Context-related biomarkers in endometrial cancer

A study with focus on obesity and genomic alterations

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Scientific environment

This work has been carried out at the Department of Clinical Science, University of Bergen, within the context of Bergen Gynaecologic Cancer Research Group. The research group consists of around 25 members, including PhD students, Post-Doctoral fellows, research fellows, technical staff, research nurses, medical students and PIs. The group has ongoing projects in the fields of tumour biology, preclinical studies, animal modelling, clinical studies and imaging studies. The diverse background of the research group members ensures fruitful collaborations and new insights.

The group is tightly linked to the Department of Gynaecology and Obstetrics at Kvinneklinikken, Haukeland University Hospital, and the collaboration with the clinic has been crucial for collection of all the tissue and data used in the publications included in this thesis.

The research group is part of CCBIO, Centre for Cancer Biomarkers, a Norwegian Centre for Excellence at the University of Bergen, led by Professor Lars A. Akslen. The focus is on tumour biomarkers, translational research and individualised therapy.

The research group has several national and international collaboration partners. We have initiated and expanded the Momatec1&2 studies, a multicentre study with participating institutions from Norway and Europe. The group is an active member in ENITEC, the European Network for individualised treatment in Endometrial cancer. Long term international collaborating institutions include the Broad Institute (Boston, USA) and MD Anderson Cancer Centre (Houston, USA).

Supervisors and mentors of this work have been (in alphabetical order) Erling A. Høivik (MS, PhD), Helga B. Salvesen (MD, PhD, Prof.), Henrica MJ Werner (MD, PhD) and Jone Trovik (MD, PhD, Prof.), all affiliated with the research group.

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My best friend Torstein: thanks for your endless patience, for your ability to make me laugh, and helping me put things in perspective. Words cannot express how grateful I am to have had you by my side during both some really tough periods and some incredibly great times these years.

Bergen, March 2017

Karen Klepsland Mauland
Abbreviations

AKT: v-AKT murine thymoma viral oncogene homolog, Protein kinase B
AR: Androgen receptor
BCR-ABL1: Fusion gene resulting from translocation of the ABL1 gene (Abelson murine leukemia viral oncogene homolog 1) to a part of the BCR (breakpoint cluster region) gene
BMI: Body mass index (kg/m2)
CA125: Cancer antigen 125
CE-CT: Contrast-enhanced computed tomography
CI: Confidence Interval
CT: Computed tomography
CTNNB1: Catenin beta-1/beta-catenin
DAB: Diaminobenzidine
DNA: Deoxyribonucleic acid
DSS: Disease specific survival
EBRT: External beam radiation therapy
EC: Endometrial cancer
EMT: Epithelial-to-mesenchymal transition
ER/ERα: Oestrogen receptor alpha
ESMO: European society for medical oncology
ESR1: Oestrogen receptor 1
FC: Flow cytometry
FDR: False discovery rate
FEF: Fresh ethanol fixed
FF: Fresh frozen
FFPE: Formalin fixed paraffin embedded
FGFR2: Fibroblast growth factor receptor 2
FIGO: International Federation of Gynaecology and Obstetrics
GSEA: Gene set enrichment analysis
H&E: Haematoxylin and eosin
HER2/neu: Receptor tyrosine-protein kinase erbB-2/cluster of differentiation 340
HU: Hounsfield units
ICD: International classification of diseases
IGF1: Insulin-like growth factor 1
IHC: Immunohistochemistry
KRAS: Kirsten rat viral sarcoma homolog
L1CAM: L1 cell adhesion molecule
LD: Liver density
LVSI: Lymphovascular space invasion
MAPK: Mitogen activated protein kinase
miRNA: Micro ribonucleic acid
MLH1: MutL homolog 1
MMR: Mismatch repair
MRI: Magnetic resonance imaging
MSH2: MutS protein homolog 2
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<td>MSH6</td>
<td>MutS homolog 6</td>
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<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>OR</td>
<td>Odds ratio</td>
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<td>OS</td>
<td>Overall survival</td>
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<td>PCOS</td>
<td>Polycystic ovary syndrome</td>
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<td>PD-1</td>
<td>Programmed cell death protein 1</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>PFS</td>
<td>Progression free survival</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>PIK3CA</td>
<td>Phosphatidylinositol 3-kinase catalytic subunit p110alpha</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>Phosphatidylinositol 3-kinase regulatory subunit p85alpha</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PMS2</td>
<td>Mismatch repair endonuclease</td>
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<tr>
<td>POLE</td>
<td>DNA polymerase epsilon catalytic subunit</td>
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<td>PP2A</td>
<td>Protein phosphatase 2A complex</td>
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<td>PPP2R3A</td>
<td>Serine/threonine-protein phosphatase 2A regulatory subunit B′′ subunit alpha</td>
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<td>PR</td>
<td>Progesterone receptor</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<td>RCT</td>
<td>Randomised controlled trial</td>
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<td>RFS</td>
<td>Recurrence free survival</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNAseq</td>
<td>RNA sequencing</td>
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<td>RPPA</td>
<td>Reverse phase protein array</td>
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<td>RR</td>
<td>Relative risk/risk ratio</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>SAM</td>
<td>Significance analysis of microarray</td>
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<td>SAV</td>
<td>Subcutaneous abdominal fat volume</td>
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<tr>
<td>SHGB</td>
<td>Sex hormone-binding globulin</td>
</tr>
<tr>
<td>STAG2</td>
<td>Cohesin subunit SA-2</td>
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<tr>
<td>TAV</td>
<td>Total abdominal fat volume</td>
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<td>TCGA</td>
<td>The cancer genome atlas</td>
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<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<td>TMA</td>
<td>Tissue microarray</td>
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<td>TP53</td>
<td>Tumour protein 53 (also p53)</td>
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<td>VAV</td>
<td>Visceral abdominal fat volume</td>
</tr>
<tr>
<td>VAV%</td>
<td>Visceral fat percentage (visceral/total abdominal fat volume)</td>
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<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>WC</td>
<td>Waist circumference</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Abstract

