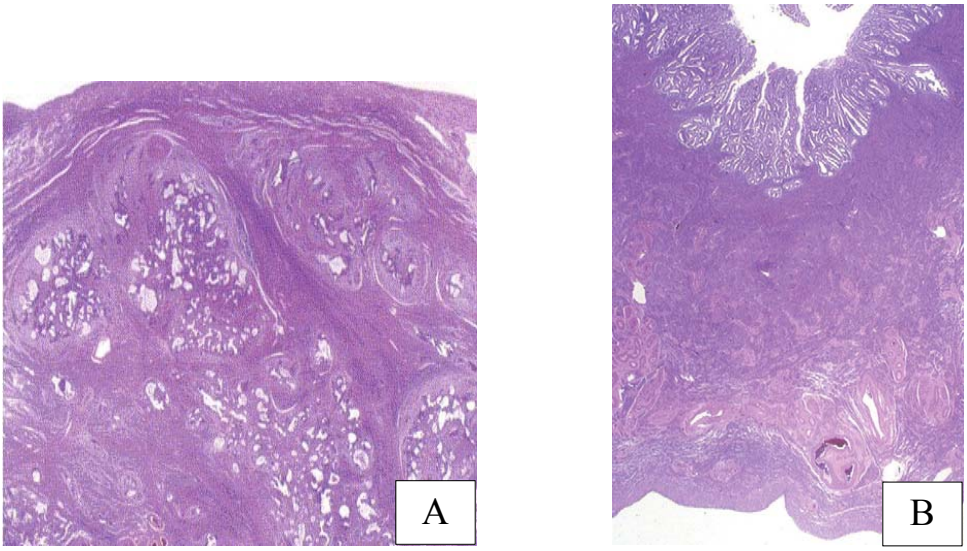
An alternative grading system has been proposed, where high grade cases included ≥ 2 of the following architectural features: 1) > 50% solid growth, 2) a growth pattern of diffuse infiltration, 3) tumor cell necrosis, as defined by areas of necrotic tumor adjacent to viable tumor tissue.[266]

The histologic subtypes serous, clear cell and undifferentiated carcinomas are included in the non-endometrioid (NE) endometrial carcinomas.[265] Serous carcinoma is the most frequent subtype amongst these cancers (3-10% of endometrial carcinomas).[64] Carcinosarcoma, also denoted Malignant Mixed Müllerian Tumor (MMMT), is a separate entity within the endometrial carcinomas. This histologic subtype is comprised by both epithelial and stromal components. Recent clinico-pathologic, immunohistochemical and genetic studies have provided evidence that carcinosarcomas most likely represent carcinomas with a mesenchymal component as a consequence of metaplasia and /or tumor progression.[267,268] Carcinosarcomas are today treated like endometrial carcinomas and are in studies often included in the non-endometrioid endometrial carcinomas.[64] All non-endometrioid carcinomas are classified as histologic grade 3 per definition and further grading is not performed.[265]

A proportion of endometrial carcinomas area denoted "ambiguous", with morphologic features overlapping with the known histologic subtypes, complicates the histologic diagnosis.[269-271] The clinical relevance to a more precise diagnosis is not yet clear.[271] Immunohistochemistry may be an aid to the histologic diagnosis and TP53 immunostaining is suggested as relevant to differentiate for example high grade endometrioid and serous carcinomas, also with add of PTEN and PR.[271,272]

Myometrial infiltration (**Figure 8A**) is recommended to be measured in mm. For cases with myometrial infiltration, the fraction of infiltration depth (mm) related to total myometrial thickness (mm) is reported.[264] As the endo-myometrial junction is not a straight line, the endometrial "tongues" of varying length towards the myometrium may mimic myometrial invasion and contribute to myometrial invasion being overdiagnosed (**Figure 8B**).[67,273] Determination of depth of myometrial infiltration is important for the distinction into FIGO stage IA vesus IB *i.e.* <50% versus ≥50% infiltration of the myometrial wall (**Table 2**).

Figure 8. A) Cancer infiltration of the myometrium. B) Endometrial cancer confined to the endometrium (Stage IA) resembling myometrial invasion by its irregular intra-endometrial growth. *Reprint with permission, from J Prat 2004.*[67]



Cervical infiltration was previously classified in the FIGO surgical staging criteria as none infiltration, epithelial involvement or stromal infiltration.[274] In the FIGO stage definitions revised in 2009, only cervical stromal infiltration is classified and treated as FIGO stage II disease.[275] Cervical involvement may occur from direct surface or stromal extension of the tumor growth from the uterine corpus, but may also be due to lymphatic spread[67] and has also been suggested as implantation from the diagnostic curettage procedure.[276,277] In the study by Jordan et al., it was denoted that only small areas of the cervical circumference was affected by tumor.[277] This points to the importance of appropriate sampling in the D&C procedure, and raises the awareness of interpreting the D&C histology report with care, regarding the diagnosis of cervical involvement.

Vascular invasion

Vascular invasion is reported to contribute independent prognostic information, and is thus recommended to be included in standard histopathology reports for endometrial carcinomas.[265] Perivascular lymphocyte infiltrates may help in identifying vessels with vascular invasion, while retraction artifacts around a tumor gland might be mistaken as vascular invasion.[67] Staining of endothelial cells with Factor VIII or CD31 facilitates recognition of vascular invasion, and blood vascular invasion may be separated from lymph vascular invasion by concurrent staining with D2-40 and CD31 antibodies.[278,279]

1.6 Therapy

The therapeutic aim for cancer has traditionally been to "cure" the patient from disease by eradicating the primary tumor and any micro-/macro metastases. This is in contrast to how we aim to treat various other diseases, e.g. hypertension and rheumatic diseases, where the overall aim is to relieve symptoms and prevent secondary harmful effects of the disease. Nearly 20 years ago, a change in the goal of cancer therapy was suggested, moving from a "*killing paradigm*" to a "*regulatory model*" where residual disease was controlled by regulation of pathways contributing to the deregulated growth.[280-282] The "regulatory model" was somehow the initial step towards the "targeted therapy" and "personalized medicine" concepts that both the scientific and clinical societies today embrace. The aim is to improve survival and quality of post-therapy life. The means are to administer appropriate therapy at the right time to the right patient groups.

Many treatment regimens in clinical use today (2013) are still based on the "killing paradigm", removing tumor surgically where possible and treating micro and macro metastases with systemic therapies and radiation.

In the following, an overview of evidence for current standard care will be given. The status for clinical implementation of the more novel and "targeted therapies" in endometrial carcinoma is presented in chapter 1.8.

Primary surgical treatment

Table 2. Surgical tumor staging accordingly the International Federation of Obstetrics and Gynecology (FIGO); 1988- and 2009 classifications.

Stage	FIGO 1988[274]	FIGO 2009[275]
I	**IA**: Tumor limited to endometrium	**IA**: Tumor with no or <50% myometrial Infiltration
	IB: Myometrial infiltration < 50%	**IB**: Myometrial infiltration ≥ 50%
	IC: Myometrial infiltration ≥ 50%	
II	**IIA**: Endocervical glandular involvement, Only	**II**: Tumor invades cervical stroma, but does not extend beyond the uterus
	IIB: Cervical stromal invasion	
III	**IIIA**: Tumor invades serosa and/or adnexa with/without positive peritoneal cytology	**IIIA**: Tumor invades the serosa of the corpus uteri and/or adnexa
	IIIB: Vaginal metastases	**IIIB**: Vaginal and/or parametrial involvement
	IIIC: Metastases to pelvic and/or para-aortic lymph nodes	**IIIC1**: Metastases to pelvic lymph nodes
		IIIC2: Metastases to para-aortic lymph nodes with/without positive pelvic lymph Nodes
IV	**IVA**: Tumor invasion of bladder and/or bowel mucosa	**IVA**: Tumor invasion of bladder and/or bowel mucosa
	IVB: Distant metastases, including intra-abdominal metastases and/or inguinal lymph	**IVB**: Distant metastases, including intra-abdominal metastases and/or inguinal lymph
	node metastases	node metastases

FIGO stage I: Hysterectomy and bilateral salpingo-oophorectomy (BSO) is the cornerstone in primary treatment of endometrial carcinomas.[283] The rationale for this therapy in early stage cases is to mechanically remove all tumor cells present, with curative intent. In young women with superficially growing lower grade endometrioid

tumors (FIGO stage IA), the ovaries may be preserved, although considered to have a potential risk for leaving behind malignant ovarian tumors from endometrial cancer spread or synchronous ovarian cancer.[283,284]

For carcinosarcomas, clear cell and serous endometrial carcinomas, omentectomy has been recommended due to the increased risk of intra-abdominal spread of these cancer subtypes similar to what is seen for serous ovarian cancer.[285,286] Although the gross inspection peri-operatively is advocated and normal macroscopic appearance predicts no omentum metastases (negative predictive value =0.94, CI=0.81-0.99),[287] omentectomy is frequently carried out as part of the primary surgical treatment to this group of endometrial cancer patients.[288] As the morbidity associated with omentectomy is considered low, this may favor the procedure in clinical practice.[288]

Lymphadenectomy: As the surgical FIGO 2009 staging classification relies on both pelvic and para-aortic lymph node status (metastatic or not),[275] both pelvic and para-aortic lymphadenectomy is recommended for complete staging.

The role of lymphadenectomy in primary surgical treatment of endometrial cancer has been heavily debated. The key controversies relate to finding the right balance between **the potential therapeutic effects** from surgery, directly or indirectly by identification of risk groups for adjuvant therapy, and **the side-effects** from the procedures.[289,290] Retrospective observational studies have shown a positive survival effect of lymphadenectomy in intermediate and high risk cases (HR for disease specific/overall survival=0.25-0.65),[291-294] while two large prospective randomized studies did not demonstrate any such effect.[295,296] Clinical practice varies from selective lymphadenectomy in patients with increased risk of nodal metastases to mandatory pelvic and para-aortic lymphadenectomy, independent of preoperative diagnosis as long as the patient's co-morbidities do not prevent such surgery.[283]

It has also been suggested that designing a prospective, randomized trial resolving the lymphadenectomy-question may not be ethical justifiable, as such a study would require

that some high-risk patients would not be given adjuvant therapy.[289] An alternative approach is to develop methods for more accurate prediction of negative lymph node status amongst patients with tumors confined to uterus that would not benefit from lymphadenectomy[297,298]

FIGO stage II: Extended or radical hysterectomy including excision of para-cervical and parametrial structures and a larger vaginal cuff is recommended to patients with cervical epithelial or stromal involvement.[288] Although not justified by randomized trials, several retrospective studies demonstrate a significant survival benefit from radical hysterectomy and BSO with or without lymphadenectomy, compared to simple hysterectomy alone.[244,299-301] Radical hysterectomy is associated with more intra- and postoperative complications,[302] and should therefore be designated to the appropriate patient subgroup.

FIGO stage III/IV: Patients with primary advanced disease may benefit from delbulking tumor burden. A meta-analysis of 14 retrospective studies (672 patients with advanced or recurrent endometrial cancer) indicated that complete cytoreduction ($\leq$ 2cm tumor left) improves overall survival time.[303]

Adjuvant treatment

Adjuvant therapy is given as supplementary treatment after primary surgery. The main rationale for administering adjuvant therapy after macroscopic tumor is considered surgically removed, is to eradicate potential microscopic residual disease not removed at primary surgery, aiming to improve overall survival and disease-related symptoms. Adjuvant therapy is in clinical practice given to patients at a certain risk of disease relapse, despite being radically operated.

Risk-stratifying algorithms for classifying endometrial carcinoma patients according to their risk of recurrence after treatment for localized disease have been suggested to guide the adjuvant therapy;[237,304] the guidelines per April 2013 in Norway are presented in

Table 3. Histologic type, histologic grade and depth of myometrial infiltration are included in the risk stratification.[237] Lymph gland assessment and DNA ploidy investigation are suggested to improve the risk stratification within the intermediate risk group.

Table 3. Risk of recurrence algorithm including histologic type, histologic grade and depth of myometrial infiltration (*from Norwegian Gynecologic Oncology Guidelines 2009*)

NORWAY (2009)	FIGO IA/IB	FIGO IC
Endometrioid Grade 1 or 2	Low risk	Intermediate risk
Endometrioid Grade 3	Intermediate risk	High risk
Non-endometrioid histologic type	High risk	High risk

The National Comprehensive Cancer Network clinical practice guidelines include age >60 years, lymphovascular space invasion, tumor size, and lower uterine involvement in addition to histologic grade and myometrial infiltration to guide selection for adjuvant therapy.[304]

Women of age <60 years, FIGO I surgically staged with endometrioid histology, histologic grade 1 or 2, myometrial infiltration <50%, and no lymph vascular space invasion, are expected to have low-risk of metastatic disease and no adjuvant therapy is recommended.[237,290,304]

Adjuvant radiation therapy

Radiation therapy may be administered internally as vaginal brachytherapy or externally, directing radiation to the pelvis, para-aortic or whole abdominal areas. Four randomized controlled trials have demonstrated significantly reduced risk of local recurrence after adjuvant external radiotherapy for FIGO stage I and II cases, but no survival benefit.[305-309] Based on subgroup analyses though, a potential survival benefit amongst FIGO stage IC

patients is possible (registry based study).[310] Meta-analyses have supported an effect on survival for patients with stage IC, grade 3 tumors.[311,312] In order to see if vaginal radiotherapy was as effective as external radiation for vaginal control, a randomized trial of 427 stage I or IIA patients compared vaginal and external radiotherapy. It was demonstrated that vaginal brachytherapy was effective in ensuring vaginal control with no significant differences in local recurrences or survival for the two treatment alternatives.[309] The frequencies of gastrointestinal side effects were significantly lower for patients who received vaginal brachytherapy.[313] In view of these results, some advocate that vaginal brachytherapy should be the standard adjuvant treatment to patients regarded of intermediate risk for metastatic disease.[314] Adjuvant external radiation therapy should be chosen for patient subgroups with high risk of recurrences, as the benefits from such therapy outweigh the risk of treatment associated toxicities.[314]

Both internationally and in Norway, the use of adjuvant radiation therapy in clinical practice has declined, accompanying the increase in surgical staging.[315-318]

Adjuvant chemotherapy

There is a need for effective systemic therapies as patients with uterine confined tumors recur at a relatively high frequency (up to ~30%) after receiving adjuvant pelvic radiation therapy.[319] Systemic therapies have advantages above radiotherapy, potentially attacking tumor cells also outside of irradiated area.

The use of adjuvant chemotherapy has been increasing in Norway.[317] Platinum based chemotherapy, anthracyclines (e.g. doxorubicin, epirubicin) and paclitaxel are the classes of drugs most frequently used, both in the adjuvant and metastatic settings.[320,321] A recent Cochrane review compared the effect of adjuvant chemotherapy in addition to surgery with or without radiation, and concludes that chemotherapy is likely to improve overall survival (HR=0.74, 95% CI: 0.62-0.89) and limits the risk of extra-pelvic recurrences (HR=0.79, 95% CI: 0.68-0.92). The platinum based therapy is assumed to be a main effector.[320]

Merged data from two randomized trials demonstrated significantly increased 5-year progression-free and disease specific survival when adding chemotherapy to external radiation therapy (72%-79% and 79-88%, respectively).[322]

There are ongoing randomized clinical trials evaluating the effect of radio- and chemotherapy and combinations of these for patients with localized and advanced disease (*www.clinicatrials.gov, September 2012*). These studies may also clarify unresolved issues related to such combination therapy; the patient subgroups benefitting most from this therapy, optimal treatment algorithms and selection of patients for external or vaginal radiotherapy.[314]

Adjuvant hormonal therapy

One Cochrane review on adjuvant progestagens in endometrial cancer included studies of stage I cases as well as primary advanced endometrial cancer.[323] Progestagens was not supported as adjuvant therapy. However, the tumor progesterone receptor status was not assessed in any of the studies, likely to be of importance when evaluating the effect of a ligand binding to this receptor.

Applying adjuvant therapy is a lot about risk-definitions and ensuring that the benefits from the therapy outweigh the adverse effect, leaving the patient with the optimal quality of life, regarded the circumstances.[320]

Treatment of primary advanced and recurrent disease

The choice of therapy for women with recurrent and advanced primary disease depends on various factors, such as the localization(s) of the tumors(s), previous treatment and the general health of the woman. The clinical picture at recurrence may range from a solitary vaginal recurrence with a potential for curative treatment, to widespread systemic disease.

Surgery, radiation therapy and systemic therapy (chemotherapy and hormonal therapy) are thus all alternative therapies in the recurrent setting.

Postoperative chemotherapy (dual regimen) to women with FIGO stage III or IV cancer, surgically debulked to residual tumor ≤ 2 cm, is demonstrated to have better effect than whole abdominal radiation with respect to progression-free and overall survival, HR=0.71 (95% CI: 0.55-0.91),[324] and is today the first choice of postoperative treatment for women with primary advanced disease.[283] A rationale for combined chemotherapy and radiation has also been demonstrated,[325] and is frequently used in clinical practice, although the best regimen for such combined therapy is probably yet to be determined.[283]

No single chemotherapeutic drug is demonstrated to have clear beneficial effect in primary advanced or recurrent endometrial cancer. Doxorubicin and Cisplatin are regarded the most active drugs in mono-therapy; response rates range from 17% to 25% for Doxorubicin.[326-329] Combinations of chemotherapy are demonstrated to have better effects on disease free survival when compared to less intensive therapy regimens, although increasing toxicity is also seen with the more intense treatment regimens.[330] Combinations of Doxorubicin and Paclitaxel or Cisplatin in the recurrent setting demonstrated similar response rates and survival.[326,331]

Vaginal recurrences are primarily treated with radiation therapy in patients with no such previous treatment.[283] Isolated vaginal recurrences in surgically treated endometrial carcinoma stage I cases should be evaluated for surgical therapy and have also been successfully cured with combined whole pelvic and vaginal radiation therapy, with 5-year overall survival after diagnosis of the recurrence of 75%.[332]

As radiation therapy is not given to a previous irradiated field, radical surgery is the only curative option for women who received radiation therapy as adjuvant therapy.[283,288] Surgery in such area presents challenges with respect to poor wound healing, increased risk of infections, and secondary hemorrhage and difficult hemostasis,[333] leading to increased risk of surgical complications and long-term co-morbidities.[288] However, a

retrospective study of 61 endometrial cancer patients demonstrated that complete cytoreduction of pelvic recurrences with no residual gross disease was beneficial based on an observed longer post-recurrence survival than for patients with gross residual tumors (39 months versus 13.5 months, respectively, $P<0.0005$).[334] The not randomized design for the study should call for caution in the conclusions though.