**Background:** Endometrial cancer is the most common female pelvic gynaecologic malignancy in industrialised countries, and incidence has been increasing over the past decades. This has partly been ascribed to the increasing obesity epidemic seen worldwide, and particularly in affluent countries. Whereas increasing body mass index (BMI, kg/m²) is a known risk factor for endometrial cancer, less is known about its influence on tumour development and prognosis.

**Aims:** The aim of this study was to increase the understanding about how context-related factors, including obesity (assessed by BMI and imaging methods) and genomic alterations (assessed by DNA ploidy status), are related to molecular tumour markers and outcome in endometrial cancer. By exploring gene and protein expression data from tumours arising in different settings, we aimed to shed light on potential context-related alterations, that may improve prognostication and represent relevant targets for therapy in future clinical trials.

**Materials and methods:** For the studies included in this thesis (**Paper I-IV**), cohorts of patients treated for primary endometrial cancer at Haukeland University Hospital with thorough follow-up data and clinicopathological characterisation were used. For subsets of the patients, FFPE tissue was available for IHC analysis (**Paper I-IV**), fresh ethanol fixed tissue was used for DNA ploidy analysis (**Paper II and III**), and fresh frozen tissue was used for gene expression microarray (**Paper II-IV**) and RPPA analyses (**Paper IV**). Preoperative CT scans were used to study body fat distribution (**Paper III**).

**Results:** High BMI was significantly associated with low FIGO stage, endometrioid histology and a high level of PR expression, but not ERα expression. Women with BMI≥25 had significantly better endometrial cancer survival compared to women with BMI<25 in univariable analysis, however not significant in multivariable analysis. Applying overall survival as outcome measure, increasing BMI independently predicted worse survival (**Paper I**).
Aneuploidy was significantly associated with high age, high FIGO stage and high grade, non-endometrioid histology and ER/PR negativity, and independently predicted reduced survival. In ER/PR negative tumours, aneuploidy independently predicted recurrence and lymph node metastasis. A nine-gene prognostic ‘aneuploidy signature’, linked to low expression of chromosome 15q genes, was identified and validated in TCGA data (Paper II).

Abdominal fat volumes were strongly positively correlated with BMI and waist circumference, and inversely correlated with liver density. High fat volumes and BMI were associated with low grade endometrioid tumours and PR and AR positivity, but not ERα positivity. The visceral fat percentage, VAV%, was not correlated with BMI or total abdominal fat volume, however, high VAV% was associated with high age and aneuploidy, and independently predicted reduced survival. Tumours from patients with low VAV% showed enrichment of gene signatures related to inflammatory and immunogenic signalling (Paper III).

In endometrioid endometrial cancers, BMI was significantly correlated with a signature of hormone receptor expression, as well as PR and phospho-ERα (S118) levels. BMI was negatively correlated with RTK- and MAPK-pathway activation, and particularly phospho-MAPK (T202 Y204) level. In the subset of FIGO stage 1, grade 1-2 tumours, non-obese patients had significantly reduced survival compared to obese patients, associated with higher level of MAPK- and RTK-pathway activation. The obese patients had higher phospho-ERα (S118) levels, and showed enrichment of gene signatures related to oestrogen signalling, inflammation, immune signalling and hypoxia (Paper IV).

Conclusions:

BMI and imaging based estimates of obesity are associated with clinicopathological markers of less aggressive endometrial cancer (Paper I, III and IV).

High BMI is associated with PR and AR but not ERα expression (Paper I, III and IV).
Obese patients with endometrioid endometrial cancer have higher levels of phosphorylated ERα. Non-obese patients have higher levels of phosphorylated MAPK (Paper IV).

High BMI is associated with improved DSS in univariable, but not multivariable analysis, and worse OS in multivariable analysis (Paper I). Increasing VAV% independently predicts reduced DSS (Paper III). Obesity is associated with improved DSS in patients with assumed excellent prognosis (Paper IV).

Gene sets linked to inflammation and immune activation are enriched in tumours arising in patients with low VAV%, and equally in tumours arising in obese patients with FIGO stage 1, grade 1-2 tumours (Paper III and IV).

DNA ploidy is a robust prognostic marker in endometrial cancer, and aneuploidy independently predicts reduced DSS. In patients with ER/PR negative tumours, aneuploidy independently predicts increased risk of lymph node metastases and recurrence (Paper II).

A nine-gene aneuploidy signature is associated with reduced survival and low expression of chromosome 15q genes (Paper II).
List of publications


*: these authors contributed equally

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1. Introduction

This thesis focuses on endometrial cancer, which arises from the epithelial lining of the uterus, the endometrium. This group comprises the vast majority of uterine cancers.\(^1\) Primary malignant tumours of the *corpus uteri* include epithelial tumours, mesenchymal tumours and mixed epithelial/mesenchymal tumours.\(^1\)

1.1 Epidemiology of endometrial cancer

In epidemiology, registry data based on ICD-codes are commonly used. ICD10-code 54, uterine cancer, comprises both epithelial, mesenchymal and mixed tumours. Thus, the crude numbers for endometrial cancer alone are somewhat lower than what is reported in registry based studies. Between 1970 and 2000, 3.4% of registered uterine cancers in Norway were uterine sarcomas or adenosarcomas,\(^2\) and in the Nordic countries the incidence of sarcomas has been reported to be relatively stable between 1978 and 2008.\(^3\) Thus, observed overall changes in incidence and survival in uterine cancer mainly reflect changes in endometrial cancer incidence and survival, and we will hereafter refer to the disease as endometrial cancer.

1.1.1 Incidence

Endometrial cancer is the most common gynaecologic malignancy in industrialised countries.\(^4\) It is the fourth most common cancer in Norwegian women, after breast, colorectal and lung cancer.\(^5\) According to the Cancer Registry of Norway, 779 new cases were registered in 2015, and the incidence has been increasing over the past decades (Figure 1).\(^5\) The age-standardised incidence rate (Norwegian standard) was 27.9 cases per 100,000 person-years in the period 2011-2015, compared to 19.4 per 100,000 in 1981-1985 and 11.1 per 100,000 in the period 1956-1960.\(^5\)
Endometrial cancer predominantly affects postmenopausal women, and in Norway the highest age-specific incidence rate is seen in the age group 75-79 (Figure 2). However, it also affects premenopausal women in around 14% of cases, some still in reproductive age.

Figure 1: Age-standardised incidence rate of uterine cancer per 100,000 person-years in Norway, in five-year intervals, from 1956-2015. Figure adapted from Cancer in Norway 2014.

Figure 2: Age-specific incidence rates of uterine cancer per 100,000 person years and five-year age group, in Norway during the period 2011-2015. Figure adapted from Cancer in Norway 2014.
1.1.2 Survival

Endometrial cancer is generally associated with a favourable prognosis. The five-year relative survival was 83.4% in Norway in the period 2010-2015 including all disease stages.\(^5\) Patients with localised disease had an excellent prognosis, and $>95\%$ of the patients were alive after five years.\(^5\) There has been an increase in survival from 1974 to 2015; for all stages considered in total, and for patients with localised disease and distant metastasis (Figure 3). For patients with localised disease, the observed survival improvement may in part be a result of increased rates of staging lymphadenectomies, leading to improved detection of patients with metastatic lymph nodes that were previously assumed to have localised disease. Once the disease has spread outside the uterus, prognosis is considerably reduced with five-year survival rates of 59\% and 36\% for patients with regional and distant metastases, respectively.\(^5\)

![Figure 3: Five-year relative survival (%) for uterine cancer according to disease stage and period of diagnosis (1976-2015). Figure adapted from *Cancer in Norway 2014*.\(^5\)](image)

1.2 Aetiology and risk factors for endometrial cancer

Aetiology means study of causation, or origination. Correlation and association does not necessarily imply causation, although epidemiological correlations or associations
are often the starting point to search for causative factors. Cancer development is a complex, multifactorial process, and for the majority of cases single causes cannot be identified.

1.2.1 Acquired factors

Endometrial cancers are presumed to largely occur sporadically, i.e. there is no known hereditary cause (hereditary causes are discussed below). Endometrial cancers have traditionally been divided into two groups after the classification by Bokhman from 1983: Type 1 tumours (approximately 80% of all cases), associated with a hyper-oestrogenic environment, often preceded by endometrial hyperplasia, and typically of endometrioid histology; and Type 2 tumours, associated with endometrial atrophy, more oestrogen independency and less differentiated, often of non-endometrioid histology.\textsuperscript{8,9}

The ‘unopposed oestrogen hypothesis’ is a long-standing theory for endometrial carcinogenesis. It originally emerged from epidemiological observations indicating that endometrial cancer incidence was associated with conditions related to increased level or prolonged exposure to circulating oestrogens,\textsuperscript{10-12} which influences the balance of proliferation, differentiation and apoptosis in the endometrium if unopposed by progesterone.\textsuperscript{13} Such conditions include low age at menarche and high age at menopause, nulliparity, exogenous oestrogen use without opposing progesterone, and anovulatory menstrual cycles/polycystic ovary syndrome (PCOS).\textsuperscript{14} These conditions have been associated with increased risk of endometrial cancer, particularly for Type 1 tumours.\textsuperscript{9} Use of the selective oestrogen receptor modulator tamoxifen, acting as a partial ER agonist in endometrial tissue, is therefore also associated with increased risk for endometrial cancer.\textsuperscript{15}