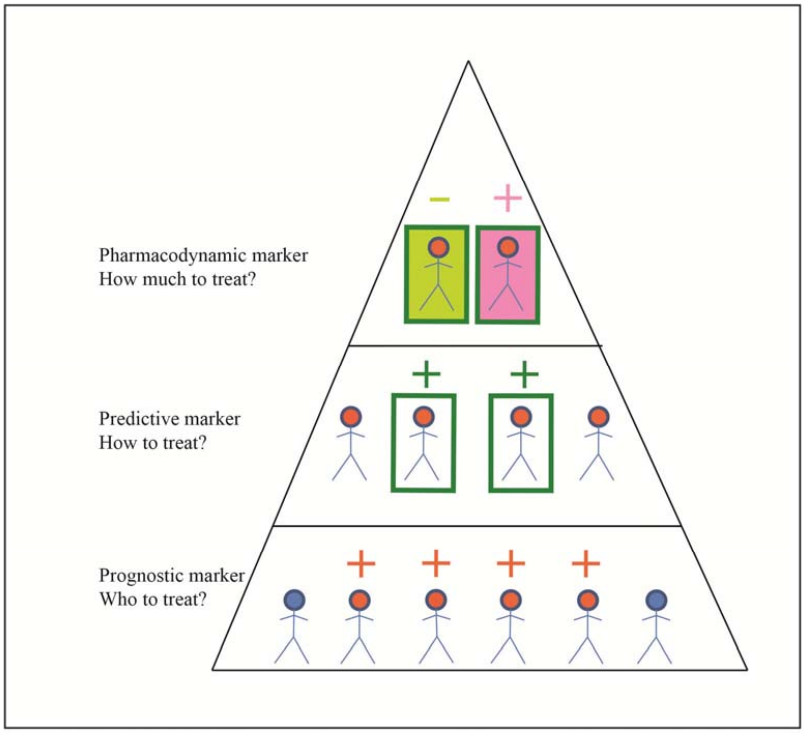
The effect of various hormonal therapies (e.g. progestagens and anti-estrogenic drugs) to advanced and recurrent endometrial carcinoma is evaluated in several studies, reporting varying effects, from no effects to response rates up to 30%, with median overall survival 7-11 months.[335,336] A recent Cochrane review evaluating the effect of hormonal therapy in advanced and recurrent endometrial carcinomas states that the evidence from randomized controlled trials per 2010 is inadequate to conclude in this question. Several of the studies included have reported response to hormonal treatment, independent of the tumor expression of progesterone and/or estrogen receptors.[336] In studies evaluating response according to hormone receptor status, patients with receptor positive primary tumors have been reported to respond better than patients with receptor negative tumors.[337,338]

1.7 Biomarkers in endometrial cancer

The National Institute of Health Biomarkers Definitions Working Group defined a biomarker as a "Characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[339]

Cancer biomarkers are ideally used to aid in decisions related to diagnosis and treatment: "Who to treat" (prognostic markers), "How to treat" (markers predicting therapy response); and "How much to treat" (pharmacodynamic markers), as illustrated in a hierarchy in **Figure 9**.

Figure 9. Types of cancer biomarkers and their clinical application for therapy decisions. "+" denotes "marker positive" and implies next level biomarker assessment.



A *prognostic biomarker* identifies groups amongst cancer patients that are likely to experience recurrence and poor outcome. Such patient groups may potentially benefit from more extensive therapy to reduce the risk of recurrent disease and thereby improve outcome/survival if the treatment is effective. Biomarkers that predict therapy response to a specific therapy are named *predictive biomarkers*. When the appropriate drug for the appropriate patient is selected, *pharmacodynamic biomarkers* may further assist in selecting the optimal dose of a specific drug to further improve the benefit from the treatment and reducing side effects.

From the biomarker definition given by the NIH Biomarkers Definitions Working Group, both clinical, histopathologic, imaging and molecular data might be relevant as biomarkers in cancer.[339]

Prognostic biomarkers

Clinical factors

Nulliparity is associated with reduced survival, also in multivariate analyses adjusting for clinico-pathologic variables (HR 1.5-2.8).[340,341] High age is also suggested as an independent prognostic marker in endometrial carcinomas,[165,342] and high histologic grade and non-endometrioid histologic subtype are more frequently occurring in patients of higher age. This, together with the relative decrease in complete surgical staging as well as less aggressive therapy, both in the adjuvant and metastatic setting,[234] may contribute to explain the prognostic impact of age.

Histopathologic factors

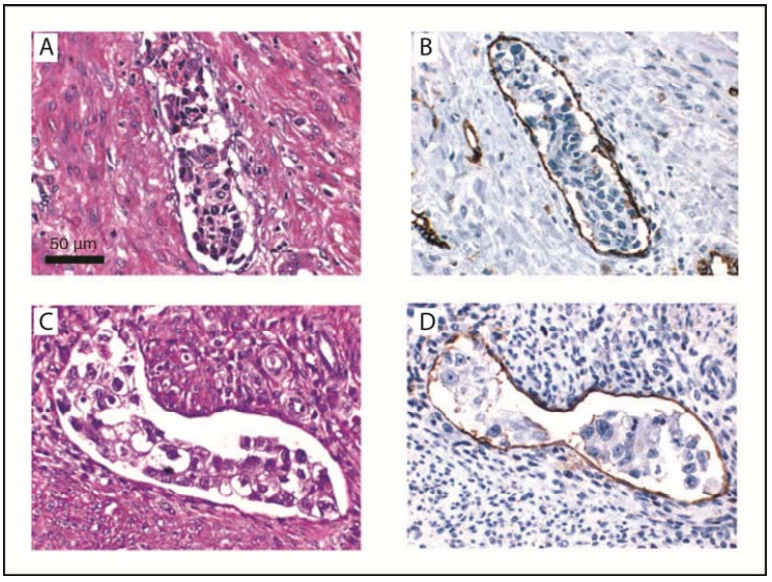
Non-endometrioid endometrial carcinomas are associated with poor survival. Patients with these tumors experience 35-45% 5-year survival, compared to 75-83% survival in patients with endometrioid subtype.[28,29,234,343] The 5-year survival is also decreasing with increasing histologic grade in endometrioid cases, and is in one study reported to be 98%

for grade 1 cases, 89% for grade 2 cases and 67% for grade 3 cases.[155] High histologic grade is associated with reduced survival, also when adjusting for other clinico-pathologic variables.[234,344-346]

Deep myometrial cancer infiltration (>50%) is associated with poor survival independently of FIGO stage and histologic subtype, and is also associated with lymph node metastasis.[155,342,347,348]

Vascular invasion is a strong prognostic marker in endometrial cancer.[155,220] Also, separate registration of lymph vascular invasion or blood vessel invasion (**Figure 10**) has demonstrated independent survival impact for each of these factors in multivariate survival analyses, as well as the strongest impact from the latter[279,349]

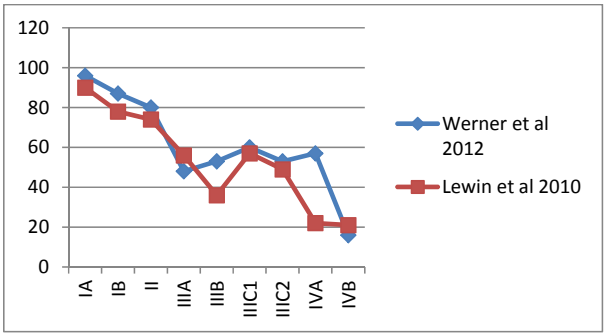
Figure 10. Blood vascular invasion (CD31 positive vascular cells surrounding tumor cells, A-B) and lymph vascular invasion (D2-40 positive vascular cells surrounding tumor cells, C-D). *Reprint with permission, from Mannelqvist et al 2009.*[279]



High mitotic count, a measure of increased tumor cell proliferation, is associated with prognosis in endometrial cancer.[155] Presence of tumor infiltrating lymphocytes are shown to be associated with improved overall survival, independent of age, FIGO stage, myometrial infiltration, histologic subtype and grade suggesting the importance of immunologic reactions to tumor.[350]

FIGO stage is by far the strongest prognostic marker in endometrial carcinoma.[234] The stages are defined by histopathologic assessment of depth of myometrial infiltration, presence of cervical infiltration and spread to lymph nodes and other organs in the pelvis and beyond (**Table 2**, Chpt 1.6).[274,275] A multicenter study of more than 1200 patients supports that the FIGO stage 2009 classification has improved prediction of prognosis, and demonstrates that FIGO stage has independent prognostic impact also amongst FIGO stage I cases in multivariate survival analyses, when adjusting for histologic subtype and grade.[351] The same study reports on 5-year survival of the various FIGO stages, ranging from 96% for stage IA to 16% for stage IVB, also demonstrated in a large register based study of more than 80 000 patients (**Figure 11**).[352]

Figure 11. 5-year survival (%) from the different FIGO stages (2009); the numbers according to two studies.[351,352]



Molecular markers

The molecular markers ER, PR, TP53, p16 and Ki67 are the prognostic biomarkers assessed by IHC most studied in endometrial carcinoma. Genomic analyses including DNA ploidy and copy number alterations and mutations have been increasingly studied the last decades, with a focus on PTEN, PIK3CA, KRAS and Her2, as elucidated below.

Steroid hormone receptors

Both estrogen and progesterone influence endometrial cell proliferation. The receptors of these ligands, estrogen receptor α (ERα), ERβ and progesterone receptor (PR) as well as GPER are studied in relation to their prognostic impact in endometrial cancer. An association between low ERα expression and survival was first demonstrated more than 30 years ago.[353,354] These findings are validated in several studies, also in multivariate survival analyses,[123] although not yet included in the routine assessment directing therapy in endometrial cancers.[30] Low ERβ2 IHC expression is shown to associate with high FIGO stage and high Ki67, but not to survival.[355] Low GPER expression is demonstrated to be associated with poor survival,[228,356] also within the subgroup of ERα positive cases.[228] Patients with low or absent tumor PR expression are associated with poor outcome compared to cases with PR expression.[123,357]

Cell cycle and proliferation markers

High expression of the cell proliferation marker Ki67 is demonstrated to be associated with clinico-pathologic variables of aggressive tumor phenotype,[156,165,358] also with independent impact on survival when adjusted for clinico-pathologic variables and other molecular markers.[165,359,360] High mitotic count, assessed by the cell proliferation marker PHH3 has been demonstrated to be independently associated with reduced survival.[157] High S-phase fraction (large proportion of cells in S-phase) is also associated with poor outcome.[361-363] Low expression of p21, an inhibitor of cyclin-dependent kinases implicated in the cell cycle regulation, is associated with reduced survival in univariate but not multivariate survival analyses.[165,364]

DNA ploidy

Aneuploidy, defined by an abnormal number of chromosomes in the cells, may reflect the general chromosomal aberrations experienced in cancer cells. DNA aneuploid tumors are demonstrated to be associated with a clinico-pathologic phenotype of aggressive cancer and reduced survival,[148,365,366] also in subgroups of FIGO stage I tumors.[367,368] DNA ploidy is also independently associated with prognosis in multivariate survival analyses (**Table 4**).[369-371] DNA index > 1.2 is in a recent study associated with further reduced survival and distant metastases, compared to better survival and local recurrences in tumors with DNA index 1.06-1.2.[365]

Table 4. DNA ploidy as prognostic marker: Overview of recent studies exploring the prognostic importance of DNA ploidy in endometrial carcinoma (studies from year 2000).

Authors[a]	Year[b]	Prognostic impact[c]	MV[d]	FIGO included in MV[e]
Sorbe B.[371]	2012	UV, MV[h,i]	*	*
Pradhan et al.[365]	2012	UV[f,h,j]		
Song et al[370]	2011	UV, MV[k]	*	*
Mangili et al[369].	2008	UV, MV[h]	*	
Susini et al.[366]	2007	UV, MV[i,k]	*	*
Osmanagaoglu et al.[372]	2005	UV[h]	*	*
Terada et al.[373]	2004	UV[h,k]		
Santala et al.[374]	2003	UV, MV	*	*
Lundgren et al.[375]	2003	UV[i]		
Mangili et al.[376]	2002	UV, MV	*	
Lundgren et al.[377]	2002	UV, MV[k]	*	*
Jhala et al.[378]	2001	UV	*	*
Nagai et al.[379]	2000	UV[h]	*	

Footnotes:[a] Reference. [b] Year of publication. [c] Prognostic impact shown in Univariate (UV) or multivariate (MV) survival analyses. [d] MV survival analysis performed. [e] FIGO 1988 or 2009 included in MV survival analyses. [f] Endometrioid FIGO I/II, only. [g] Endometrioid FIGO I, only. [h] Overall survival. [i] Disease specific survival. [j] Progression free survival. [k] Recurrence free survival.

Hogberg et al. included DNA ploidy, myometrial invasion and a histopathologic score in a panel defining low or high risk endometrial carcinoma (serous and clear cell histology excluded).[380] The high risk group, including DNA aneuploid cases, demonstrated significantly reduced survival and 50% of the progressions in this group were distant metastases, pointing to the importance of developing effective systemic therapies to this group.

Tumor suppressor genes and markers of oncogenic pathways

Mutations of TP53 and high IHC expression of TP53, a surrogate marker for TP53 mutations, are overlapping in 76 % of endometrial carcinomas,[381] and are associated with high FIGO stage and other markers for aggressive clinico-pathologic phenotype and reduced survival in this cancer type.[165,167,382-384]

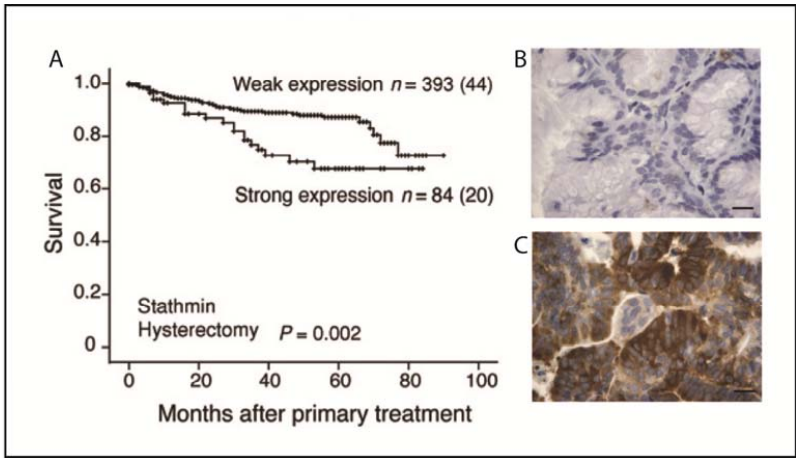
Inactivation of the tumor suppressor p16 as estimated by loss of protein expression is associated with poor prognosis in endometrial cancer. Deletions, point mutations and promoter hypermethylation are potential mechanisms of inactivation, and present in endometrial carcinomas.[117,166,167]

Mutations in the tumor suppressor PTEN are the most frequent genetic alterations detected in endometrial carcinomas. Inactivating PTEN mutations are in the majority of studies of endometrial cancer associated with type I cancers, better survival and non-aggressive clinico-pathologic features such as endometrioid histology in particular.[64,103,104,385,386] Loss of PTEN function is described as an early event in the carcinogenesis from normal endometrium to endometrial cancer, being present also in atypical endometrial hyperplasia.[387,388]

The oncoprotein Stathmin was initially suggested as a marker for PTEN loss in a breast cancer study[389] and is suggested as a surrogate marker for PI3Kinase activation. Stathmin is recently identified as a strong prognostic marker in endometrial cancer.[298,390] High Stathmin IHC expression (**Figure 12**) is associated with clinico-pathologic features of aggressive disease and reduced survival, also in multivariate survival analyses, adjusted

for traditional clinico-pathologic variables such as age, FIGO stage, histologic subtype and grade. High Stathmin expression in tumor tissue from pre-operative curettage also predicted lymph node metastases.[298]

Figure 12. Reduced disease specific survival (A) in cases with Stathmin-high (C) compared to Stathmin-low (B) immunostaining. *Reprint with permission, from Trovik et al.*[298]



Alterations of various PI3K pathway members such as PIK3CA mutations and amplifications have been identified in endometrial carcinomas.[64,74,79,101,116] Furthermore, Salvesen and colleagues demonstrated that amplification of the 3q26 region, harboring PIK3CA, was associated with reduced survival in this cancer type.[79]

Her2 protein overexpression is associated with reduced survival, also in various models of multivariate survival analyses, [107,391-393]although other studies have not positively validated this finding.[63] Her2 overexpression and amplification is more frequently occurring in non-endometrioid cancer,[108,394] and more prevalent in serous carcinomas as compared to the other histologic subtypes within non-endometrioid carcinomas.[395] Her2 amplification is also shown to be associated with reduced survival in univariate survival analyses.[393] Morrison et al demonstrated in a study of 483 endometrial carcinomas that

concurrent Her2 overexpression and amplification was independently associated with reduced survival when adjusted for clinico-pathologic factors such as age, histologic grade and FIGO stage.[396]

EGFR overexpression is in some studies associated with reduced survival,[108,397] although other studies have not demonstrated this association.[398]

KRAS point mutations are found both in endometrial hyperplasia and endometrial carcinoma, indicating that KRAS might be important early in the carcinogenic development from hyperplasia to endometrial cancer.[399] A recent study of 414 primary endometrial carcinomas and 61 metastatic lesions found KRAS amplification and high KRAS mRNA, but not KRAS mutations, associated with reduced survival.[71] Also , in this study KRAS mRNA expression and the frequency of KRAS amplifications increased from primary to metastatic lesions.

Biomarkers predicting therapy response

Hormone receptor (ERα and PR) expression is suggested as markers predicting therapy response to hormonal therapy, such as Tamoxifen and progestagens. Recently two large literature reviews have evaluated hormonal therapy in the metastatic setting in endometrial cancer.[335,336] Both studies conclude that trial design overall has been too unstructured and very few studies have stratified according to ERα and PR status when evaluating therapy response. However, a few studies have assessed the response according to hormonal receptor status and support ERα and PR as predictive markers for hormonal therapy.[337,338] However, randomized controlled trials, stratified according to ERα and PR status are recommended to conclude in this matter.[336]

Only approximately 10% of the presently ongoing clinical trials on targeted therapy in endometrial cancer are biomarker restricted (**Tab 8**, Chpt 1.8). As the focus on the importance of more molecularly based clinical trials and development of predictive

biomarkers in parallel is increasing, hopefully data from these and similar clinical trials will be explored in the search for valid markers predicting therapy response and further tested in new biomarker restricted clinical trials.

In four of the 14 published phase I/II trials on targeted therapies in endometrial cancer, (**Table 9**, Chpt 1.8, hormonal therapy studies not included), potentially predictive markers have been assessed in the search for markers to select patients with response. In the study of the EGFR inhibitor erlotinib in 32 recurrent or metastatic endometrial cancer patients, none of the EGFR markers (IHC, amplification by FISH, mutations status) were associated with erlotinib response.[400] In a study of mixed cancer types (in total 136 patients; 15 endometrioid endometrial carcinomas), PIK3CA mutations and/or PTEN loss/mutation were suggested as predictive markers to combined bevacizumab/temsirolimus/liposomal doxorubicin, and this needs further evaluation in larger patient series.[401]

In a study on 56 advanced endometrial cancer patients, the response to the VEGF-A antibody bevacizumab was suggested to be associated with low pretreatment plasma VEGF-A.[402] However, the question whether VEGF-A is a potentially predictive marker for bevacizumab remains open. Her2 alterations (IHC expression and amplification by FISH) were evaluated as a potential predictive marker to the anti-Her2 antibody trastuzumab in 34 endometrial carcinoma patients. No major tumor response was seen, and neither Her2 IHC expression nor Her2 amplification was associated with survival.[402] Two recent studies including endometrial cancer and various other cancer types, have suggested the H1047R PIK3CA mutation as predictive marker for response to PI3K/mTOR inhibitors.[73,403]

Gene expression signatures as biomarkers

Global gene expression data may have a stronger potential to reflect the complexity of cancer biology as compared to the detection of single gene alterations, and also for

identifying markers for more complex biological processes taking place in the cancer cells. When taking the global expression pattern into account, we somehow compensate for the lack of knowledge regarding "the complete picture" of specific signaling pathways and the phenotypic consequences including potential compensatory mechanisms derived from their de-regulation.

Gene expression arrays have the last ~15 years been increasingly applied in translational cancer research. Some of the first array studies within this research field demonstrated that gene expression data could identify both known and new molecular cancer subclasses with similarities in terms of biological behavior.[404,405] In addition to identifying molecular phenotypes in various cancer types, such as breast, bladder and colorectal cancer,[406-410] the transcriptional alterations underlying the tumors examined have demonstrated to be powerful with respect to creating classifiers predicting cancer recurrences in breast and colorectal cancer,[411-413] and to identify alterations in functional pathways and thereby suggesting relevant targets for therapy.[414]

Analyses of the global transcriptional pattern in cancer have identified molecular subclasses associated with prognosis. The number of genes differentially expressed between classes has in many studies been reduced to a limited number of genes with maintained prognostic information presented as prognostic *gene expression signatures*. Such signatures (*i.e.* gene sets) might be regarded as *metagenes* with respect to expression value, and a *signature score* is calculated to evaluate the *metagene expression value*.[415] Other signatures than gene expression signatures have been published in cancer (*e.g.* sets of miRNAs, proteins or methylation sites unified by various means into the "signature").[416-418] Similarly, such signatures have been developed to predict response to specific treatment regimens.[419-421]

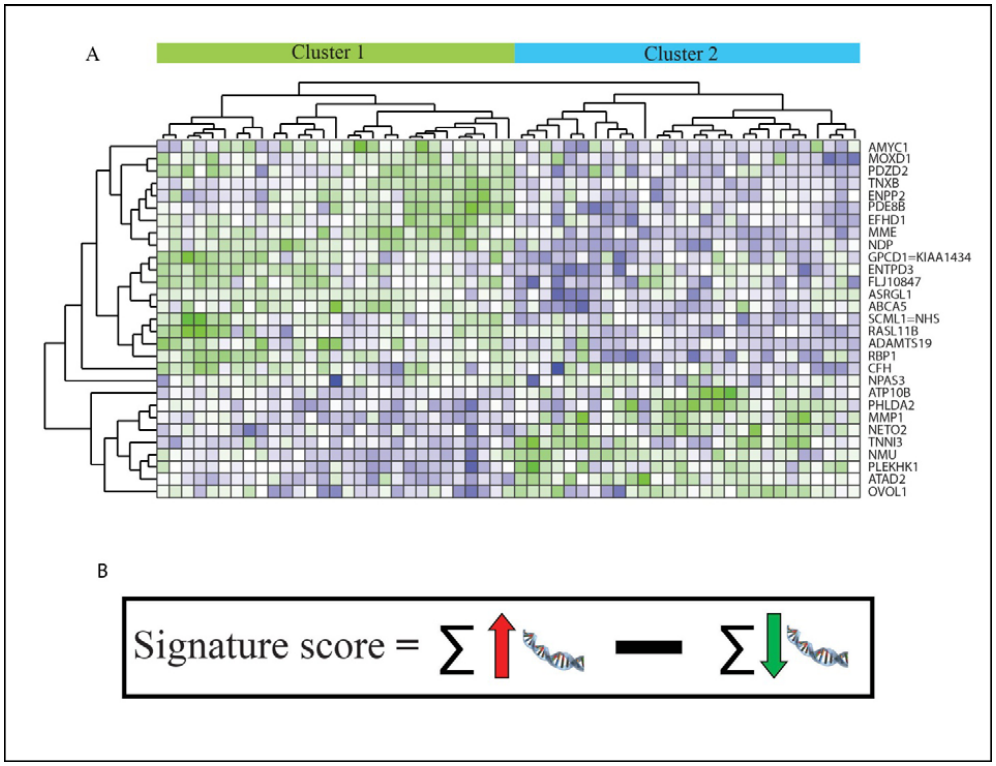
Different methods for estimating gene expression signature scores have been presented.[415,422] One method applied in several studies is presented by Huang et al:[415] the gene expression values are normalized by subtracting a common mean from each

expression value before these are scaled to the same standard deviation. The *signature score* is calculated by subtracting the sum of normalized expression values of genes expressed lower in one of the classes examined from the sum of expression values expressed higher in the same class, as illustrated in **Figure 13.**

Databases of gene expression patterns according to various pathway and therapy specific perturbations are published.[423,424] Gene-expression data from cancer samples can be mapped to such databases and analyzed according to how well the cancer sample expression data overlap with specific perturbations. Such tools may reveal connections between biological perturbations and tumor phenotypes that have yet been unknown. Other platforms, facilitating the gene expression exploration (both human and cell line data), are also publically available and may be a valuable tool in the search for transcriptional alterations pointing to targets and response to specific drugs.[425-427]

A few gene expression signatures have been published for endometrial cancer. The first studies applying gene expression microarrays in endometrial cancer examined transcriptional alterations between endometrioid and non-endometrioid tumors.[428,429] It was demonstrated that genes involved in endometrial homeostasis were higher expressed in endometrioid tumors, and genes involved in the regulation of mitotic spindle checkpoint were expressed higher in non-endometrioid cancer. Based on this, they further found that amplification of STK15 (=AURKA) was present only in non-endometrioid carcinomas.[428] Risinger et al compared normal endometrium with cancer of endometrioid and non-endometrioid histology and pointed to IGF-1and a potential relation to PTEN/AKT, and summarized that some genes are altered in a histology subtype-specific manner, while other genes are similarly altered in endometrioid and non-endometrioid tumors.[429] Both a knowledge-based study, comprising 492 endometrial carcinoma related genes in an array,[430] and two studies of type I endometrial carcinomas, applying gene expression microarray technology covering 15-25 000 genes, have revealed transcriptional alterations pointing to biological processes previously unknown in this cancer subtype,[431] and also a signature predicting survival is suggested.[432]

Figure 13. Illustration of one method to generate a gene expression signature score. A: Heat map showing genes differentially expressed between 2 clusters. B: A sum expression score is generated, subtracting the sum of down-regulated genes from the sum of up-regulated genes in one class/cluster.



Salvesen et al reported a 29-gene signature that separated two clusters with different clinico-pathologic and molecular phenotypes as well as significant prognostic differences.[79] A high signature score was associated with PI3K pathways alterations, suggesting patients with high signature score to be candidates for PI3K inhibitors. A study comparing transcriptional differences between endometrioid, serous and mixed mullerian

uterine carcinomas revealed distinct transcriptional patterns for each of the different histological subtypes.[433]

A BMI-1 driven signature reported by Glinsky et al.[434] was demonstrated to impact survival in endometrial carcinomas, more than BMI-1 mRNA expression.[435] In a study by Mannelqvist and colleagues, an 18-gene signature derived from genes differentially expressed between endometrial carcinomas with and without vascular invasion, demonstrated prognostic impact.[133] Furthermore, expression levels for selected angiogenesis related genes were demonstrated to be expressed higher in tumors with vascular invasion and gene sets representing epithelial-mesenchymal transition, wound response and VEGF activity were also enriched in tumors with vascular invasion.

Experimental models have been used to create gene expression signatures mimicking activation of oncogenic pathways.[415,436] It is demonstrated that such signatures might associate with prognosis and predict therapy response in cancer.[436,437] The endometrial carcinoma 29-gene signature[79] and the 18-gene vascular invasion signature[133] are examples of signatures that indicate associations with druggable targets (PI3K, VEGF) but their potential as predictive markers for relevant inhibitors needs further testing in clinical trials.

1.8 Personalized medicine; targeted therapies and predictive markers

"Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression." (*www.cancer.gov; National Cancer Institute, USA, October 2012*)

By directing therapy towards molecules specifically altered in cancer cells and to little extent altered in normal cells, the aim for the therapeutic strategy has been that the therapy will effectively kill the harmful cancer cell growth, while having less harmful effects in the normal cells. We have moved into the time for more personalized or stratified medicine, aiming to give the right drug to the right patient, at the right time. This therapeutic strategy has been increasingly focused the last decades. While this approach presently is being explored in large scale, the translation of research findings into clinical use has been somewhat more cumbersome, and the conventional therapies such as chemotherapy and radiotherapy still play the major role after surgery in the handling of endometrial cancer patients, with none of the new targeted therapeutics yet approved for standard clinical care, contrasting the improvements implemented for the cancer types like breast-, renal-, colorectal- and lung cancers (*www.fda.gov*).

Estrogen receptor α (ERα) was the first identified molecular target relevant for targeted therapy in cancer. An ERα antagonist (Tamoxifen) was developed and given to ERα positive breast cancer patients, and was the first and a very promising example of targeted cancer therapy. Other drugs that interfere with the binding of estrogen to ERα, drugs that interfere with synthesis of estrogen (aromatase inhibitors), and drugs promoting destruction of ERα (fulvestrant) have later been FDA approved for ERα positive breast cancer (*www.fda.gov*). The cloning and functional description of Her2 (ERBB2) was another early discovery, relevant for targeted cancer therapy in breast cancer. A monoclonal antibody is developed against Her2 (trastuzumab), also in clinical use today. Both ERα and Her2 are examples of markers associated with prognosis and prediction of response in breast cancer, and integrated as markers for treatment stratification in standard clinical breast cancer therapy today.

The majority of clinical studies evaluating the effect of new targeted therapies are per today performed according to a standard set-up starting with Phase I studies and continuing to Phase II and III studies (**Table 5).**[438]

Table 5: Overview of standard focus of clinical trials in oncology (a*fter deBono 2010*).[438]

PHASE	SIZE	FOCUS
Phase I	20-60	Define safety, tolerability, MTD
		Describe dose-limiting toxicity
		Evaluate pharmacokinetic and pharmacodynamic relationships
Phase II	30-200	Evaluate anti-tumor activity
		One cancer type; no stratification according to molecular tumor alterations
		Often non-randomized in oncology studies
Phase III	400-2000	Designed to show a statistical benefit in clinical outcome, preferably overall survival
		Usually unselected patient population; one tumor type

In addition, post-marketing surveillance after clinical implementation of new therapy is performed as *phase IV* studies. These studies are carried out for specific disease subgroups, after the drug is approved for human use. Long-term effects of the drug in study might be assessed, and the drug effects amongst specific patient subgroups (*e.g.* pregnant women) may be explored, along with drug-interactions.

What is a relevant target?

Relevant targets for cancer therapy are ideally molecules that have an important role in cancer initiation, for cancer progression or for the metastatic tumor cells to "thrive" in the environment where they settle. Genetic addictions and other vulnerabilities and dependencies in tumor cells are exploited in the search for relevant molecular targets for therapy.[439]

We know there are multiple genetic and epigenetic aberrations in cancer. However, the concept "oncogene addiction" has been introduced describing a particular dependency in the cancer to one or a few genes to maintain a "cancer phenotype"[440,441] In the search for relevant druggable targets, the "oncogene addiction" theory has been driving the strategy for a long period,[442] and this approach has successfully contributed to the development of several important FDA approved drugs (*e.g* Trastuzumab/Her2, Gefitinib/EGFR).

More recent studies in targeted cancer therapy have provided the concepts of "'non-oncogene addiction",[443,444] and "synthetic sickness/lethality".[445-447] "Non-oncogene addiction" genes are suggested as important in the search for relevant targets in cancer therapy as several of these genes and pathways are essential in the development and maintenance of the cancer phenotype, although not regarded as "drivers" in these processes.[444] Inhibition of their corresponding proteins may thus impair cancer cell survival or other major important processes for the cancer to "thrive". Luo and colleagues suggested categorization of "non-oncogene addiction" genes into: 1) *tumor intrinsic* and 2) *tumor extrinsic*, where the genes support the oncogenic state by functioning within the tumor cell or through stromal and vascular cells surrounding the tumor, respectively.[444] It is suggested advantageous to target the supportive cells as these are genetically more stable than the tumor cells, and also less likely develop drug resistance.[444] The viability of normal cells is also to a large extent not dependent on such "tumor extrinsic" genes.

The concept of "synthetic sickness/lethality" might be seen as an entity within the concept of "non-oncogene addiction". "Synthetic sickness/lethality" takes advantage of co-occurring gene alterations that together will impair cell viability, even if each defect alone would not affect the cell "fitness".[445] One textbook example of this is the demonstration of PARP inhibitors inducing synthetic lethality and thereby selective cytotoxicity to breast cancer cells with concomitant BRCA1 or BRCA2 mutations, also demonstrated in clinical studies.[448,449] Both "non-oncogene addiction" and "synthetic lethality" are being explored in the search for relevant drug targets (**Table 6**).[439]

Table 6. Studies where "non-oncogene addiction" and "synthetic lethality" are exploited in targeted therapies.[a]

Agent	Target	Reference
17AAG[b]	HSP90	450
1MT, MTH_Trp[b]	IDO	451
5-fluorouracil[c]	DNA	452
AP12009	TGFβ2	451
AZD2281/AG014699[b]	PARP1	453,454
Bevacizumab[c]	VEGF	455
Bortezomib	Proteasome	456
Celecoxib	COX2	451
Cisplatin and analogs[c]	DNA	457
Mapatumumab/lexatumumab	TRAIL receptor	458
Methotrexate	DHFR	459
Paclitaxel/Vinblastine[c]	Mitotic spindle	460
PF-00477736[b]	Chek1	461
Rapamycin/temsirolimus[c]	mTOR	462
Sorafenib/Sunitinib[c]	Multiple kinases	455
Topotecan/Irinotecan[c]	TOP1	463

Footnotes: [a] Adapted from Luo et al 2009,[444] [b] Tested in endometrial carcinomas, [c] Tested in any solid tumors, www.clinicaltrials.gov (March 2013)
HSP 90; Heat shock protein 90**.** IDO; indoleamin 2,3-dioxygenase. DNA; deoxyribonucleic acid. TGFβ2; Transforming growth factor β2. PARP1; Poly(ADP-ribose) polymerase 1. VEGF; Vascular endothelial growth factor. COX2; Cyclooxygenase-2. TRAIL; TNF-related apoptosis inducing ligand (=tumor necrosis factor (ligand) superfamily, member 10, TNFSF10). DHFR; Dihydrofolate reductase. Chk1; Checkpoint kinase 1. mTOR; Mechanistic target of rapamycin. TOP1; Topoisomerase 1.

It has been shown to be a challenging task to identify a target that has such an important role in human cancer that directing therapy towards it has the potential to "cure the cancer". When studying patients and tumor tissues from patients, the best studies we do today are association studies; we study the association between a marker (a potential target) and for example histopathologic variables and clinical parameters, including outcome (*e.g.* risk of recurrence or progression and survival). The majority of these

patients are not treated with any drug considered to specifically aim for the target investigated. How are we able to identify molecules that are crucial for the cancer to "thrive"? In cell lines and mouse models, we do this by engineering functional models considered to mimic molecular changes seen in patient samples. But how well do the results from these functional models reflect the situation in the cancer patients?

Genome-scale RNA interference (RNAi) screenings of cell lines are supported as a robust method to identify candidate targets. Use of genome-wide RNAi screens has been able to point to genes that are important for the specific process studied (*e.g.* drug resistance) without being for example mutated, as will be valid for many of the "non-oncogene drivers".[444] This is an advantage of the method above the next generation sequencing that takes place in larger and larger scale in cancer research: non-mutated, but nevertheless important genes will not be caught in the latter investigation. A limitation of the RNAi screen is that per today it is only performed in cell lines.

Cancer cell lines and mouse models have been found to be useful tools when studying specific cell biological mechanisms. By interfering with the genotype and molecular phenotype (*e.g.* overexpressing or knocking down a gene), we are able to read how these forced changes may lead to other expected or unexpected molecular alterations. Cell line studies have often been the starting point for the targeted drugs that have been developed, and are together with mouse models regarded of importance when identifying and testing how to influence relevant targets for therapy.[464] Still, cell lines are somehow artificial cancer models, as these models mainly consist of only one or a few cell types. The tumor microenvironment surrounding the cancer cells is demonstrated to be of importance for the cancer growth, but is rarely accounted for in the models. Thus, important interaction between cancer cells and surrounding microenvironment cells that takes place in a tumor may go undetected. It is today recognized that cell line studies have limitations as cancer models, for example when studying therapy response.[439]

In the majority of preclinical cell line models presented until now, too few cell lines have been studied to be able to evaluate drug response in relation to tumor heterogeneity, regarding both the various cancer subtypes and the genetically heterogeneity seen.[465] Recently, two comprehensive studies on cell line panels were published; 639 and 947 cell lines were molecularly annotated by sequencing candidate genes, assessing copy number variations (CNV) and gene expression data. The cell line data were linked to drug sensitivity data of a range of compounds, and the studies suggested new drug sensitivity biomarkers.[425,426] The data generated in these studies is made publically available (http://www.broadinstitute.org/ccle; http://www.cancerrxgene.org/). Comments to these works have highlighted the important contributions of the studies as examples of how such large, diverse cell line panels together is a model that match the molecular tumor heterogeneity we experience.[466-468] Despite limitations of cell lines in culture, in terms of being models for human cancer, studies of such large cell line panels are also able to point to cancer cell vulnerabilities and thereby new relevant targets for therapy combined with markers predicting therapy response.[466,467]

Genetically engineered mouse models and xenograft models of mice transplanted with human tumor tissue are promising models when validating molecular findings from patient tumor tissue and functional cell line studies.[464] These models may allow investigation of factors involved in malignant transformation, invasion and metastasis, as well as testing for response to therapy. In human tumor xenografts, the human tumor cells may be transplanted into immunocompromised mice (to avoid rejection) under the skin or in the orthotopic model, into the organ type the tumor originated from.

Important to remember, when interpreting the results from mice studies in light of the human cancer biology; *mice are not men*. Limitations such as lack of tumor diversity are seen in some mouse models of cancer, and introducing genomic instability might aid to resolve this problem.[465] Also, the lack of an adequate responsive immune system in the immunocompromised mice, might be a weakness when examining the muse models, and aiming to mirror the cancer biological processes in human.

A key challenge is to identify important drivers in the carcinogenic process and how to target these processes. If such a potential target is suggested based on pre-clinical models, a next step may be to investigate further the presence of the candidate marker in patient tumor tissues in relation to patient outcome. If the identified marker is enriched in samples from patients with aggressive disease and poor survival, it is more likely that you will be able to recruit sufficient number of cases to test for effects from targeting the alteration in a metastatic setting. Still, a crucial question is whether the alteration only "co-associates" to the aggressive phenotype OR drives the aggressiveness of the tumor. In the first case, targeting this marker will probably not be helpful. The inevitable ultimate step to determine the relevance of the target for new therapeutics is to evaluate if drugging the target will lead to therapeutic effects in patients in a controlled clinical trial.[439] Studies that has led to FDA approved targeted therapies have pointed to molecular alterations of the cancer cells not shared by normal cells, being potential targets for therapy with specific drugs. Some examples of such studies are listed in **Table 7**.