Obesity is a recognised risk factor for several cancer types, with the strongest association seen for endometrial cancer.\textsuperscript{16,17} It has been suggested that at least 30-40% of endometrial cancer cases in Europe can be attributed to obesity,\textsuperscript{18-20} and the numbers
are even higher in North America, ranging from 48-57% \textsuperscript{20,21}. Thus, the increasing obesity epidemic may therefore at least partly explain the increasing endometrial cancer incidence seen over the past decades. Underscoring this, a striking similarity is seen between the curves reflecting endometrial cancer incidence (Figure 1) and the increased prevalence of overweight/obesity, shown for US females (Figure 4). A similar increasing prevalence of overweight and obesity has been reported for Norwegian women, and in the period 2006-2008, 61% of women included in the HUNT3 Study (The Nord-Trøndelag Health Study) were overweight or obese \textsuperscript{22}.

![Figure 4](image.png)

**Figure 4:** Age-adjusted prevalence of overweight and obesity in US females (aged 20-74) from 1960 to 2014. Overweight (BMI 25-30), obesity (BMI$\geq$30) and extreme obesity (BMI$\geq$40) are displayed. Figure adapted from Centers for Disease Control and Prevention data \textsuperscript{23}.

Obesity is commonly evaluated by body mass index (BMI, kg/m\textsuperscript{2}). The World Health Organisation (WHO) has defined criteria for under-weight (BMI<18.5), normal weight (BMI 18.5-24.9), overweight (BMI 25.0-29.9), and obesity (BMI$\geq$30) \textsuperscript{24}. It has been estimated that women with BMI$\geq$40 (Class 3 obesity by WHO criteria) have a relative risk (RR) of 6.36 for endometrial cancer development compared to women with BMI in the range 20-24, adjusted for diabetes, smoking status, alcohol use, physical activity and hypertension \textsuperscript{25}. Diabetes mellitus, independent of obesity, has also been associated with an increased risk of endometrial cancer \textsuperscript{26}. 
Factors shown to reduce endometrial cancer risk include use of continuous combined hormone replacement therapy postmenopausally,\textsuperscript{27} use of oral contraceptives premenopausally,\textsuperscript{28} high parity\textsuperscript{14} and prolonged periods of breast feeding,\textsuperscript{29} all linked to relatively higher levels of progesterone, counter-balancing the effects of oestrogen. Intrauterine device use, both levonorgestrel-containing and non-hormone containing, has also been associated with reduced risk.\textsuperscript{30,31} Physical activity reduces endometrial cancer risk,\textsuperscript{32} and emerging data suggest that patients who have undergone bariatric surgery reduce their risk of developing endometrial cancer.\textsuperscript{33} These data all underline that many of the risk factors for endometrial cancer are to some extent modifiable.

1.2.2 Hereditary factors

Approximately 3-5\% of endometrial cancers are thought to be caused by inherited genetic changes.\textsuperscript{34,35} The most common genetic predisposition syndrome, Lynch syndrome, or hereditary non-polyposis colorectal cancer (HNPCC), is reported with a prevalence ranging 1.8-2.1\% in unselected endometrial cancer subgroups,\textsuperscript{36-38} but with a higher prevalence in younger patients.\textsuperscript{39} The life-time risk of developing endometrial cancer is estimated to 40-60\% for women with Lynch syndrome.\textsuperscript{40} Lynch syndrome is characterized by autosomal dominant inherited germline mutations in DNA mismatch repair (MMR) genes; \textit{MLH1}, \textit{MSH2}, \textit{MSH6} and \textit{PMS2}.\textsuperscript{41,42} The MMR proteins are involved in repair of base-pair mismatches, and normally function to eliminate insertion/deletion loops, caused by slippage of DNA polymerase during replication. In presence of defective MMR, repetitive DNA sequences called microsatellites tend to undergo a high level of genetic alterations, known as microsatellite instability (MSI), resulting in high overall mutational burden and increased risk for cancer development.\textsuperscript{43} Lynch syndrome is associated with higher risk for a range of cancer types, including colorectal, endometrial, gastric and ovarian cancer.\textsuperscript{44}
1.3 Clinical features and diagnosis

1.3.1 Symptoms of endometrial cancer

The classical presenting symptom of endometrial cancer is abnormal vaginal bleeding, reported in more than 90% of all patients. Although bleeding is present in most cases, premenopausal abnormal bleeding (menorrhagia/metrorrhagia) is associated with a low overall risk of endometrial cancer: in a meta-analysis of premenopausal women with abnormal uterine bleeding, 0.33% had endometrial cancer. Postmenopausal uterine bleeding, however, should be considered “cancer until proven otherwise”, as it is reported to be caused by endometrial cancer in 5-10% of cases, and the risk increases with increasing age and the presence of additional risk factors. Patients with advanced stage disease may also experience symptoms such as abdominal pain, oedema in the lower extremities and weight loss, related to metastatic disease.