Table 7. Examples of FDA approved targeted therapies, and reference studies *(www.cancer.gov/cancertopics/factsheet/Therapy/targeted).*

Drug	Trade Name	Primary target(s)	Primary indication
Signal transduction inhibitors			
Trastuzumab	Herceptin	Her2	Her2 positive metastatic breast cancer[469]
Imatinib	Gleevec	ABL, cKIT	BCR-ABL translocation-driven CML[470]
Gefitinib	Iressa	EGFR	EGFR-driven NSCLC[471]
Erlotinib	Tarceva	EGFR	NSCLC; pancreatic carcinoma[472]
Cetuximab	Erbitux	EGFR	Head and neck squamous cell carcinoma [473]
			KRAS wt colorectal cancer[474]
Panitumumab	Vectibix	EGFR	KRAS-wt colorectal cancer[475]
Temsirolimus	Torisel	mTOR	Renal cell carcinoma[476]
Lapatinib	Tykerb	ERBB2/EGFR	Her2-positive breast cancer[477]
Everolimus	Afinitor	mTOR	Giant cell astrocytoma;[478] renal cell carcinoma[479]
			PNET[480]
Crizotinib	Xalkori	ALK	ALK-rearranged NSCLC[481]
Vemurafininb	Zelboraf	BRAF	V600E BRAF-mutated melanoma[482]
Angiogenesis interference			
Bevacizumab	Avastin	VEGF	Metastatic colorectal cancer[483]
Sorafenib	Nexavar	VEGFR	Hepatocellular carcinoma;[484]
			renal cell carcinoma[485]
Sunitinib	Sutent	VEGFR	renal cell carcinoma;[486] PNET[487]
Ziv-aflibercept	Zaltrap	VEGF	Metastatic colorectal cancer[488]
Modulate protein function			
Vorinostat	Zolinza	HDACs	Cutaneous T-cell lymphoma[489]
Bexarotene	Tagretin	RXR	Cutaneous T-cell lymphoma
Tretinoin	Vesanoid	RAR	
Induce apoptosis			
Bortesomib	Velcade	proteasome	muliple myeloma;[490]
Modulate immune response			
Ipilimumab	Yervoy	CTLA-4	unresectable or metastatic
			melanoma[491]
Rituximab	Rituxan	CD20	B-cell non-Hodgkin lymphoma;[492]
			chronic lymphocytic leukemia[493]

Footnotes:*FDA approved in combinatorial therapy (*www.fda.gov*). FDA=Food and Drug Administration (USA), CML= chronic myeloid leukemia. NSCLC= non-small-cell lung cancer. VEGF= vascular endothelial growth factor. VEGFR=vascular endothelial growth factor receptor. GIST=gastrointestinal stromal tumor. PNET=pancreatic neuroendocrine tumor. wt= wild-type. CTLA-4=cytotoxic T-lymphocyte-associated antigen-4. RXR= retinoid X receptor. RAR=retinoic acid receptor.

Targeted therapies in endometrial cancer

For endometrial cancer, the number of ongoing clinical trials are far less (~350) than for ovarian- and breast cancer; >1300 and >4500 studies, respectively, based on all reported clinical trials at the www.clinicaltrials.gov *(October 2012*). For endometrial cancer, several clinical trials on targeted therapies are ongoing. These are mainly phase I and II trials, and are exploring effects of PI3K/mTOR pathway inhibitors, PARP and angiogenesis inhibitors (**Table 8**).

Table 8. Overview of ongoing clinical trials on targeted therapy in endometrial cancer (www.clinicaltrials.gov*, October 2012*).

Target	Biological agent	ID	Additional therapy	N	Biomarker Restriction
AKT	MK2206	NCT01307631		90	PIK3CA mut.
AKT	MK-2206	NCT01312753		90	PIK3CA mut.
AKT/MEK	GSK2141795/GSK1120212	NCT01138085		125[a]	
AKT/MEK	GSK110183/GSK1120212	NCT01476137		335[a]	
ALK1 ligands	Dalantercept	NCT01642082		52	
Angiopoetin ½	AMG386/trebananib	NCT01210222		55	
ER	Endoxifen	NCT01273168		72[a]	ER/PR pos
ER/PR	BN83495/MA	NCT00910091		73	
ErbB3	MM-121	NCT01209195	P	24[a]	
FGFR2	TKI258	NCT01379534		80	
Her2	Lapatinib	NCT01454479	I	24	Her2 pos
Her2	Trastuzumab	NCT01367002	C/P	100	Her2 pos
mTOR	Ridaforolimus	NCT00770185		30	
mTOR/CYP19A1	Everolimus/Letrazole	NCT01068249		42	
mTOR	Ridaforolimus	NCT01256268	C/P	28[a]	
mTOR	temsirolimus	NCT01155258	VD	19[a]	
mTOR	temsirolimus	NCT00982631	PLD	30[a]	
mTOR/angiopoetin 1/2	temsirolimus/AMG386	NCT01548482		42[a]	
mTOR/ER/PR	Temsirolimus/MA/TC	NCT00729586		84	
mTOR/Notch	temsirolimus/RO4929097	NCT01198184		30[a]	
PARP	olaparib	NCT01237067	C	66[a]	
PARP ½	MK-4827	NCT00749502		113[a]	
PARP ½	veliparib	NCT01366144	C/P	276[a]	
PI3K	XL147	NCT01013324		88	
PI3K	BKM120	NCT01397877		56	

Contd. Table 8

Target	Biological agent	ID	Additional therapy	N	Biomarker Restriction
PI3K	XL147	NCT00756847	C/P	74[a]	
PI3K	GSK2636771	NCT01458067		150[a]	PTEN loss
PI3K	BKM120	NCT01068483		83[a]	
PI3K/mTOR	PF-04691502/PF-05212384	NCT01420081		269	
PI3K/mTOR	DS-7423	NCT01364844		66[a]	
PI3K/mTOR	GDC-0980	NCT01455493		50	
PI3K/mTOR	PKI-587	NCT00940498		85[a]	
PR	MPA	NCT01594879	LNG	39	
PR/AMPK	LNG/metformin	NCT01686126		111	
RTKs	E7080	NCT01111461		133	
RTKs	Dasatinib	NCT01440998		15	
VEGF	Bevacizumab	NCT00879359	C/P	31	
VEGF/mTOR	Bevacizumab/Temsirolimus	NCT00977574	C/P/I	330	
VEGFRs/PDGFRs/KIT	Sunitinib malate	NCT00478426		30	
VEGF	Bevacizumab	NCT00513786	C/P	38	
VEGFR/FGFR/PDGFR	BIBF 1120/nintedanib	NCT01225887		55	
VEGFR/FGFR	Brivanib alaninate	NCT00888173		43	
VEGFR	cediranib maleate	NCT01132820		54	
VEGF-A/VEGF-B/PlGF	ziv-aflibercept	NCT00462826		43	
VEGF/mTOR	Bevacizumab/temsirolimus	NCT00723255		43	
VEGF/mTOR	cediranib/temsirolimus	NCT01065662		50[a]	
VEGF/mTOR	bevacizumab/temsirolimus	NCT01010126		299[a]	
VEGFRs/PDGFRs/KIT	sunitinib malate	NCT00813423	HC	20[a]	
CYP19A1	Letrozole	NCT00997373		50	

Footnotes: ID= Clinicaltrial.gov identifier. N= Number of patients to be included. [a] Other cancer types included in the study. C/P=Carboplatin/Paclitaxel. I= Ixabepilone. VD= Vinorelbine ditartrate. PLD= Pegesylated Doxorubicin. LNG= levonorgestrel (intra uterine system). HC= Hydroxychloroquine

Published and preliminary study results (conference/abstract presentations) from targeted therapies in endometrial cancer mainly report on studies targeting mTOR and VEGF/VEGFR (**Table 9**). The response rates are overall modest, and the studies could potentially have benefited from biomarker stratification, as further discussed below. Only

a few of these studies have assessed molecular alterations in pre-treatment biopsies and evaluated therapy response according to such information.[400-402,494]

Table 9. Published phase I/II trials on targeted therapy in endometrial cancer (per *October 2012*, except hormonal therapy covered in Chpt 1.6).

Ph	Drug	Target	N[a]	Prior therapy	Add ther	CR (%)	PR (%)	SD (%)	median PFS (range)[b]	Ref
II	Cetuximab	EGFR	30	≤ 4 CT		0	5	10		495
II	Erlotinib	EGFR	32	CT=0		0	12.5	46.9	3.7 (2-36)	400
II	Trastuzumab	Her2	33	UL CT		0	0	40	1.8 (range NR)	494
II	Temsirolimus	mTOR	54	A) CT=0 ≤1 HT		0	A) 14	A) 69	5.1 (3.7-18.4)	496
				B) 1 CT		0	B) 4	B) 46	B) 4.3 (3.6-4.9)	
II	Everolimus	mTOR	28	≤ 2 CT		0	0	43	4.2 (1.8-9.3)	497
II	Deforolimus	mTOR	27	≤ 2 CT			7	26	NR	498
II	Ridaforolimus	mTOR	130	≤ 1 CT		0		35	3.6	499
I	Temsirolimus	mTOR	11	≤ 2 CT	C/P	0	91	NR	NR	500
I	Bevacizumab/ Temsirolimus	VEGF-A/ mTOR	15	CT	LD	40[c]	53[d]		NR	401
II	Bevacizumab	VEGF-A	52	≤ 2 CT +/- RT		2	12		4.2 (range NR)	402
II	Aflibercept	VEGF/PlGF	44	≤ 2 CT		0	7	32	2.9 (0.3-18)	501
II	Sunitinib	VEGFR	20	≤ 1 CT		0	15	25	NR	502
II	Sorafenib	VEGFR	55	≤ 1 CT		0	4	42	2.3 (range NR)	503

Footnotes:

Abbreviations: Ph=Clinical trial phase, Add ther = additional therapy, CR= complete response, PR= partial response, SD = stable disease, PFS = progression free survival, HT= hormonal therapy, CT = chemotherapy regimens, NR = not reported, LD = liposomal doxorubicin, C = carboplatin, P = paclitaxel, HT = hormonal therapy regimens, RT=radiation therapy, ref.=reference, NL=no limitations. [a] number of endometrial cancer patients evaluable, [b] PFS= Progression free survival (months), [c] CR or PR, [d] SD≥ 6 months/PR/CR.

Why has there been a limited effect of targeted therapies so far?

"*All models are wrong, but some are useful*" *(George Box 1979)*

The lack of biomarker stratified clinical trials might have been one of the "strategic mistakes" done in the approach to testing for effect from molecularly targeted therapeutics in cancer treatment. Arguments for not applying a molecular basis for treatment stratification have been uncertainties regarding the link between the presumed target and the actual drug tested, often found to have multiple target activities. Also, preclinical models have been difficult to translate into adequate clinical trial design in humans. Still, important arguments for improved trial design are that the development and testing of drugs is extremely expensive, and with the large number of drugs in the pipeline to be tested, unselected testing of all drugs will not be able to recruit sufficient number of patients. Drug companies might have hoped for better overall response rates when testing new drugs in clinical trials without biomarker stratification, but the experience so far and costs related to this approach may limit this in the future. The idea of targeted therapeutics is that the drug targets a specific molecular phenotype that is important to the tumor cell. If the patient receiving the drug does not have tumor(s) with this specific molecular phenotype, it is not likely that she will respond to the drug.

How to consider targeted therapy in the metastatic setting, when available results from molecular analyses is present only for the primary tumor? If the primary tumor is positive for the marker of interest, one pragmatic approach is not to biopsy any metastases because a negative result will not prevent therapy from being given. A negative metastasis with respect to the marker in question is not reassuring for any metastasis being negative for the marker. As clones in primary tumor have demonstrated to be positive for the marker, these may also metastasize. However, if the primary tumor was negative for a marker that would indicate therapy if positive, biopsies from metastatic lesions are recommended if achievable, as a positive marker in a metastatic lesion would lead to therapy being given. Per today we are not taking into account the challenge of intratumor heterogeneity or

tumor cell evolution.[504,505] These are issues that also have to be addressed properly in relation to targeted therapy the coming years.

We know the biology is complex, and the biology of cancer cells maybe even more intricate, as genomic instability is one of the hallmarks of the cancer cells,[10] and which genes and pathways that are overactive may switch as the tumor develops.[504] When knowing this, isn't it likely that mono-targeting a receptor tyrosine kinase is insufficient for a large proportion of the patients? Combinatorial therapy in cancer has mainly been focused to combinations of chemo/radiation and a molecular targeted drug. Even in the case of Trastuzumab to Her2 positive breast cancer, regarded as an example of success in the history of targeted therapies, 25-30% of Her2 positive cases progress on Trastuzumab and chemotherapy.[506] The concern of drug resistance, both *de novo* and acquired resistance has probably been one of the reasons for limited effect of targeted therapies on overall survival. This issue is now being addressed and in some studies the drug combinations are designed trying to overcome the problem of resistance. Berns and colleagues demonstrated by RNAi screen of untreated breast cancer cell lines versus cell lines treated with Trastuzumab that low expression of PTEN was associated with lack of therapy response.[507] In line with this, a phase I/II study on everolimus (mTOR inhibitor) and Trastuzumab to patients with Her2 overexpressing metastatic breast cancer demonstrated promising results [506]

As targeted drugs are often given in combinations with conventional therapy, it might be of importance how the drugs are combined. A phase III trial on adjuvant chemotherapy and tamoxifen to breast cancer patients demonstrated that the two drugs given sequentially gave a favorable hazard ratio, although not statistically significant, as compared to when the drugs were given concurrently.[508] Depending on the rationale for giving targeted drug(s) in combination with chemo or radiation therapy or combinations of several targeted drugs, the timing for drug delivery is probably of importance, and understudied per today.[509]

2. AIMS OF THE STUDY

2.1 Background

Endometrial cancer is diagnosed early and has in general good prognosis. The more important it is to diagnose and treat the poor-prognosis cases. Therapy of endometrial carcinoma patients per today is to a large extent empirically based. Improvements on therapeutic strategies with more personalized focus are needed; ***what*** kind of therapy (conventional versus targeted) should be given ***when*** (*e.g.* localized versus systemic disease), and ***to whom*** (all patients versus defined risk groups)? It is well known that development of new therapies to appropriate patient groups is a demanding and time-consuming task exemplified by the identification of Her2 as target and development of Trastuzumab for patients with Her2 positive breast cancer. Preparing the ground for later clinical studies, by combining clinico-pathologic and molecular data from preclinical studies and cancer patients, is an important step to individualize therapy in cancer.

The incidence of endometrial cancer is increasing[5] and the prognostic categorization used in clinical practices today is suboptimal for separating patients according to survival.[28-30] Despite a focus on translational research in endometrial cancer for some decades, it has proven difficult to bring new biomarkers to the clinic to improve prognostication and prediction of therapy response in this cancer type.[30,64] The type I/II categorization is used world-wide, based originally on a clinical-descriptive study 30 years ago,[27] although molecular characteristics for the two groups have been related to microsatellite instability and PTEN, p16, TP53, ERα, PR and PI3K pathway alterations.[64] Endometrial cancer is behind other cancer types as breast, lung and colorectal cancer in clinical application of molecular classification of tumors to select patients for targeted therapy.[30,64] Better tumor biological understanding of subgroups, applicability of prognostic markers in a routine clinical setting, and identification of targets for therapy including markers predicting response to such, are important to improve personalized treatment strategies to benefit the

endometrial carcinoma patients.

2.2 General aim

The main goal of this project was to study biomarkers potentially associated with endometrial carcinoma progression, to assess their potential as prognostic markers and explore on targets for therapy associated with pathologic expression of these markers. By this, we aimed to provide a rationale for further testing of candidate markers as prognostic and predictive markers in clinical trials.

A second aim has been to focus on biomarker implementation through an important step in the stair-case from research to clinical use; *biomarker validation* in independent patient series and in a routine clinical setting.

2.3 Specific aims

1. To investigate the prognostic impact of DNA ploidy in endometrial carcinoma in a routine diagnostic compared to a research setting. (**Paper I**).

2. To explore whether low ERα expression, associated with poor prognosis in endometrial carcinomas, was reflected in transcriptional signatures pointing to tumor biological processes important for cancer progression, and with a potential as targets for new therapy (**Paper II**).

3. To study how pStathmin(S38) immunohistochemical expression correlated with prognosis in endometrial cancer. Also, we wanted to explore on transcriptional changes associated with pStathmin(S38) expression to uncover tumor phenotypic alterations associated with pStathmin(S38) and to potentially suggest targets and

predictive markers for novel therapeutics in aggressive endometrial carcinoma (**Paper III**).

4. To validate prospectively in independent patient series the ability for a previously defined endometrial cancer *risk of recurrence* signature to predict disease relapse in general and amongst presumed low risk groups in particular. We also wanted to assess the correlation between the signature score and potential measures for PI3K signaling, hormone receptor status and other potential targetable factors involved in the process of tumor progression (**Paper IV**).

3. MATERIAL AND METHODOLOGICAL CONSIDERATIONS

"*Whether you can observe a thing or not depends on the theory which you use. It is the theory which decides what can be observed.*" (*A. Einstein* 1879-1955)

3.1 Patient series and tissues

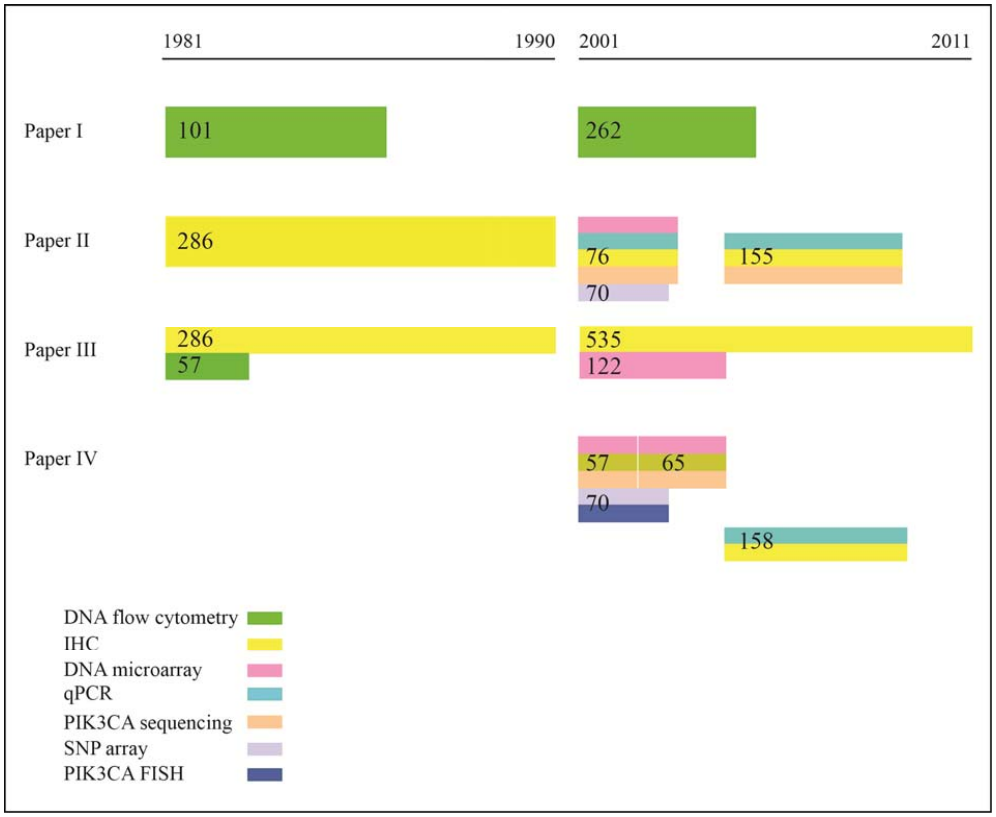
Overall, two major patient series are studied in this project; 1) A retrospectively collected series of 286 cases, all verified endometrial carcinoma patients diagnosed in Hordaland County in the period 1981-1990. Hordaland County covers approximately 10% of the Norwegian population[510] and has the same age-adjusted incidence rate for endometrial cancer as the whole Norwegian population.[4] Initially 316 patients were diagnosed with endometrial cancer in Hordaland County this time period. Twelve of these were excluded due to altered diagnosis after histopathologic revision, and 5 more cases were diagnosed by cytology only and therefore also excluded. For the remaining 299 cases, paraffin blocks from primary tumor were accessible for 286 patients.[117] This series is described as a population based series and has been extensively studied and described.[104,117,120,155,390] 2) From 2001 onwards, patients from several Norwegian and European gynecologic oncology centers have consented and been prospectively enrolled in the MoMaTec study (http://www.clinicaltrials.gov/ct2/show/NCT00598845). Patients from Haukeland University Hospital have been studied in this project, and were enrolled from May 2001 to January 2011. An overview of the patients studied and tissues/methods applied is given in **Figure 14, Table 10** and **Table 11**.