Preoperative investigations aim to support the decision on the best treatment for the patient, by determination of the histopathological subtype, estimation of the infiltration depth into the myometrium, and potential infiltration into the cervical stroma, neighbouring organs as well as distant metastasis.

1.3.2 Preoperative histology

Histological assessment is a cornerstone in cancer diagnostics. An endometrial biopsy can be obtained in an outpatient setting, and the Pipelle is considered the most accurate biopsy tool with estimated sensitivity ranging from 91-99%. However, negative or inconclusive results should be interpreted with caution since lack of sufficient material for diagnosis has been demonstrated to be more frequent with this method compared to dilatation and curettage. Curettage is therefore recommended if the endometrial biopsy is inconclusive, but this procedure requires full anaesthesia.
1.3.3 Preoperative imaging

Transvaginal ultrasound is routinely used in the evaluation of women with abnormal uterine bleeding. In a meta-analysis, endometrial thickness > 3mm was suggested as cut-off value requiring further examinations to exclude EC, with pooled sensitivity of 98%.

For the evaluation of myometrial infiltration and cervical stromal infiltration, pelvic contrast-enhanced (CE) MRI is considered superior to CT and transvaginal ultrasound. However, modest inter-observer agreement has been reported, and the diagnostic performance is variable between studies.

CE-CT is widely used for preoperative detection of lymph node metastases and distant spread. PET/CT has been shown to outperform CE-CT in detection of lymph node metastasis, with reported sensitivities of 57% versus 29%, respectively, however this not performed as a part of routine diagnostics in most centres.

1.3.4 Histopathology

Final histopathological diagnosis is obtained after surgical removal of the tumour. Around 80-85% of endometrial cancers are classified as endometrioid carcinomas, typically displaying a glandular structure. Non-endometrioid histological types include serous carcinomas (3-10% of cases), clear cell carcinomas (2-3% of cases) carcinosarcomas (<2% of cases) and undifferentiated carcinomas. Carcinosarcomas are composed of both an epithelial and a mesenchymal component; these tumours are however thought to be of monoclonal, epithelial origin.
Figure 5: Endometrioid carcinomas: grade 1 (A), grade 3 (B). Non-endometrioid carcinomas: serous carcinoma (C), clear cell carcinoma (D). All pictures: 400x magnification.

Endometrioid carcinomas are graded histologically according to solid growth pattern. Grade 1 tumours are well differentiated with a glandular pattern and $\leq 5\%$ solid growth, grade 2 tumours have less well-defined glands and 6-50% solid growth, and grade 3 tumours are poorly differentiated with hardly recognisable glands and $>50\%$ solid growth.$^1$ Non-endometrioid tumours are high grade by definition.$^9$ However, the distinction between histological subtypes may be difficult, and studies have shown relatively poor accordance between experienced pathologists both in distinguishing grade 2 and particularly grade 3 endometrioid carcinomas from non-endometrioid tumours, and also in determining the histological subtype within the non-endometrioid tumours.$^{56,57}$
1.3.5 FIGO staging

Endometrial cancer is staged surgically according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria, revised in 2009.58

**Stage I**  
**Tumour confined to the uterus**

- Ia: No or <50% myometrial invasion
- Ib: ≥50% myometrial invasion

**Stage II**  
**Tumour invades cervical stroma, but does not extend beyond the uterus**

**Stage III**  
**Local and/or regional spread of the tumour**

- IIIa: Tumour invades serosa and/or adnexa
- IIIb: Vaginal and/or parametrial spread
- IIIc: Metastasis to pelvic and/or para-aortic lymph nodes
- IIIc1: Metastasis to pelvic lymph nodes
- IIIc2: Metastasis to para-aortic lymph nodes

**Stage IV:**  
**Tumour invades bladder and/or bowel mucosa, and/or distant metastasis**

- IVa: Tumour invades bladder and/or bowel mucosa
- IVb: Distant metastasis and/or inguinal lymph node metastasis

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**Table 1:** Endometrial cancer staging according to the FIGO 2009 criteria, adapted from Pecorelli, (2009).58 Figures are modified and reprinted with permission from Cancer Research UK/Wikimedia Commons.
1.4 Tumour biomarkers

1.4.1 Biomarkers definition

The Biomarkers Definition Working Group has defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. Biomarkers can be measured in a variety of samples, including blood, urine, tissue and images; in fact, anything that is quantifiable in a patient may potentially serve as a biomarker. Biomarkers can be single, such as serum CA125 level which is used for detection and treatment monitoring in ovarian cancer, or a panel, i.e. a signature, of for instance gene expression levels, miRNA expression levels, methylation sites or protein expression levels. Various classifications of biomarkers exist, and an important distinction is prognostic and predictive biomarkers.

1.4.2 Prognostic biomarkers

Prognostic biomarkers provide information about cancer outcome, regardless of therapy. Such markers may be useful to select patients who need further treatment, but do not necessarily predict response to the therapy.

In endometrial cancer, FIGO stage, histological subtype and grade are long known strong prognostic markers, used to support decisions on therapeutic strategies. However, as mentioned, histopathological evaluation has not always shown good reproducibility among pathologists. In addition, around 15-20% of assumed low risk tumours recur. Additional histopathological and molecular prognostic biomarkers have been extensively studied to improve identification of high-risk patients. For example, lymphovascular space invasion (LVSI), oestrogen and progesterone receptor (ER/PR) expression, TP53 expression, KRAS amplification, DNA ploidy status and LICAM expression are all biomarkers shown to have
independent prognostic value, corrected for standard histopathological variables. A 29-gene expression signature has also been shown to add independent prognostic information in endometrial cancer, and particularly to identify a subgroup of aggressive tumours among presumed low-risk cancers.\textsuperscript{62,77} Preoperatively identified biomarkers may serve to i.e. better identify patients with low risk of lymph node metastasis, where extensive surgical treatment could potentially be omitted, and examples include ER/PR expression\textsuperscript{78} and DNA ploidy status.\textsuperscript{79,80}

1.4.3 Predictive biomarkers

Predictive biomarkers identify patients who will most likely respond to a therapeutic intervention.\textsuperscript{60} An illustrative example of a successful predictive marker is the Philadelphia chromosome in chronic myelogenous leukaemia. This translocation (t9;22) creates a constitutively activated fusion protein, BCR-ABL1. Presence of BCR-ABL1 predicts response to tyrosine kinase inhibitors, which have revolutionised the treatment of these leukaemia patients.\textsuperscript{81} Many patients who previously suffered premature death now have almost no reduction in life-expectancy or quality of life. Other examples of predictive markers in clinical use include HER2/neu amplification as a predictive marker for trastuzumab response in breast cancer,\textsuperscript{82} as well as ER/PR expression as predictive markers for response to hormonal therapy in breast cancer.\textsuperscript{83}

No predictive markers are clinically used in endometrial cancer. Hormonal therapy has been associated with better response rates if hormone receptors are present,\textsuperscript{84} but receptor status is currently not routinely assessed before initiation of treatment. Clinical trials incorporating biomarkers and biopsies in the treatment stratification are needed to identify and validate predictive biomarkers that may predict response to targeted therapies.\textsuperscript{54}
1.5 Tumour biology and molecular characteristics

1.5.1 Genetic basis for cancer

Cancer can broadly be defined as diseases involving abnormal cell growth and the ability of cells to cross normal tissue barriers, and is considered a disease involving alterations in the genome of cells. The main mechanisms for genetic changes are mutations, deletions, amplifications and translocations. In addition, epigenetic changes may affect the activity of gene transcription and thus also play a role in malignant neoplastic growth. Genes involved in malignant transformation are typically described as oncogenes; genes of which constitutive activation may ultimately lead to cancer development, or tumour suppressor genes; genes of which loss of function may enable cancer development.

1.5.2 The Hallmarks of cancer

Virtually all mammalian cells have similar molecular machineries regulating proliferation, differentiation and death. Evidence built over the past decades suggests that cancer development is a multistep process requiring several genetic alterations accumulated over time, affecting these tightly regulated machineries. A handful of cellular acquired capabilities have been described as “rules governing the transformation of human cells to malignant cancers”, known as the hallmarks of cancer (Figure 6). The hallmarks were originally described by Hanahan and Weinberg in 2000, and extended in 2011 with two new hallmarks and two enabling characteristics (tumour-promoting inflammation and genome instability & mutation).
These characteristics are thought to be shared by most cancer types in different degrees, however, it should be kept in mind that this is a conceptual framework rather than the full explanation of the complexity of all human cancers.

1.5.3 Endometrial cancer in light of the hallmarks of cancer

Although each tumour harbours its individual combination of genetic changes, and thus represents a unique biological entity, some alterations are reported with a higher frequency in endometrial cancer: the following sections serve as an illustration of how such commonly altered genes, pathways and processes may enhance its formation and growth.

Sustained proliferative signalling: PI3K pathway alterations

The PI3K pathway regulates cell growth, proliferation, differentiation, migration, apoptosis, protein synthesis and glucose metabolism. Alterations in this pathway
are reported to occur in about 70% of all endometrial cancers. Normal PI3K pathway activation is initiated by binding of ligands to receptor tyrosine kinases (RTKs), resulting in phosphorylation of a regulatory subunit, e.g. p85α/PIK3R1, and activation of a catalytic subunit, e.g. p110α/PIK3CA. This increases PIP₃ production, and subsequently downstream activation of AKT and mTOR. PTEN negatively regulates intracellular PIP₃ levels and thus slows down pathway activity. PIK3CA mutations and amplification, as well as PIK3R1 mutations, are all frequent in endometrial cancer. Such alterations may lead to constitutive activation of these pathway members, and consequently disrupted regulation of cell growth and proliferation. Likewise, loss of PTEN by mutational inactivation, deletion or epigenetic silencing, leads to loss of its inhibitory activity. Thus, the PI3K-pathway alterations exemplify how cells may achieve sustained proliferative signalling in endometrial cancer.