Clinical data

For both major cohorts studied, clinical data covering parity, menopausal status, height and body weight, age at primary diagnosis, date of diagnosis, type of primary and adjuvant treatment, FIGO stage (according to 1988 criteria for the retrospective cohort and 2009 criteria for the prospective cohort, except for **Paper I** where FIGO 2008 was applied), and information about lymph node sampling were collected from patient files.[511] Imaging diagnostics was retrieved from routine radiological reports for both cohorts.

Complete follow-up information was recorded, including date and site of recurrent and/or progressive disease and therapy of such, and patient survival with status at last date of follow-up. Supplementary follow-up information was collected from out-patients medical doctors responsible for parts of the follow-up.[511] Last date of follow-up for the retrospective cohort was June 30th 2004. For the prospective cohort, the follow-up month was November 2008/**Paper I**, November 2010/**Paper II**, June 2011/**Paper III and IV**.

Figure 14. Overview of the Haukeland based patient series and methods applied in the project



IHC: immunohistochemistry, qPCR: quantitative polymerase chain reaction, SNP: single nucleotide polymorphism, FISH: Fluorescence *in situ* hybridization.

The primary treatment for both patient cohorts has been hysterectomy and bilateral salpingo-oophorectomy. Radical hysterectomy (ad modum Wertheim-Meigs) has been performed in cases with cervical/paracervical infiltration identified pre-/peroperatively. In the retrospective cohort, lymph node sampling was not routinely performed, but enlarged nodes suspect of malignancy were biopsied. Lymph node sampling has increasingly from 2001 been part of the standard surgical treatment recommended to endometrial carcinoma patients.[237,317] Patients not suitable for surgical treatment were staged by clinical examination, curettage and x-ray/CT results. These patients were offered radiotherapy or hormonal therapy in the retrospective cohort and radiotherapy, chemotherapy and/or hormonal treatment in the prospective cohort. Adjuvant radiotherapy was part of the standard treatment in the period 1981-90; brachytherapy to cases with myometrial infiltration <50%, external pelvic radiation to cases with deep myometrial infiltration and/or high histologic grade.[317] In the prospective cohort, external radiation therapy and chemotherapy has been given as adjuvant treatment to patients with high risk of recurrence (e.g. non-endometrioid histology, high histologic grade and deeply infiltrating tumors).[237] Only a few cases received adjuvant chemo-radiation in the prospective cohort.

TABLE 10: Comparison of clinico-pathologic data in the retrospective and complete prospective series.

	Hordaland population 1981-1990 Retrospectively collected N=286 n (%)	**Haukeland population 2001-2010 Prospectively enrolled N=620 n (%)**	**P[a]**
Mean age, years (range)	65 (33-92)	66 (28-94)	0.6
Menopausal status			
Pre-/perimenopausal	43 (15)	75 (12)	0.2
Postmenopausal	242 (85)	545 (88)	
FIGO 1988/2009			0.5
I-II	230 (81)	521 (83)	
III-IV	55 (19)	108 (17)	
Histologic type[b,c]			< 0.001
Endometrioid	257 (90)	486 (78)	
Non-endometrioid	29 (10)	134 (22)	
Histologic grade[c]			< 0.001
1-2	218 (76)	399 (65)	
3	68 (24)	216 (35)	
Myometrial infiltration			0.5
< 50%	146 (62)	377 (64)	
≥ 50%	90 (38)	210 (36)	
Lymph node metastases	Not assessed		
Absent		426 (69)	
Present		55 (9)	
Not examined		139 (22)	
Recurrence[d]			
No	224 (83)	465 (85)	0.6
Yes	46 (17)	85 (15)	

Footnotes: Missing cases: Menopausal status 1 case and FIGO stage 1 case retrospective series, histologic grade 5 cases in the prospective series. Myometrial infiltration data available for 236 cases in the retrospective series and 587 cases in the prospective series. [a] Chi square test applied except for comparison of age where Mann-Whitney U test applied. [b] Carcinosarcomas included in the non-endometrioid group for the prospective series. [c] Revised pathology diagnosis. [d] Without metastatic disease at primary diagnosis.

External gene expression data set

Gene expression data are generally required to be added to publically available repositories when published first time (e.g. GEO, *http://www.ncbi.nlm.nih.gov/geo/*, ArrayExpress, *http://www.ebi.ac.uk/arrayexpress/*). For this study, a data set comprising 111 endometrial cancers with basic clinico-pathologic data available were used. These samples were run at Affymetrix U133 Plus2 arrays. The data set had been downloaded and normalized in a previous work in our group. Individual probes were then sequenced matched against AceView (NCBI35),[512] to construct transcript level probe sets.[79] A quality check on updated clinico-pathologic data, confirmed 111 endometrial carcinomas that were included in **Paper II**.

The Cancer Genome Atlas (TCGA) data

Collaborators in the project have analyzed gene expression levels from RNA sequencing in the 29-gene Endometrial Carcinoma Recurrence Score in 333 patient tumors.[513]

Tissues

Snap frozen fresh tissue

In **Paper II, III and IV** fresh tissue that was snap frozen after surgery was used for mRNA expression analyses (DNA oligonucleotide microarray and qPCR): 76 (**Paper II**) and 122 primary tumors (**Paper III and IV**) were analyzed on DNA oligonucleotide microarrays. Also, mRNA was extracted from 19 metastatic lesions (**Paper IV**). In **Paper II and IV**, qPCR was applied for 155 and 158 cases, respectively.

Fresh ethanol fixed tissue

Fresh ethanol fixed tissue was used for DNA ploidy analyses in **Paper I** and **Paper III**.

FFPE samples

Tissue samples fixed in formalin and embedded in paraffin were retrieved from the archives of Department of Pathology, The Gade Institute, Haukeland University Hospital. FFPE samples were applied for immunohistochemical studies (**Paper II and III**), and for data previously published.[120,124,155,228,298]

3.2 Histopathologic data

Histopathologic data such as histologic type, pattern of spread, histologic grade and myometrial infiltration was recorded.[511] For the retrospective cohort and a subset of 76 cases from the prospective series (described in **Paper II, III and IV**), histopathologic data were revised by two experienced pathologists (I.M.S. and L.A.A.).[155] For the prospective series apart from the subset of 76 cases, data were retrieved from routine pathology reports. Histologic subtyping and determination of histologic grade was reported according to WHO criteria.[265] Myometrial infiltration was evaluated by microscopy and was measured as vertical tumor thickness in the myometrium divided by the full thickness of the myometrium (measures in mm). The number of mitoses (mitotic count) was counted in 10 high-power fields (x400) by selecting areas with the highest histologic grade and highest mitotic activity ("hot spots"). Vascular invasion was defined as present if invasion in more than one vessel or vascular space was seen.[155]

In the retrospective cohort, carcinosarcomas were not included as these were regarded and treated as a sarcoma subtype in this time period. This histologic subtype of endometrial cancer was in the late 90's suggested to be of monoclonal epithelial origin and as a consequence, patients with carcinosarcomas have been treated as high-risk endometrial carcinomas in the prospective cohort,[237,514] and were therefore included amongst the non-endometrioid cases in this patient series. When excluding the carcinosarcomas (n=24) from analyses as performed in **Table 10**, there is still a significant difference in the

distribution of histologic subtypes for the two cohorts (more non-endometrioid cases in the prospective cohort, P=0.008) and thereby as expected, also more cases of high histologic grade in the same cohort.

Table 11. Patient series, tissue types, methods applied and biomarkers assessed in the different papers.

Paper	Patient cohort/N	Tissue	Biomarker	Method	Evaluable/N
I	1981-90/286	Fr. ethanol fixed	DNA ploidy	Flow cytometry	101
	2001-2007/427	Fr. ethanol fixed	DNA ploidy	Flow cytometry	262
II	1981-90/286	FFPE	ERα	IHC	266
			E-cadherin	IHC	284
			P-cadherin	IHC	276
			β-catenin	IHC	286
			Catenin p120	IHC	276
	2001-2010/620				
		Fr. frozen		DNA microarray	76
			ESR1	qPCR	155
			E-cadherin	qPCR	155
			P-cadherin	qPCR	155
			N-cadherin	qPCR	155
			β-catenin	qPCR	155
			Catenin p120	qPCR	155
			α-catenin	qPCR	155
			3q26 amplif.	SNP array	70
			PIK3CA mut.status	PIK3CA seq.	245
	External gene expression data/111			DNA microarray	
III	1981-1990/286	FFPE	pStathmin(S38)	IHC	256
			Ki67	IHC	196
			PTEN	IHC	279
			Mitotic count	Morphology	286
			S-phase	Flow cytometry	64
	2001-2010/620	FFPE	pStathmin(S38)	IHC	518
			3q26 amplif.	SNP array	70
			PIK3CA copy no.	FISH	66
			PIK3CA seq.		245
		Fr. frozen		DNA microarray	122

Contd. Table 11

IV	2001-2010/620		Signature score	DNA microarray	122
		Fr. frozen	Signature score	qPCR	158
			E-cadherin	qPCR	155
			P-cadherin	qPCR	155
			N-cadherin	qPCR	155
			β-catenin	qPCR	155
			Catenin p120	qPCR	155
			α-catenin	qPCR	155
IV	2001-2010/620	Fr. frozen	3q26 amplif.	SNP array	70
			PIK3CA copy no.	FISH	66
			PIK3CA seq.		245
	TCGA data		Signature score	RNA seq.	333

Abbreviations: Fr.=fresh, FFPE=Formalin fixed and paraffin embedded, mut.status=mutation status, Copy no.=copy number, seq.=sequencing, TCGA=The Cancer Genome Atlas

3.3 Immunohistochemical methods

Immunohistochemical staining is widely used to assess protein expression in routine pathology diagnostics and research. A morphologic description is pivotal for the diagnosis of cancer. Immunohistochemistry may be a relevant addition to the morphologic examination as IHC may reveal cellular localization and degree of expression of epitopes under study, and may point to functional changes in tumor tissue. IHC is demonstrated to be an important tool in biomarker studies.[515]

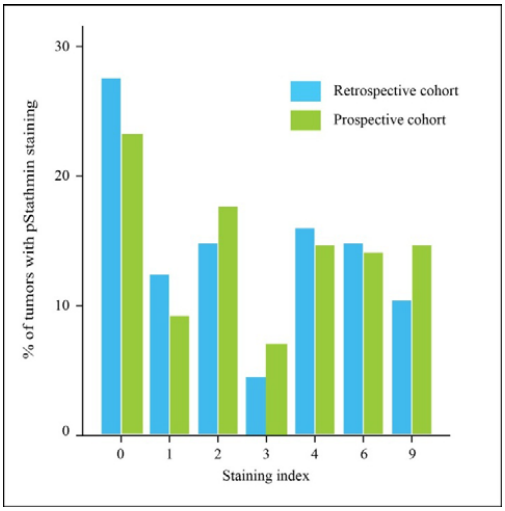
Tissue sections and tissue microarray (TMA)

FFPE sections of 5µm were used for immunohistochemistry (IHC), taken either from standard paraffin blocks or from TMA blocks. The technology of tissue microarray (TMA) was first described by Battifora in 1986,[516] but use of the technique took off when Kononen et al developed a device for rapid, good-quality TMA production.[517,518] By using TMA, the experimental performance will be more uniform, there is an economization of both tissue and material used as well as time spent to evaluate large sample cohorts.[519] Comparison of IHC staining evaluation from studies on TMAs and standard sections have

been done for various cancer types, with good correlation between results,[520-522] although some studies have suggested a problem of selection bias since the area assessed is of larger impact when assessing location-dependent markers or markers that exhibits large degree of expression heterogeneity.[523,524] In general, 2 or 3 cores each of diameter 6µm are recommended.[522,525,526] The TMA method has been applied in our research group since 2000.[107,120,527]

It is recognized that FFPE tissue maintains antigenicity for several decades.[522] However, researchers are recommended to ensure that staining intensity is not associated with the storage time of the archival material.[517] We did not find any association between storage time of the FFPE tissue (categorized into 5-year periods) and staining index for pStathmin(S38) (IHC data from **Paper IV**), when IHC staining was performed simultaneously for samples in the two major patient cohorts (**Figure 15**, P=0.09). In a previous study by our group, no significant associations between staining index and fixation time was demonstrated for the biomarker Stathmin.[298]

Figure 15. Comparison of the staining intensity in the two cohorts studied for pStathmin(S38) to evaluate potential effects from FFPE tissue block archival time on antigenicity.



Staining protocols: ERα and pStathmin(S38)

How well the staining reflects the protein levels of the marker of interest depends amongst other factors on the antibody used (how specific the antibody binds to the epitope under study), the staining protocol used, and how the staining is evaluated.

IHC assessment of the biomarkers ERα and pStathmin (S38) was performed on 5µm thick TMA sections of FFPE primary tumor samples. Previously published IHC data included in the study was assessed on standard or TMA sections.[104,120,154,228,298] Sections were de-waxed in xylene and rehydrated in alcohol in appropriate concentrations. In tissue formalin fixation, chemical bonds are created between proteins, potentially masking the epitope. Epitope retrieval can be achieved enzymatically (e.g. proteinase K, pepsin, trypsin) or by heat, the latter applied for the IHC studies in this project and also recommended used in IHC of FFPE tissue to minimize the epitope level variance due to variation in fixation time.[528] Peroxidase activity block was performed by S2032 DakoCytomation. Thereafter, samples were incubated with antibodies according to optimized and/or establish protocols (**Table 12**) developed under supervision of senior engineer Gerd Lillian Hallseth and consultant pathologist prof. Lars A. Akslen. EnVision+(K4006) DacoCytomation was applied for detection, with enzyme labeled polymer of the secondary antibody and DAB+ chromogen. Finally, the slides were counterstained with haematoxylin (Dako S2020). The IHC data except of ERα and pStathmin(S38) were performed by other researchers in the groups of prof. Salvesen and prof. Akslen and previously published as part of other studies (see references for primary publication for each marker in **Table 12 – part B**). The IHC protocol for pStathmin(S38), except for evaluation of the staining, was performed in the lab of OvaGene Oncology (Irvine CA, USA) under supervision of William Ricketts (scientist, founder and scientific officer at OvaGene Oncology). Tissue samples expected to express the epitope were used as positive controls. Negative controls were achieved by omitting the primary antibody, using diluent only.

Table 12. Immunohistochemical protocols. A: New to this project, B: previously published.

A.

Antibody[a] Provider/Clone	**Epitope retreival**	**Dilution**	**Incubation[b]**	**Detection**	**TMA/ Std. slide**
ERα Dako/ER50	Tris-a EDTA MV 10' 750W, 20' 350W	1:50	30 min	EnVision	TMA
pStathmin(Ser38) Cell Signaling/D19H10	Citrate, Bond Epitope Retreival Solution 1	1:200	15 min	BondPolym Refine detect	TMA

B.

E-cadherin[120] Zymed Laboratories/HECD-1	Citrate MW 10' 750W, 15' 500W	1:400	Overnight	LSAB	Standard
β-catenin[120] BD Transduction Lab./14	Citrate MW 10' 750W, 15' 500W	1:800	25 min	LSAB	Standard
P-cadherin[120] BD Transduction Lab./56	TRS MW 10' 750W, 15' 500W	1:100	60 min	EnVision	TMA
Catenin p120[120] BD Transduction Lab./98	TRS MW 10' 750W, 15' 500W	1:3200	60 min	EnVision	TMA
Stathmin[298] Cell Signaling/3352	Citrate MW 10' 750W, 15' 350W	1:50	1hr	EnVision	TMA
Ki67[154] Dako/A-047	Citrate MW 7.5' 750W, 5' 500W	1:50	1 hr	LSAB	Standard

Footnotes: [a] References to previously published data. [b] Incubation at room temperature if not otherwise specified. **A)** IHC method for ERα and pStathmin(Ser38) applied in **Paper II and III**. **B)** IHC for antibodies primarily evaluated and published in other studies. Abbreviations: LSAB=Labeled StreptAvidin Biotin. TMA=tissue microarray.

Evaluation of staining

Various staining evaluation methods are applied in biomarker studies; *e.g.* percent stained of evaluable area and an H-score weighting differently weak to strong nuclear staining in the same sample.[529] The National Cancer Institute and European Organisation for Research and Treatment of Cancer suggested the "Reporting Recommendations for Tumor Marker Prognostic Studies" (REMARK).[515,530] In our research group, a staining index (SI) method for staining evaluation has been established in the group of L.A Akslen[531,532] and has been applied over several years and in many studies, covering various cancer types.[298,355,531-536] The staining index considers both staining intensity and the proportion of tumor cells with positive staining reaction. SI (0-9) is the product of staining intensity (0-3) and proportion immunopositive tumor cells (0%=0, <10%=1, 10-50%=2, >50%=3). The method is subjective and semi-quantitative. Intra- and interobserver reproducibility have been evaluated in several studies, with good outcome.[120,298,531,533,537] Also, in this project a subset of random TMA slides covering 210 cases was evaluated for interobserver agreement for pStathmin(S38) staining evaluation between 2 of the authors on **Paper III** (H.B.S. and E.W.), blinded for each other's evaluation results. Complete categorical agreement was found in 88% of the samples (Kappa value=0.7) which is considered good agreement between observers.[538]

By including two staining measures (staining intensity and area stained), the SI method potentially better quantitates the amount of epitope bound by the antibody, as compared to evaluation of only one staining measure. This principle is also similar to the FDA-approved criteria for HER2 assessment used in routine diagnsotics of breast cancer (Hercep Test).[539] However, assessment of the SI applied in independent laboratories is yet to be done and such a "two-point" score index, and in particular the more subjective staining intensity measure, may contribute to reduced inter laboratory reproducibility, as seen for other score methods.[540] A score method less subjectively influenced could potentially be more valuable in a routine setting. Follow-up studies comparing the score methods applied in the research setting and the routine setting should in that case be

performed. Digitalized image analysis including data on morphology is presented,[541,542] and time will show how well such methods perform in routine pathology.