Evasion of growth suppression and apoptosis: TP53 mutations

Tumour suppressor genes are often categorised as “gatekeeper genes” and “caretaker genes”. Gatekeepers directly regulate tumour growth by inhibiting cell growth (cell cycle progression) or promoting cell death (apoptosis), whereas caretakers are typically involved in maintaining genome stability, for example by induction of DNA repair. TP53 is a classic example of a tumour suppressor gene, and it has both gatekeeper and caretaker functions. This transcription factor is a critical node in the response to DNA damage and cellular stress, and is able to activate processes leading to DNA repair, cellular senescence or apoptosis. Endometrial cancers, particularly serous tumours, frequently carry TP53 mutations, leading to loss of tumour suppressor activity. Aberrant TP53 function is thus one example of how endometrial cancers may evade growth suppression and apoptosis.

Tissue invasion and metastasis: epithelial-to-mesenchymal transition

Through a consecutive series of adaptive changes, malignant cells invade adjacent tissue, and eventually break away from the primary tumour to enter the lymph or blood vessels and metastasise to neighbouring organs and/or distant sites. Cancer cells are
suggested to acquire invasive abilities through activation of epithelial-to-mesenchymal transition (EMT). This is a developmental regulatory program by which the cellular phenotype is altered towards a more mesenchymal morphology, allowing motility, invasiveness and resistance to apoptosis. During EMT, loss of epithelial cell-cell adhesion molecules, including for instance E-cadherin is common. Reduced E-cadherin expression is frequently observed in endometrial cancer, and associated with deep myometrial invasion and vascular invasion. Gene signatures indicating EMT-activation through Sonic Hedgehog, TGF-β and Wnt-signalling pathways, developmental genetic programs that are also involved in the EMT process, have been associated with reduced survival in endometrial cancer, supporting a role of EMT in aggressive disease. Also, L1CAM overexpression, although not a classical member of the EMT pathways, has been associated with tissue invasion, metastasis and poor prognosis in endometrial cancer, and has been suggested as a potential EMT-marker.

**Angiogenesis**

In order to grow, all cells need continuous supply of nutrients and oxygen, delivered by the blood vessels. Formation of new blood vessels becomes necessary once the tumour size exceeds 1-2 mm, and occurs through a process called angiogenesis. This is a normal physiological process, tightly regulated through a balance between pro- and antiangiogenic factors, seen during e.g. wound healing and menstrual cycle. However, tumours may induce an angiogenic “switch”, by overexpression of proangiogenic factors and/or downregulation of anti-angiogenic factors, facilitating the formation of new blood vessels. One prototypic pro-angiogenic factor is VEGF-A. High VEGF-A expression has been linked to increased microvessel density and adverse outcome in endometrial cancer. Bevacizumab, a compound targeting VEGF-A is currently in clinical use for other gynaecological cancer types, including ovarian cancer and cervical cancer.
Genome instability describes the unstable genetic make-up of cancer cells, and the term encompasses both a high rate of mutations, chromosomal instability, and epigenetic instability.\cite{111,112} Genome instability is thought to allow cancer cells to acquire properties that give them survival advantages through various mechanisms, therefore considered as an enabling characteristic of cancer cells.\cite{90} MSI is one example of genome instability,\cite{111} and is seen in approximately a third of endometrial cancers.\cite{93} Sporadic MSI cases (non-Lynch syndrome) are thought to occur by acquired somatic mutations or promoter hypermethylation in DNA MMR genes.\cite{111} Chromosomal instability describes a high rate of gains or losses of whole chromosomes, chromosome arms or chromosomal segments (focal alterations), suggested to arise through mitotic errors or chromosomal rearrangements (deletions, amplifications, translocations).\cite{112} Such alterations may lead to aneuploidy. Ploidy is originally a cytogenetic term, describing the number of homologous chromosomes in a cell ($n=$the haploid number); a normal somatic human cell is diploid, containing 23 pairs of chromosomes ($2n$). The term aneuploidy is used to describe cells with a chromosome number that is not a multiple of $n$.\cite{113} Aneuploidy is frequently observed in endometrial cancer, and has repeatedly been associated with tumour aggressiveness and poor prognosis.\cite{71,73,79}

**1.5.4 The role of the micro- and macro-environment**

Development and progression of a tumour is not only dependent on its genetic make-up, but also the cellular biological context, characteristics specific to the individual patient, and environmental influences.\cite{114} The tumour micro-environment is composed of components surrounding the tumour cells, and includes tumour associated fibroblasts, extracellular matrix, vascular and lymphatic cells, adipocytes and immune cells, which are important in initiating angiogenesis, inflammation, cell growth and metastasis.\cite{90,115,116} The increasing understanding of the importance of the tumour microenvironment in cancer development and progression is reflected in the updated Hallmarks of cancer, as three of four newly introduced concepts in the 2011 version
were related to this (deregulating cellular energetics, avoiding immune destruction and tumour-promoting inflammation). Also, systemic factors derived from the “macro-environment” including hormones, inflammatory mediators and plasma lipoproteins are increasingly recognised as factors influencing tumour development and growth, although the complex relations between systemic signalling and local tumour promoting effects are incompletely understood. In the next section, obesity is discussed as an example to illustrate how systemic factors may promote endometrial carcinogenesis.