3.4 Gene expression data

Cancer associated transcriptional alterations being important to cancer development are often preceded by genomic aberrations (e.g. copy number gain/loss, mutations). The genomic and the accompanying transcriptional alterations may potentially point to targets for therapy and may also predict therapy response. Gene expression data has also proved useful to classify cancer into molecularly defined subtypes,[412] and the gene expression technology is regarded an important tool in cancer research.[543]

DNA Oligonucleotide microarray

In DNA oligonucleotide technology, total RNA is extracted and reversely transcribed to complementary DNA (cDNA) before amplified to cRNA. Fluorescently (Cy3) labeled ribonucleotides are incorporated in the cRNA in the amplification process. Labeled cRNAs are hybridized to complementary probes on a microarray slide. The strength of the fluorescent signal from a spot after hybridization depends on the amount of the sample cRNA that binds to the probes at this spot. Each spot signal was read by scanning the arrays using Agilent Microarray Scanner Bundle. The spot signals are processed by various measures (adjusting for background signal, replacing missing values, filtering spots and normalizing signal data) and thereafter represents the gene expression level for the genes present at the spots. In our study, the final gene expression levels were determined using J-Express (*www.molmine.com*) and BRB-ArrayTools (*http://linus.nci.nih.gov/BRB-ArrayTools/*).

One-channel microarray technology is applied in this study (Agilent Whole Human Genome Microarrays 44k (Cat.no. G4112F)), where only one fluorescent dye is added to the samples and one sample is added per array. The expression levels for each gene are

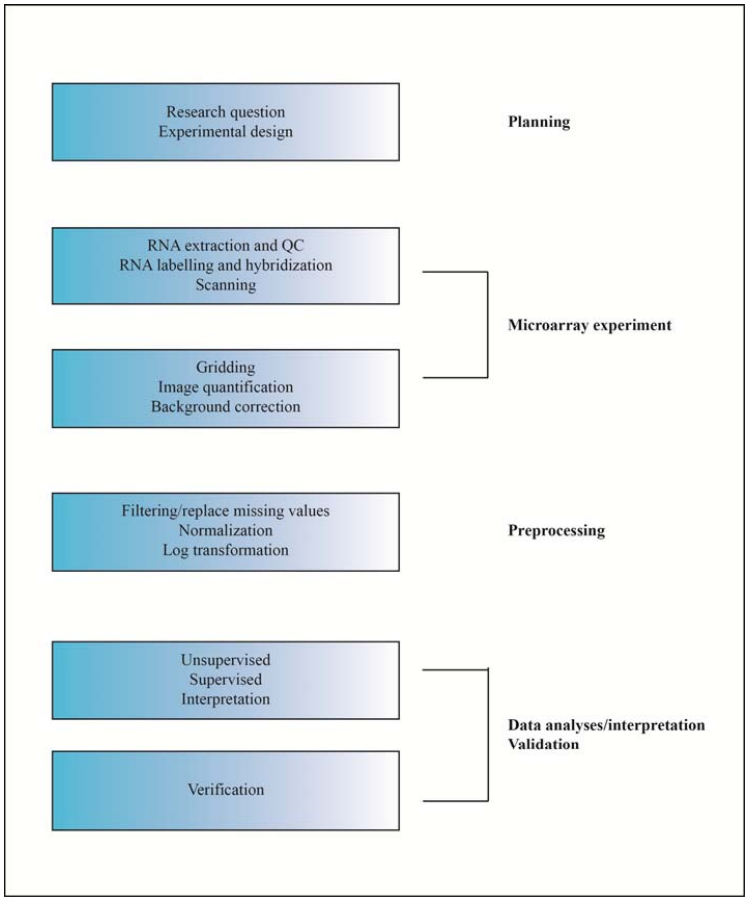
indicated as a relative hybridization level for the current target. The expression values are not true measures for abundance, but are merely used to relate expression levels between classes; to compare expression levels between groups of samples processed in the same experiment.

Gene expression profiling by microarray is today widely used in the search for transcriptional alterations and network-based interactions in cancer (**Figure 16**).[543] The first DNA microarray study was described in 1995, assessing 45 genes in a plant (*Arabidopsis thaliana*).[544] Both the microarray technology and areas of application have since then been extensively developed and standard gene expression microarray experiments assess thousands of genes; in the arrays we have applied, 44 000 probes cover 20-22 000 genes.

Microarrays are performed on "simple" and "complex" systems, such as a cell lines and human cancer tissue, respectively.[545] Sampling complex systems makes is more difficult to conclude on the origin of signals; does the increased expression of the gene of interest come from the epithelial or stromal cells, or from the vasculature interspersed amongst the other cells? Until recently, the cancer cells (the cancer epithelial cells when studying a carcinoma) have been the main focus in cancer research both when trying to reveal mechanisms for initiation and progression of cancer as well as targets for therapy. To optimize human cancer tissue sampling for genome wide analyses (e.g. SNP array, gene expression array), it has been regarded "good practice" to increase the proportion of the epithelial component by sampling cancer tissue with high tumor cell content, microscopically judged or by laser capture dissection (LCD) of epithelial cancer cells. In our microarray studies, the majority of samples have tumor content >80% (microscopically judged in frozen sections immediately adjacent to sections used for DNA and RNA extraction). A study by Halle et al demonstrated an association between high tumor cell content in snap frozen tissue and high histologic grade and other features of aggressive endometrial carcinomas and reduced survival.[546] This demonstrates the

importance of biomarker validation in routine settings before applying research results in routine pathology and clinical practice. As tumor stroma has gotten extensively more attention in translational cancer research, separate gene expression assessment of these cells might be performed by LCD.[547,548]

Figure 16. Pipeline of microarray analyses (*Adapted from*[545]).



Probe selection

Probe selection for whole gene expression microarrays, aiming for optimal specificity and sensitivity, often occurs through specialized design programs.[549] Optimal probe design is

a compromise between specificity and sensitivity:[550] Long oligonucleotide probes (50-80 mers) are likely to cross-hybridize[551,552] while short oligonucleotide probes (25-30 mers) are more likely to present with low signal intensity.[553] The Agilent 44k arrays apply oligonucleotide probes, each consisting of approximately 60 nucleotides.

Probes are designed with an "intelligent" purpose. However, it is recognized amongst users of microarray technology and analyses, that there are "good" probes and "bad" probes. An example: the ESR1 gene at the Agilent 44k array is covered by three different probes, each of them covering ~60 nucleotides of the ESR1 gene that in total is ~ 413k base pairs long. In our data, one of the probes does not associate significantly with ERα protein expression (by IHC) or with ESR1 mRNA by qPCR. The two other probes associate both with estrogen receptor α protein and mRNA (qPCR) expression (**Paper II**). We further found that the one "outsider probe" did not vary to any extent between samples, while the other two probes did. Are we dealing with a "bad" probe? Or is the lack of association between this probe and ERα protein expression pointing to a biological explanation for this finding? One approach, if there are more than two probes, may be to examine how the expression values for each probe relates to the other probes, potentially adding an argument for these probes being particularly relevant as compared to a probe which expression did not associate with other probes for the candidate gene.

Bioinformatics

The field of bioinformatics has developed rapidly as the high-throughput analyses have become daily practice for many researchers. Bioinformatic tools are highly valuable when compiling clinical data and expression information about 44 000 probes covering 20-22 000 unique genes for each tumor sample run on the arrays, and when trying to see some of the "stories" within the large amount of data. Such tools may aid to extract trustworthy and biologically meaningful information from the microarrays. Before data analysis, the raw data must be pre-processed. Background intensity and spots with low or difficult detectable signals must be removed.

Normalization

Normalization is a major step before comparing gene expression values between arrays. There is likely an intensity imbalance between RNA samples not due to biological differences and true gene expression alterations between samples, but for reasons such as imbalance in RNA input or differences in uptake of dye.[554] The goal of normalization is to ensure similar distribution of expression values across arrays and will facilitate the comparison of expression values between samples.[554,555]

There are several different normalization methods – some more recognized than others. A way to find an appropriate normalization method is to test various methods and see how they affect the data and output. We have used median over entire array (**Paper II**) and quantile normalization (**Paper III and IV**).[556] By normalizing data, we reduce the risk of false positive findings, but simultaneously introduce an increased risk of false negative findings, as we may dilute differences that were real, but adjusted so that the difference vanish in later analyses. This is an issue especially for genes with a low expression fold change between classes.

Analyses of transcriptional alterations between samples

Microarray analyses can be divided into *unsupervised* and *supervised* analyses. The former require no supplementary information to the expression data to be performed. The latter is driven by sample characteristics, typically in 2 groups (*e.g.* "positive" versus "negative" molecular phenotype, high versus low tumor stage).

Unsupervised analyses and class discovery:

By unsupervised analyses, without guidance by additional data except for the gene expression data itself, the aim of the methods is to find patterns in the expression profiles where no predefined class is presented.

Hierarchical clustering is an example of unsupervised analysis. This method aims to group together similar objects based on measures of similarity and dissimilarities between the objects.[557] Hierarchical clustering requires specification of a *similarity metrics* and a *linkage*. The *similarity metric* describes how similar two samples are by reflecting the distance between two samples. Additional information for the distance between clusters is needed, and this is in the hierarchical cluster analyses reflected by the *linkage method* (single, average or complete linkage). Complete linkage are demonstrated to be superior for clustering genes,[558] while for clustering of samples, both average and complete linkage is proven useful.[554] Validation of the identified clusters is crucial, including validation of both biological and clinical plausibility and the level of statistical evidence.[554] The hierarchical clustering is applied in **Paper II, III and IV.**

Supervised analyses

Single genes differentially expressed between groups

Identifying genes with known function that are differentially expressed between two groups may provide better understanding of biological differences between the groups.[554] If the genes found are of unknown function, the analyses might provide insight into new gene functions. Supervised analyses require supplementary information about the groups, such as clinico-pathologic data or molecular phenotypic data. In **Paper II and III**, supervised analyses are performed based on ERα and pStathmin(S38) status.

An increased risk of false positive findings due to *multiple testing* occurs as we run 20 000 tests simultaneously on the same data, when searching for genes differentially expressed between classes. There are various methods to adjusting for multiple testing, all of them with the aim to give us certainty that the genes in our analysis output really are differentially expressed between the groups we examine, and not listed due to chance. Being very strict in the multiple testing adjustments might lead us to mask true biological effects. The adjustments will thus be a "trade-off" between too few and too many genes

correctly identified as differentially expressed between classes. It is generally accepted that adjusting to get no false positive genes in the output is a too stringent approach. If searching for single genes differentially expressed between classes, the genes identified should never the less be further validated, and elimination of potential candidate genes/biomarkers (false positives) occurs at these stages. A commonly used approach to adjust for multiple testing is to control the false positive discovery rate (FDR).[559] In the **Paper II and III** we have adjusted for multiple testing by performing the class comparison analyses "Significance Analysis of Microarray" (SAM), where the FDR adjustment method is included.[560] We have defined FDR <0.05 and FDR<0.1 as cut-off (**Paper II and III**, respectively), relatively stringent cut-off values, but for the purpose of our analyses we found it more important to reduce the risk of false positives than risk of false negatives.

Gene Sets differentially expressed between classes

To gain further insight into biological mechanisms involved, based on gene expression microarray data, is a major challenge when working on DNA microarray data.[422] Subramanian et al. pointed to a few highly relevant obstacles in how to interpret the single-gene lists into new and/or relevant biological information:[422] We may miss information about pathways alterations by single gene analyses, as the interpretation of these are heavily depending on the researcher's pre-knowledge in the field. Pathway signaling may involve large gene networks, and we should not be too focused on "large enough" fold changes of single genes in the search for biological information in our data output. Minor changes in all genes known to be involved in a signaling pathway may be of higher importance than large fold changes of a few genes.

Gene Set Enrichment Analysis (GSEA), an online available tool (*www.broadinstitute.org/gsea*), is a method that determines whether an *a priori* defined set of genes shows statistically significant differences between two classes (*e.g.* phenotypes). The Molecular Signatures Database (MSigDB) is a publically available

collection of five major classes of annotated gene sets (*www.broadinstitute.org/gsea/msigdb*) (**Table 13**) and is implemented in the GSEA.

Table 13. Overview of the 5 major classes of gene sets in MSigDB (*www.broadinstitute.org/gsea*)

Gene Sets	Description
Positional	Gene sets corresponding to each human chromosome and each cytogenetic band has at least one gene
Curated	Gene sets collected from various sources such as online pathway databases (*e.g.* BioCarta, Kegg), publications listed in PubMed and from domain experts
Motif	Gene sets that contain genes that share a cis-regulatory motif that is conserved across the human, mouse, rat, and dog genomes
Computational	Gene sets defined by mining large collections of cancer-oriented microarray data
Gene ontology (GO)	Gene sets named by their GO term and contain genes annotated by that term

The gene expression signature applied in GSEA/ MSigDB are generated in various ways, and caution needs to be drawn when interpreting the results. To draw conclusions on gene set analyses, it is crucial to understand how the gene sets and signatures in question are generated and evaluate whether the gene set as generated is relevant for the current study. Also when exploring on gene sets alterations between classes like in GSEA, it is important to adjust for multiple testing, which we did by use of FDR (See above) (**Paper II and III**). Another gene set analysis (GSA), presented by Efron & Tibshirani[561] (applied in **Paper II**) is a further development of the GSEA and suggested to present output with more strength than the statistics used in GSEA.[554]

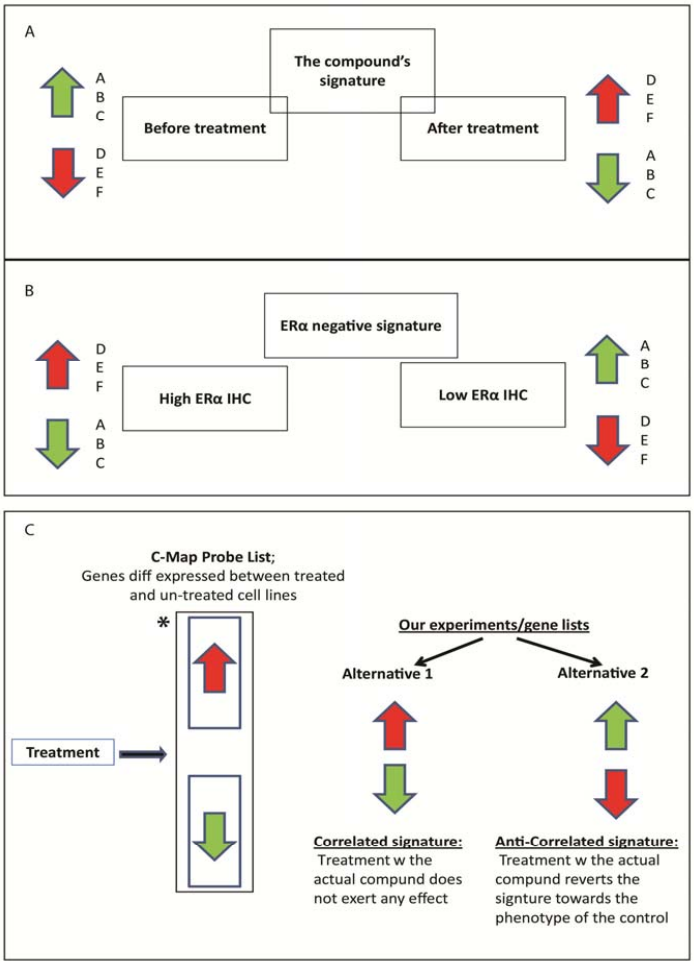
Gene expression signature score

Different approaches can be used to calculate a *gene expression signature score*, based on the expression values of the genes comprising the gene set/signature.[415,512,562] For this, we have selected an established model where the signature genes are high level mean and variance normalized, and thereafter summarized to a score by subtracting the expression sum of down-regulated genes from the expression sum of up-regulated genes.[415]

Connectivity Map

Connectivity Map is a publically available drug signatures database (*www.broadinstitute.org/cmap*),[424] and applied in **Paper II, III and IV.** The database is built up by treating different cancer cell lines with ~1300 different compounds, both FDA approved drugs and other bioactive compounds. The cell lines are treated with various compound concentrations and treatment durations. Cell line RNA is extracted and run at gene expression microarrays before and after the different therapy regimens, and genes differentially expressed between treated and non-treated cell lines comprises the "compound's signature". A gene signature from a specific study (*e.g.* our ERα negative gene expression signature, **Paper II**) is correlated to the compound signatures and given a "connectivity score", indicating whether our signature of interest is positively or negatively associated with the various compound signatures (**Figure 17)**. A ranked list of compound signatures with significant positive or negative correlation with our signature of interest is given.

Figure 17. Connectivity Map; schematic workflow. **A.** Genes differentially expressed between a cell line before and after treatment with one specific compound constitute the "*compound signature*". Arrows up/down denotes genes higher/lower expressed in one cell line, compared to the other cell line (non-treated versus treated). **B.** The signature of ERα negative tumors is generated the same way. **C.** When running a *candidate signature* (*e.g.* the ERα negative signature) against all the compound signatures in Connectivity-Map (as exemplified by one signature in [*]), the candidate signature may positively [Alt 1] or negatively [Alt 2] associate with the compound signatures.



Connectivity Map may serve as a hypothesis generating tool that aims to associate gene expression data, small molecules and disease states.[424] By correlating a gene expression signature of interest to the drug signatures in the database, hypotheses of targets for therapy and pathophysiological processes can be derived. Limitations are present, such as few cell lines included in the test panel, limited number of concentrations tested, and maybe not a good enough correction for false positive findings, as the database has been extended from the first 164 compounds to more than 1300 compounds today. Also, it is important to remember that the hypotheses generated will need to be further tested to conclude on associations and potential relevance for new therapeutics. Due to the relative uncertainty related to where a signal of interest originates from, together with the issue of false positive findings due to multiple testing, validations of results from gene expression microarray studies are recommended.

Quantitative polymerase chain reaction and relative quantitation of gene expression

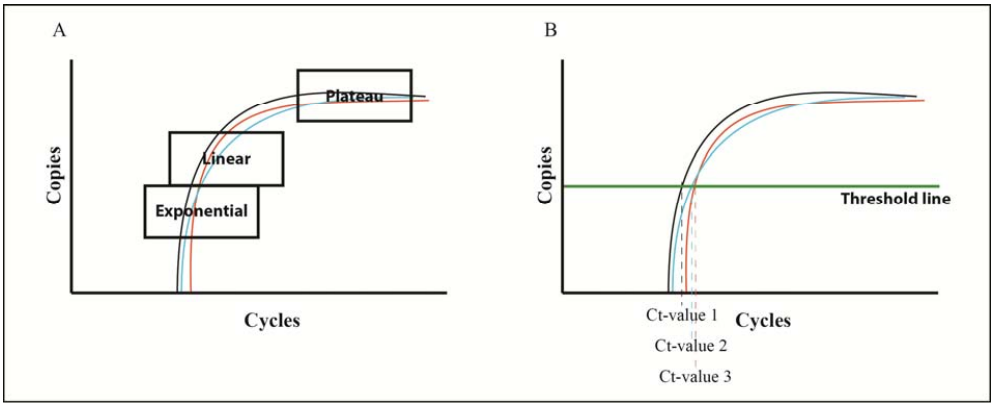
In polymerase chain reaction (PCR), total RNA is extracted from fresh frozen tumor tissue. RNA is thereafter converted to cDNA for hybridization with DNA oligonucleotide primers for gene(s) of interest. The cDNA product is doubled in cycles, aided by DNA polymerase and cycling of the primer and sample temperature. The PCR reaction consists of three different phases: 1) *the exponential phase* with a doubling of product at every cycle; 2) *the linear phase* - the reaction slows down*; 3) the plateau*, where no more products are being made (**Figure 18**).

Unlike traditional PCR, which measures the amount of accumulated PCR product at the end of the PCR cycles, real-time qPCR measures PCR products as they accumulate during amplification. Two values are calculated during the qPCR run: 1) A threshold line/level of detection at which reaction reaches a fluorescent intensity above background in the exponential phase of amplification, 2) The PCR cycle number at which the sample reaches this level, called "Cycle threshold" (*C*t) (**Figure 18-B**). The higher the RNA input

to the qPCR reaction, the fewer cycles are needed before the sample will reach its copy number threshold level and the numbers of cycles (Ct value) needed to reach this level is lower than for a sample with lower RNA input.

In this project, we used qPCR to validate the mRNA expression of ESR1 and SERPINE-1 (= PAI-1) from the DNA microarray (**Paper II**). We also assessed candidate genes linked to EMT by qPCR (**Paper II**) and the expression levels of the 29 genes in the *endometrial cancer recurrence score*, **ECARS** (**Paper IV**).

Figure 18: Schematic picture of the copy number increase per cycle and the three phases of the qPCR (A) and examples of three genes with different cycle threshold (Ct) values.



The qPCR was run in TaqMan© Low Density Arrays (LDAs). The LDAs are microfluidic cards with user-defined primers and probes, detecting mRNA levels of single genes from tumor. A sample of complex, atypical endometrial hyperplasia was used as calibrator. The genes' expression levels were estimated based on the delta-delta-Ct method for relative quantitation of expression.[563]

The most optimal choice for calibrator for the relative quantitation is a sample where the gene expression variance across samples is low. The atypical hyperplasia was an appropriate calibrator for the genes assessed in these studies. GAPDH and ACTB were run as endogenous controls. We assessed which of the genes GAPDH and ACTB that was least associated with clinico-pathologic phenotype in the patient series assessed and GAPDH was applied in the relative quantitation as this varied least between samples. Several studies have reported on a panel of endogenous control in qPCR studies, selecting the best endogenous control for the specific experiment, the control gene depending on the experiment and the type of tissue being examined.[564-566] With this knowledge in mind, our studies could potentially have benefitted from running a larger panel of endogenous controls, selecting the most appropriate one for each marker and study.

3.5 DNA analyses

Primary endometrial carcinoma tissue was examined by Single Nucleotide Polymorphism (SNP) arrays in a previous publication from the research group.[79] These SNP array data were applied in this project (**Paper II, III and IV**).

PIK3CA exon 9 and 20 was sequenced primarily in a study by Birkeland et al.[71] These data were also applied in this project (**Paper II, III and IV**).

PIK3CA copy number in primary tumors was examined by Fluorescent *in situ* hybridization (FISH) by project collaborators (F. Holst and R. Simon) (**Paper III and IV**).

3.6 Statistical methods

Statistical analyses

Statistical analyses have been performed, using SPSS 15 (Statistical package for social sciences 15.0) or PASW 18.0 (IMB, New York, USA). Associations between categories have been assessed by Pearson's chi-square test, replaced by Fisher's exact test when estimated expected count was <5. Comparison of continuous variables between categories was examined by Mann-Whitney U test. The Spearman rank correlation test was used for correlation analyses between two continuous variables.

Comparing the survival pattern between groups (disease-specific, recurrence free survival) was performed by log-rank (Mantel-Cox) test. The Kaplan-Meier plot was applied to visualize survival and lifetime data. Multivariate survival analyses were performed by Cox' proportional hazard regression method and the likelihood ratio test. Each variable was examined by log-minus-log plot before incorporation into the multivariate regression model to determine if the variable was appropriate to be included in the analysis. Unadjusted and multivariate adjusted hazard ratios (HR) were estimated. Tests for interactions were performed if regarded pertinent. Time of entry was similar to time of diagnosis, at primary surgery. The end-point was first recurrence or death from endometrial carcinoma, when assessing risk of recurrence or death from disease, respectively. All statistical tests were considered statistical significant if $p<0.05$ (except for the DNA microarray analyses, see separate section).

Cut-off determination

We scored the samples blinded for clinical data and outcome measures of the patient series under study, as recommended by the REMARK guidelines.[530] To dichotomize continuous and ordinal/nominal variables (gene expression and IHC data), the variables were categorized by median and quartiles, as a quartile based cut-off (also for ERα) has been demonstrated useful and robust in various biomarker studies in previous publications

from the group,[167,298,355,435] and is also a recommended approach without the risk of over fitting the cut-off point to the data examined.[567] The number of events in the quartile groups was considered, and groups with similar survival pattern were eventually merged,[568] rendering median or upper/lower quartile the cut-off point for the IHC variables assessed (**Paper II and III**), **Table 14**, while the median was chosen as cut-off for the endometrial carcinoma recurrence score (**Paper IV**).

Table 14. Overview of IHC staining; localization of staining and cut-off definition.

Antibody	Standard slide or TMA	Staining localization	Cut-off; percentile group/SI (high)
ERα	TMA	Nuclear	Lowest quartile/SI >0[a]
pStathmin(S38)	TMA	Cytoplasmic	Highest quartile/SI ≥6
Previously published data			
E-cadherin[120]	Standard slide	Membranous	Median/SI >3
β-catenin[120]	Standard slide	Membranous	Median/SI >2
P-cadherin[120]	TMA	Membranous	Highest quartile/SI ≥6
Catenin p120[120]	TMA	Membranous	Lowest quartile/SI ≥3
Stathmin[298]	TMA	Cytoplasmic	Highest quartile/SI =9

Footnotes: SI (high) = staining index defining "high expression". TMA= Tissue microarray. [a] SI ≥2 in prospective validation series (n=155, **Paper II**) and in qPCR validations series (n=158, **Paper IV**).

3.7 Approvals

This study has been approved by the Norwegian Data Inspectorate (961478-2), the Norwegian Social Science Data Service (15501) and the local Institutional Review Board (REKIII nr 052.01).

4. MAIN RESULTS

Paper I

The prognostic impact of DNA ploidy was investigated in endometrial carcinomas in a research setting (n=101) and compared to a routine diagnostic setting (n=262), with aneuploidy detected in 25% and 21%, respectively. Aneuploidy was associated with higher age at diagnosis, non-endometrioid histology and high histologic grade in both series studied. DNA ploidy was correlated to poorer survival in both uni- and multivariate survival analyses, adjusting for age, histologic subtype, histologic grade and FIGO stage, also in the routine diagnostic setting. Within the subgroup of FIGO stage I cases only, DNA ploidy was still associated with survival outcome in univariate survival analysis, when merging both data sets. Within FIGO stage I cases of the routine diagnostic series, DNA ploidy showed only borderline independent association with survival in multivariate survival analysis, adjusting for age, histologic subtype, histologic grade and myometrial infiltration. In this study, we found the research and routine diagnostic series to be comparable, with no significant differences in distribution in the standardly applied clinico-pathological variables.

Paper II

We investigated 4 independent patient series and found ERα level to be an independent prognostic marker in endometrial carcinoma; low ERα expression was associated with age, non-endometrioid histology, high histologic grade, and high FIGO stage, as well as poor survival. The same pattern was seen in patient subgroups comprising cases with expected good survival, such as endometrioid grade 1 or 2 tumors only. ERα maintained its independent association with prognosis in multivariate survival analyses, adjusting for age, histologic subtype, histologic grade and FIGO stage.

Transcriptional differences based on ERα status revealed pathways, single genes and transcription factors linked to epithelial-mesenchymal transition (EMT) enriched in ERα negative tumors. These findings were validated in an external gene expression data set. The association between low ERα expression and EMT was further validated in two independent patient series; low ERα expression was significantly correlated to pathologic mRNA expression of the cell adhesion E-cadherin, catenin p120, catenin α and catenin β expression levels. Further, in a population based series, ERα negative immunohistochemical expression was associated with pathologic protein expression of E-cadherin, P-cadherin, β-catenin and catenin p120, as well as deep myometrial infiltration and vascular invasion.

A generated EMT gene expression signature demonstrated that high signature score significantly correlated with aggressive endometrial cancer and poor survival in univariate and multivariate survival analyses, adjusting for age, histologic subtype, histologic grade and FIGO stage.

ERα-low tumor status was also significantly correlated to various markers for PI3K pathway alterations including amplification of the 3q26 region harboring PIK3CA, PIK3CA/PTEN/AKT1 mRNA levels, high PI3K activation score and high Stathmin expression, although ERα did not significantly associate with presence of PIK3CA mutations in exon 9 or 20. When querying a drug signature database (Connectivity Map) for compound signatures significantly correlated to the gene expression signature of low ERα expression in two independent patient series, PI3K/mTOR inhibitor signatures were in both series top ranked amongst the negatively correlated compound signatures.

Paper III

The immunohistochemical expression of pStathmin(S38) associated with a clinico-pathologic phenotype; high pStathmin(S38) was correlated to features of aggressive

tumors, as non-endometrioid histology, high histologic grade, and high FIGO stage. High pStathmin(S38) also correlated with shorter disease specific and recurrence free survival, in both the investigation and validation cohorts. Within subgroups of presumed good prognosis, high pStathmin(S38) was correlated to reduced survival. Furthermore, pStathmin(S38) was independently associated with prognosis in multivariate survival analyses of tumors confined to uterus, when adjusting for myometrial infiltration, histologic subtype and histologic grade. pStathmin(S38) and Stathmin expression levels were significantly correlated but pStathmin(S38) was stronger associated with disease specific survival than Stathmin, when assessing both markers in the Cox' survival analysis, adjusting for histologic subtype, histologic grade and myometrial infiltration.

The gene expression pattern related to pStathmin(S38) expression was analyzed, and gene sets related to cell cycle progression were observed enriched in pStathmin(S38)-high cases. pStathmin(S38) also correlated with a panel of established markers for tumor cell proliferation: Ki67, mitotic count and S-phase fraction, whereas high Stathmin level associated with high proliferation assessed by Ki67 and mitotic count.

A pStathmin(S38)-high gene expression signature was negatively correlated with drug signatures representing effect of PI3K/mTOR and HSP90 inhibitors. Furthermore, high pStathmin(S38) correlated significantly with several potential markers for PI3K activation.

Paper IV

In this study, we validated the ability of the previously defined 29-gene signature[79] to predict recurrence free and disease specific survival. The 29-gene signature score, now assessed by qPCR, validated to identify patients with increased risk of recurrence, also in patient subgroups with presumed favorable outcome. The 29-gene endometrial carcinoma recurrence score (ECARS) was also associated with clinico-pathologic data of aggressive

endometrial cancer. Also, the two patient clusters defined by the signature were associated with reduced survival when assessed by oligonucleotide microarray data in an independent cohort (n=65) and in the cohort originally identifying the signature (n=57), now with new generation oligonucleotide microarrays. ECARS also validated to predict overall survival in 324 cases from The Cancer Genome Atlas (TCGA) database.

High ECARS was associated with vascular invasion and measures for EMT. Also, the signature score was significantly associated with potential measures for PI3K pathway activation; amplification of the 3q26 region harboring PIK3CA, high PIK3CA copy number assessed by FISH, high Stathmin, high PIK3CA mRNA, and high PI3K activation score.

We also assessed how ECARS and our EMT score (**Paper II**) were distributed in metastatic (n=19) compared to primary tumors. Both the ECARS and EMT score were significantly higher in the metastatic lesions.

5. DISCUSSIONS OF RESULTS

Endometrial carcinoma is a heterogeneous disease often early diagnosed and with overall good prognosis. However, 15-20% of patients with localized disease at diagnosis experience recurrences, with increased risk of cancer-related death. Also, patients may experience reduced quality of life due to early and late toxicity, specifically related to the cancer treatment. To better balance the risk of both under- and over-treatment, there is a need to develop methods to improve the identification of patients with high risk of recurrences and to tailor surgical adjuvant therapy. Today, prognostication is based largely on histopathologic parameters and surgical staging.[30] In the locally advanced or metastatic setting, more effective and targeted systemic therapies are needed, as well as markers predicting response to such treatment.

This study is based on four publications (**Paper I-IV**), focusing on validation of the prognostic potential of selected biomarkers in independent cohorts and in a routine setting. Furthermore, we explore new biomarkers and relate these to tumor biological processes and potentials for targeted therapies by exploring drug signatures associated with biomarkers present in aggressive endometrial cancer in particular. By this, we suggest markers to identify patient groups with higher risk of recurrence and cancer-related death and also provide a rationale for potential relevant targets for therapy and predictive markers in endometrial carcinoma. We also add knowledge to parts of the tumor biology underlying aggressive endometrial carcinomas.

5.1 Improved identification of patients with high risk of recurrence and cancer-related death

Current clinical decision making in the treatment of endometrial carcinomas mainly relies on surgical FIGO stage, histologic subtype and histologic grade.[30] Several biomarkers

have been demonstrated to predict survival in endometrial carcinoma, although none are yet applied clinically for risk stratification.[30,148]

After the first identification of a prognostic biomarker, the next step recommended is validation in independent cohorts before prospective validation in a routine setting. Eventually, prospective validation of clinical applicability for treatment stratification is necessary before application in routine practice.[569]

A prognostic impact of DNA ploidy is well-established in endometrial carcinoma, the aneuploid tumors being associated with reduced survival, also when adjusting for standard clinico-pathologic variables as age, FIGO stage, histologic subtype and histologic grade.[148] In this study, we demonstrate DNA ploidy as a robust prognostic marker (**Paper I**), identifying aggressive endometrial carcinomas also when adjusting for standard clinico-pathologic variables applied for prognostication in the routine setting today.[30] We further demonstrate the robustness of DNA ploidy as a prognostic marker when assessed in the routine setting in a pathology laboratory, as compared to the research setting, an important step in the staircase from research to clinical application.[569,570]

Low ERα expression has been associated with features of aggressive endometrial carcinoma for several decades.[123,353-355] Still, the marker is not routinely integrated to tailor surgical therapy or adjuvant treatment in endometrial cancer.[237,336] We report a strong relation between low estrogen receptor expression (both ESR1 mRNA and ERα IHC expression) and features of aggressive disease and reduced survival (**Paper II**). For the primary investigation and retrospective validation series, ERα staining index (SI) was 0 for the ERα-low cases, representing truly ERα negative cases. For the prospective validation cohort, the ERα-low cases were identified by SI≤3, the same cut-off point also seen for a large multi-center study on ERα immunostaining in curettage specimens (*Trovik et al, unpublished data*). Less inter-observer variation is expected when reporting on the levels of an immunomarker where the cut-off is based on no expression versus any

expression, as compared to the cut-off point based on "weak" versus "strong" expression. If bringing ERα as prognostic biomarker closer to clinical application in endometrial cancer, further studies are needed to define ERα-low cases.

Biomarker signatures (*e.g.* sets of gene or protein expression values or mutation status for a range of genes) are suggested to be of value for improved molecular classification and identification of risk-groups, as published in specific cancer types,[411-413,571] and also recently demonstrated for endometrial carcinoma.[79,572,573] Gene expression signatures are favored as prognostic markers, potentially reflecting a more complex part of the biology in "one go", as compared to single protein biomarkers.[543] Two gene expression signatures (MammaPrint and Oncotype Dx) are approved for clinical utility in breast cancer, predicting risk of recurrence and metastatic disease, and utilized to direct adjuvant therapy to high-risk cases.[412,574] A previously published 29-gene endometrial carcinoma signature, associating with potential measures for PI3K activation and predicting disease relapse,[79] was here validated in three prospectively collected patient series, independent from the series originally identifying the signature (**Paper IV**). The signature validated to associate with features of aggressive endometrial carcinoma and to predict risk of recurrence, also in patient subgroups with expected good prognosis, hence named Endometrial Carcinoma Recurrence Score (ECARS).

Stathmin, a known microtubule destabilizer, is previously reported as a prognostic marker in breast and urothelial cancer,[575,576] and high Stathmin is found to predict lymph node metastases, features of aggressive disease and reduced survival in our patient series.[298] Phospho-Stathmin(S38), one of the four Stathmin phospho-sites known to inactivate the Stathmin function, is previously not studied as a prognostic marker in any cancer type,[153] although explored in experimental models, mainly in relation to the effect on microtubule formation, proliferation, cell migration and cancer invasion.[153,577,578] We here demonstrated the ability of pStathmin(S38) immunostaining to identify aggressive endometrial carcinoma, risk of recurrence and cancer-related death, also in patient subgroups with expected good prognosis (**Paper III**). pStathmin(S38) adds prognostic

information to Stathmin. As this is a first exploration of a new prognostic biomarker, although assessed in two independent patient series, further validation in cohorts from other hospitals and with IHC assessment in other labs are needed to conclude on the strength and robustness of pStathmin(S38) as prognostic marker in endometrial carcinomas, and its potential applicability in a routine clinical setting.

5.2 Epithelial-mesenchymal transition (EMT) in aggressive endometrial carcinoma

Through supervised analyses of transcriptional alterations between tumors with low versus high ERα expression (IHC), we identified an association between ERα negative tumors and EMT (**Paper II**). This was a consistent finding also validated in independent patient cohorts and by various EMT measures. Although an association between low ERα and Snail expression is previously published,[212] our study is to date the most comprehensive study to demonstrate the association between ERα negative endometrial carcinoma and EMT. Tumors with low ERα expression were also associated with deep myometrial infiltration and vascular invasion, underlining the invasive characteristics amongst the ERα-low and EMT-positive tumors in this study. Pathologically altered IHC expression of the cell adhesion/EMT markers applied in **Paper II** (retrospective cohort) are in a previous study associated with features of aggressive endometrial carcinoma and reduced survival.[120] Several recent studies have described an importance of EMT in endometrial cancer,[89,129,579,580] and a study by Tanaka et al defined EMT status as low E-cadherin and concurrent nuclear Snail expression (assessed by IHC) and demonstrated an association between EMT status, features of aggressive endometrial carcinoma and reduced survival.[580] In line with this, we identified a curated EMT gene expression signature associated with reduced cancer specific and recurrence free survival (**Paper II**). This EMT signature demonstrated a superior ability to predict poor outcome to the

mRNA expression of single genes reported to be involved in EMT, adjusting for age, FIGO stage, histologic subtype and histologic grade.

Also, high levels of the previously reported Endometrial Carcinoma Recurrence Score (ECARS) associated with several measures for EMT: high EMT score, altered mRNA expression of a panel of cell adhesion/EMT markers, deep myometrial infiltration, vascular invasion, and a vascular invasion gene expression score, supporting that high levels of ECARS also reflects presence of EMT and tumor invasiveness (**Paper IV**). Some of the single genes in ECARS are in the literature linked to tumor invasive properties, while other genes are more linked to tumor cell proliferation, potentially contributing to catch various aspects of the tumor biologic processes involved to develop the aggressive features seen in ECARS-high cases.

From supervised analyses identifying differentially expressed genes and gene sets between ERα negative and positive tumors, a signature indicating TGF-β signaling was enriched in ERα negative tumors. Furthermore, PAI-1 (=SERPINE-1) mRNA expression, a suggested marker for TGF-β signaling,[134] was significantly higher expressed in ERα negative cases, also validated in an independent patient cohort. High PAI-1 expression is shown to be associated with features of aggressive endometrial carcinoma and reduced survival.[135-137] TGF-β is regarded a major player in EMT and the invasive and metastatic process,[127,581] and our findings may imply a role for TGF-β also in aggressive endometrial cancer, as supported by other studies.[129,130]

Is low ERα contributing to drive EMT activation? Breast cancer cell line studies have demonstrated that loss of ERα induces Snail and Slug expression, E-cadherin is subsequently repressed and a mesenchymal-like phenotype and increased invasive properties have been observed.[582,583] A similar causality may be active in endometrial carcinoma. Also, it may well be that not low ERα in itself but a third party is the "driver" of EMT activation in our study, and with low ERα expression only co-occurring with EMT activation. In a study by Dhasarathy et al. on breast cancer cell lines, TGF-β

signaling was demonstrated to be linked to Snail expression, and Snail had the ability to repress ERα.[584] If this is the scenario also in our study, TGF-β renders a potential key player in EMT activation in our setting. Further functional studies are needed to elucidate potential links between low ERα, TGF-β and EMT in endometrial carcinoma.

It is generally accepted that the EMT program is somehow "inactivated" once the metastatic tumor cells reside in the new microenvironment, lacking the signals from the surrounding stroma that initiated EMT in the primary tumor, thus reverting from the induced mesenchymal like back to the epithelial phenotype (mesenchymal-epithelial transition, MET).[127] However, when examining the mRNA sum score of the curated EMT signature in primary and metastatic tumors, we found the score significantly increased from primary to metastatic lesions (**Paper IV**). Genes that constitute our EMT signature may contribute in a manner facilitating the growth of the metastatic tumors in the new environment, as exemplified by deletion of catenin p120 that in a recent study was demonstrated to have a potential as a regulator of inflammation in the tumor microenvironment,[585] and thereby being potentially important for the metastatic colonization. One previous study comparing the levels of EMT markers in primary and metastatic endometrial carcinomas did not demonstrate any significant change in E-cadherin, Snail and Slug protein expression by immunohistochemistry.[580]

5.3 Potential targets and accompanying predictive biomarkers

By examining associations between drug signatures from a publically available database[424] and transcription signatures of molecular phenotypes related to biomarkers associated with aggressive endometrial carcinomas (ER negative, pStathmin(S38)-high cases), we identified targets such as PI3K/mTOR and HSP90 potentially relevant to these molecular subtypes of endometrial carcinoma (**Paper II and III**). Also, PI3K/mTOR is suggested as target in ECARS-high cases in the publication identifying this recurrence score.[79] Based on identified biological processes and signaling pathways, we also suggest

TGF-β signaling and elements of the EMT program as potentially relevant targets in aggressive endometrial cancer (**Paper II and IV**). The majority of ongoing clinical trials in endometrial carcinoma target PI3K/AKT/mTOR (see Table 8, chpt 1.8).

Both TGF-β and EMT are regarded relevant targets in cancer today,[586] and response to inhibitors of these are presently being evaluated (*www.clinicaltrials.gov*, Feb 2013).[586,587] Based on the positive correlations between the biomarkers ERα and ECARS and measures for TGF-β signaling and EMT, we suggest to explore the potential of ERα and ECARS as predictive markers to TGF-β and EMT inhibiting therapy in endometrial carcinoma. Our data provide a rational for this to be tested in relevant clinical trials (**Paper II and IV**). Inhibition of HSP90, as suggested for pStathmin(S38)-high cases (**Paper III**), is also amongst promising drugs entering clinical trials in cancer treatment today. Increased expression of HSP90 allows tumor cells to cope with an imbalanced signaling in cancer, with demonstrated enhanced cancer cell survival.[450] HSP90 inhibitors are presently in clinical trials for various cancer types (*www.clinicaltrials.gov,* December 2012). Potential measures for HSP90 activity are not assessed in this study, as done for EMT and TGF-β. Thus, a next relevant step may be to explore HSP90 expression in endometrial carcinomas.

PI3K/mTOR signaling; suggested targets and predictive biomarkers

The level of understanding of the biology related to the target and how the drug interacts with the target itself and its related biology is regarded critical in drug development.[569] This also applies to the development of relevant and robust predictive biomarkers, together with the robustness of the biomarker assay.[569] Inhibition of the PI3K signaling pathway in cancer has been in focus during the last decade,[92,588] also for endometrial carcinoma where PI3K/mTOR signaling is suggested as a relevant target.[64,79,589] It has been a challenge to identify markers for PI3K signaling activity in tumor specimens, and thereby also relevant predictive biomarkers accompanying PI3K inhibitors. PIK3CA mutations are frequently associated with PI3K activation,[72] although other PI3K pathway

alterations also are suggested as relevant measures.[590] Gene expression signatures are also indicated to reflect PI3K pathway activation,[591,592] the Endometrial Carcinoma Recurrence Scores being associated with one of these.[79]

PIK3CA mutations are recently supported as potential predictive markers in various cancer types, including endometrial carcinoma,[72,73,403,593] with the H1047R mutation being associated specifically with response to PI3K/mTOR/AKT inhibitors.[73] PIK3CA mutations are found both in endometrioid and non-endometrioid subtypes, with a tendency of higher frequency in the type I carcinomas.[64,72] We did not find a significant association between PIK3CA mutations and the biomarkers assessed in this study (ERα, pStathmin(S38) and ECARS), neither when assessing specifically the H1047R mutation. However, low ERα, high pStathmin(S38) and high ECARS are all associated with various other potential markers for PI3K pathway activation (**Paper II-IV**), and we have therefore suggested PI3K/mTOR inhibitors to these patient subsets

Amplification of the 3q26 region has been associated with type II cancer (non-endometrioid histology, high histologic grade, high FIGO stage).[79] An endometrial cancer cell line study demonstrated estradiol driven PI3K signaling through both ERα dependent and independent mechanisms,[145] supporting a potential for PI3K inhibition also in ERα negative tumors, supporting our findings and suggestions (**Paper II**). Phosphorylation of Stathmin is suggested linked to the PI3K pathway,[594] supported by functional studies demonstrating Stathmin phosphorylation by PAK1.[595] This supports relevance to our finding of an association between pStathmin(S38)-high cases and potential measures for PI3K pathway activation (**Paper III**).

By our study, we propose a rationale for exploring the potential of ERα, pStathmin(S38) and ECARS as predictive markers for inhibitors of the PI3K pathway in endometrial carcinoma. In breast cancer, patients with the triple negative (ERα, PR and Her2 negative) and basal-like molecular phenotype is frequently included in clinical trials of various targeted therapies, not always due to a well-defined target demonstrated in this cancer

subtype, but simply because the triple negative/basal-like cancer is an aggressive subtype. The same approach could potentially be applied for ERα negative endometrial carcinomas, also supported by the molecular findings associated with the low ERα in these tumors.

Combinatorial therapy (strategies)

Activated signaling pathways are complex processes, and often more than one pathway may activate a downstream target and affect the 'end-stage' biological processes driving the cancer disease. If targeted mono-therapy blocks for example a receptor tyrosine kinase, alternative signaling pathways may be activated leading to resistance.[596] This, together with the challenges of intratumor heterogeneity,[504] tumor cell evolution[509] and drug resistance (*de novo* and acquired) require development of more complex regimens including combinatorial therapy.[597] Combined drug therapies may be classified as "horizontal" (across two pathways) or "vertical" (within one oncogenic pathway).[597] Targeting a receptor and a protein downstream in the transduction pathway, such as done in dual inhibition of PI3K and mTOR, is an example of such "vertical" inhibition.[593] There are several combinations of molecularly targeted drugs in phase I trials today,[597] and a "vertical" combination of Everolimus (mTOR inhibitor) and Trastuzumab (Her2 inhibitor) given together with Vinorelbine (anti-mitotic chemotherapy) is today in phase III trials in Her2 positive breast cancer. A combination of molecularly targeted drugs and conventional chemotherapy therapy has proven useful in several cancer types, including breast and colorectal cancer.[598,599] This combinatorial strategy has partially been driven as a means to gain regulatory approval for drugs in diseases where conventional therapy is established.[597]

We identify several potential targets for therapy in aggressive endometrial carcinoma (**Paper II-IV**). As EMT and also TGF-β itself are suggested important in drug resistance,[600] these are potentially relevant targets for therapy in combinatorial treatment. HSP90 is required for correct folding of AKT1 and PDK1,[601] and is as such important to

PI3K signaling. Combining HSP90 and PI3K/mTOR inhibitors could be relevant in endometrial carcinoma. A study in metastatic gastric cancer is about to be started with such a therapeutic regimen (*www.clinicaltrials.gov*, ID: NCT01613950). HSP90 inhibitors are designed to affect multiple oncogenic client proteins and may block several cancer-supporting signal transduction pathways[597] and are highly relevant as co-drugs in combinatorial therapy regimens. Also, other combinations of inhibition of the targets we identify in aggressive endometrial carcinomas should be further explored.

It has been suggested to apply panels of markers to better catch the biological alterations associated with therapy response to specific drugs,[569] and gene expression signatures may be examples of such.[436] In line with this, ECARS may be a relevant predictive marker also in combinatorial therapy regimens. Still, a panel of IHC markers may represent more robust and clinically applicable predictive marker, and ERα and pStathmin(S38) may contribute to such a panel.

6. CONCLUSIONS

1. DNA aneuploidy identifies aggressive endometrial carcinoma and predicts poor outcome, also in a routine clinical setting (**Paper I**).

2. Low ERα in endometrial carcinoma is associated with epithelial-mesenchymal transition, vascular invasion and PI3K alterations (**Paper II**).

3. High pStathmin(S38) associates with high tumor cell proliferation and potential measures for PI3K pathway activation in endometrial carcinomas (**Paper III**).

4. The endometrial carcinoma recurrence score (ECARS) validates to identify endometrial carcinomas with shorter recurrence free survival. ECARS increases from primary to metastatic lesions and is associated with potential measures for PI3K activation and epithelial-mesenchymal transition (**Paper IV**).

5. Low ERα, high pStathmin(S38) and high ECARS predict aggressive endometrial carcinomas and reduced survival, and may suggest treatment with PI3K/mTOR and/or EMT inhibitors in clinical trials (**Paper II, III and IV**).

7. FUTURE PERSPECTIVES

"It's not that I'm so smart, it's just that I stay with it longer." (A. Einstein, 1879-1955)

7.1 Potential ways forward

We stand back with the key questions to answer in the further development of personalized therapy: What is a relevant target? Is the target druggable? How to identify and test intelligent combinatorial therapy regimens? And last but not at all least: Which biomarkers match the right patient and the right drugs?

Both better identification of relevant targets and development of robust predictive markers are recognized as important issues to improve survival through more targeted cancer therapies.[444] Development of new drugs is time-consuming and costly. Development of robust, testable and valid biological hypotheses, where the target in question is altered in the cancer, and targetable in tumor cells more than normal cells, are suggested as a critical elements to promote successful drug development.[438]

Systems biology approach

Our understanding of how the cancer cells function in comparison to normal cells is incomplete; the biology is complex and all research done has revealed probably only parts of what is driving cancer. By combining data from various 'omics (*e.g.* genomics, transcriptomics, proteomics, metabolomics), systems biology may reveal more of the complex biological networks that are taking place in cancer cells, and potentially point to "nodes" of major importance for tumor initiation and progression.[602-604] This systems biology approach, through high-throughput profiling, computational tools and experimental validation, is ongoing, addressing the challenge of biological complexity and aid in developing "network" therapeutic strategies and intelligent combinatorial therapies.[438,464]

Fast track accelerated biomarker testing and drug approval

The development of Trastuzumab took ~25 years to be approved for adjuvant therapy to Her2 positive cases after the Her2/neu gene first was identified (*www.fda.gov*).[605] To ensure faster progression from target discovery to clinical trials and clinical implementation for promising drugs, a "Fast Track" accelerated approval and priority review has been introduced. This will accelerate the availability of new drugs for patients with deadly and disabling diseases (*www.fda.gov*). One successful example of this is the approval of vemurafinib to patients with BRAF mutated melanomas only 9 years after the BRAF mutation was first published.[439,606] Furthermore, a new set-up for clinical trials has been proposed; biomarker driven and hypothesis testing trials, where the three phases of the trials are more flexible and adaptive in their performance, depending on clinical and molecular data gathered:[438]

Phase I/Proof of mechanism: Determine the optimal dose (range and schedule) to achieve sufficient target blockade.
Phase II/Proof of concept: Evaluate the antitumor activity and the potential of predictive biomarkers in selected populations.
Phase III/Pharmacogenomics: To identify which patients needs which therapy doses/schedules by assessing inter-patient variability

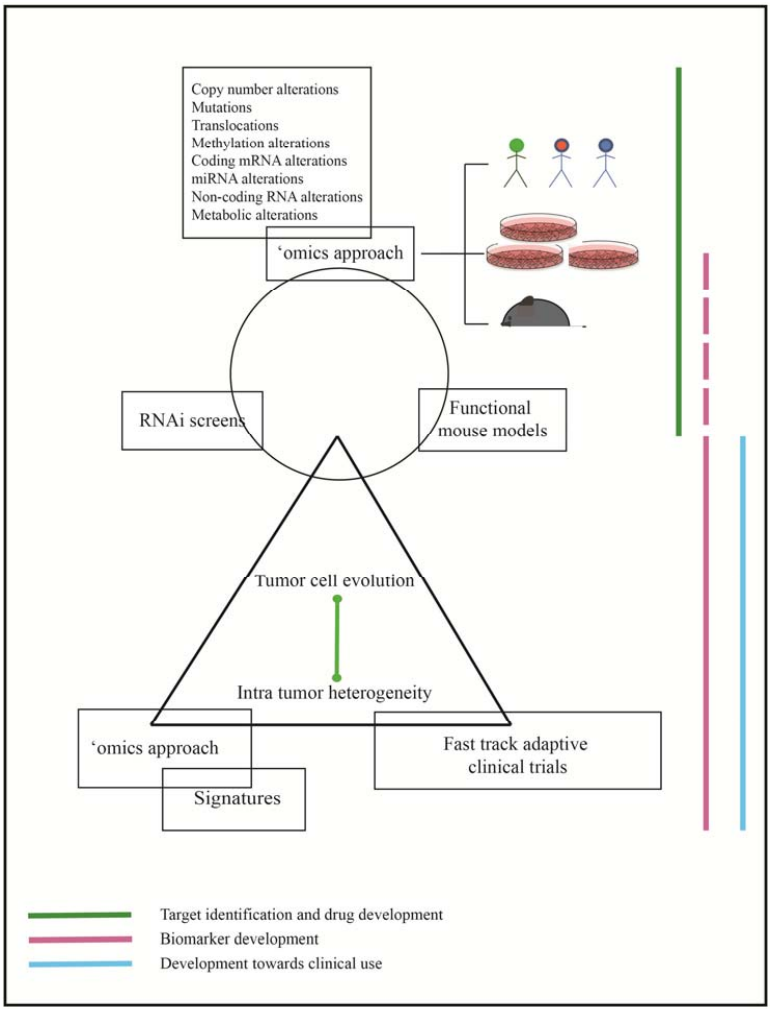
Alternative models of clinical trials have recently been published, enriching for patients with tumors of a molecular genotype.[403,593] Phase II-III trials have been presented with the aim to match the drug(s) studied with molecularly defined tumor subsets, not only to patients with advanced disease.[607-609]

"Joint efforts"

By combining the RNAi screen of cancer cell lines and an 'omics approach to human tumors and large cell line panels, together with functional validation in appropriate models, and also taking the potential strength of signatures into account (both for pointing to targets and as predictive markers), together with "fast track" adaptive designed clinical

trials, we may be approaching good input to and improved output from the strategies to personalize cancer therapy. Also, accounting for the intratumor heterogeneity and tumor cell evolution that takes place, will be important elements in the future strategies of personalized medicine (**Figure 19**).

Figure 19. Schematic overview of applied and suggested methods for target identification, drug and biomarker development and a suggested path to clinical use.



7.2 Suggestions to follow-up studies

"… something is happening here, but you don't know what it is. Do you, Mr. Jones?" (*Bob Dylan,* 1941-)

In the following are suggestions of follow-up studies to this PhD project. These are probably tiny steps in the large puzzle of "how to fight cancer", but to re-phrase Neil Armstrong: *a small step from a study might lead to a giant leap for mankind*…:

Clinical implementation of biomarkers to define risk for recurrence

DNA ploidy is repeatedly demonstrated as prognostic marker in endometrial cancer. A prospective multicenter study exploring on the ability of DNA ploidy to predict outcome, and the potential of DNA ploidy to identify patient subgroups that per today are regarded "good prognosis cases" (defined by standard clinico-pathologic data) could be a valuable add to individualize surgery and the risk stratification for adjuvant therapy. Also, concurrent assay exploration would be a relevant addition to such a study. Assessment of the ability of DNA ploidy to predict response to radiation therapy and also other therapies would be relevant elements of studies exploring the potential of DNA ploidy as prognostic and predictive marker.

We and others have demonstrated that low ERα expression is associated with aggressive endometrial cancer and reduced survival. The time has come to perform a prospective implementation study on ERα as prognostic marker in endometrial cancer, preferably in a multicenter setting. Exploration of immunostaining methods and determination of cut-off (positive/negative) would be important elements in such a study.

Identification of new targets for therapy

As a follow-up on the link between TGF-β/PAI-1 and aggressive endometrial cancer demonstrated in **Paper II,** it would be interesting to explore on the role of these and other microenvironment elements in endometrial cancer progression, and their potential as

targets for therapy. Furthermore, the functional role of ERα in this setting would also be relevant to study.

Further exploration of individual genes in the 29-gene ECARS (**Paper IV**), their association with the cancer hallmarks and their ability to drive cancer progression may contribute to target development and further strengthen the potential of ECARS as predictive marker.

Identification of predictive markers for response to therapy

Based on transcriptional tumor alterations, we have shown that ERα, pStathmin(S38) and ECARS status may suggest different targets for treatment, including PI3K/mTOR. A natural follow-up to this is would be to assess the potential of these markers to predict response to PI3K/mTOR inhibitors. A first step could be to assess the levels of the markers in tumor samples from clinical trials on the mentioned inhibitors. If ERα, pStathmin(S38) and/or ECARS status predict response, a "second level" study would be to enrich for endometrial cancer patients with present relevant markers to in randomized clinical trials of the candidate inhibitor. This will potentially increase the likelihood of significant effect from given therapy in randomized clinical trials with smaller sample sizes. Furthermore, we suggest similar exploration in clinical trials of the ability of pStathmin(S38) to predict response to HSP90 inhibitors.

As Stathmin has been suggested as a marker for response to Taxanes in breast cancer,[610,611] it would be interesting to investigate the potential of pStathmin(S38) alone and together with Stathmin to predict response to Taxanes in endometrial cancer and other cancers.

ERRATA

Material and Methodological considerations: Figure 14, page 94.Number of cases with IHC in 2001-2011 patient series: “534” should read “518”

Reference #498: “N.C, S.M, P.S et al: A phase II trial of the mTOR inhibitor AP23573 as a single agent in advanced endometrial cancer. J Clin Oncol, ASCO Ann Meet Proc 2007.:2518Sabstract 5516, 2007” should read “Colombo N, McMeekin S, Schwartz P, et al: A phase II trial of the mTOR inhibitor AP23573 as a single agent in advanced endometrial cancer. J Clin Oncol, ASCO Ann Meet Proc 2007:Abstract No 18S, 2007”

Reference # 513: “(TCGA) TCGA: Integrated Genomic Characetrization of Endometrial Carcinoma. Nature 2013, *in press*” should read “(TCGA) TCGA: Integrated Genomic Characetrization of Endometrial Carcinoma.”

Reference # 559: “Y.B, Y.H: Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of The Royal Statistical Society 57:11, 1995” should read ”Benjamini Y, Hochberg Y: Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of The Royal Statistical Society 57:289-300, 1995”

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