Obesity and endometrial carcinogenesis: proposed mechanisms

The obesity-related increased endometrial cancer risk has been linked to unopposed oestrogen exposure: in postmenopausal women, the adipose tissue is the major source of oestrogens, converting circulating androgens to oestrone and oestradiol by aromatization. Also, the obesity-related increase in insulin levels results in reduced hepatic sex hormone-binding globulin (SHBG) production, further increasing systemically bioavailable oestrogen levels. Mechanistically, oestrogen exerts mitogenic effects on the cells, both via binding to the oestrogen receptor, a nuclear receptor that among others activate transcription of pro-proliferative genes such as IGF1, but also by activation of membrane bound oestrogen receptors and RTKs, that may directly stimulate endometrial proliferation through activation of the PI3K- and mitogen activated protein kinase (MAPK)-pathways. Oestrogen may also have direct mutagenic effects: genotoxic oestrogen metabolites have been shown to induce DNA damage, and thus cause genetic instability.

Increased insulin signalling is another suggested obesity-related carcinogenic mechanism. Insulin is thought to mediate its effects both directly on the (pre)neoplastic cells by activation of the insulin receptor, leading among other to increased activity in the PI3K pathway. Also, it may indirectly affect proliferation via changes in hormone metabolism secondary to hyperinsulinemia, resulting in increased IGF1 signalling.

Finally, the adipose tissue, particularly the visceral adipose tissue, is a metabolically active endocrine organ in itself, producing a range of inflammatory mediators.
Increased levels of pro-inflammatory cytokines and leptin, as well as reduced adiponectin levels have been observed in endometrial cancer patients compared to healthy controls,\textsuperscript{121,122} suggesting that systemic inflammatory signalling may contribute to endometrial carcinogenesis. A graphical representation summarising the main postulated mechanisms and mediators involved in obesity-related carcinogenesis is presented in Figure 7.
Figure 7: Schematic representation of three main mechanisms hypothesised to link excess adiposity and cancer risk. Dashed arrows indicate indirect actions. Δ4A, Δ4-androstenedione; 17β-HSD, 17β-hydroxysteroid dehydrogenase; E1, oestrone; E2, oestadiol; IGF1, insulin-like growth factor I; IGF1R, IGF1 receptor; IGFBP, IGF-binding protein; IL, interleukin; IR, insulin receptor; LR, leptin receptor; NF-κB, nuclear factor-κB; SHBG, sex hormone-binding globulin; T, testosterone; TNF, tumour necrosis factor. Figure reprinted with permission from Nature Publishing Group.
1.5.5 Molecular classification of endometrial cancer

Molecular alterations in Type 1 versus Type 2

Attempts have been made to describe molecular alterations associated with the two prototypical subtypes of endometrial cancer, Type 1 and Type 2, which were mainly histologically and epidemiologically defined in the original publication. A selection of these alterations is summarised in Table 2. However, this dualistic model has been criticised for being too simplistic, and various definitions have been used in different studies. As already mentioned, particularly grade 3 endometrioid tumours are considered heterogeneous, also reflected in the fact that these tumours have been classified as both Type 1 and Type 2 in different studies. As noted from the table, overlapping molecular characteristics are seen between the two types.

<table>
<thead>
<tr>
<th>Tumour marker</th>
<th>Alteration</th>
<th>Frequency in Type 1</th>
<th>Frequency in Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN(^{125,124})</td>
<td>Mutation, LOH, loss of expression</td>
<td>37-77%</td>
<td>0-11%</td>
</tr>
<tr>
<td>PIK3CA(^{54,123})</td>
<td>Mutation</td>
<td>30-53%</td>
<td>20-42%</td>
</tr>
<tr>
<td>PIK3CA(^{54})</td>
<td>Amplification</td>
<td>2-14%</td>
<td>46%</td>
</tr>
<tr>
<td>PIK3R1(^{124})</td>
<td>Mutation</td>
<td>43%</td>
<td>12%</td>
</tr>
<tr>
<td>KRAS(^{99})</td>
<td>Mutation</td>
<td>26%</td>
<td>2%</td>
</tr>
<tr>
<td>FGFR2(^{54,123})</td>
<td>Mutation</td>
<td>11-16%</td>
<td>1%</td>
</tr>
<tr>
<td>CTNNB1(^{125})</td>
<td>Mutation</td>
<td>36%</td>
<td>0-5%</td>
</tr>
<tr>
<td>E-Cadherin(^{103})</td>
<td>Loss of expression (LOH, promoter hypermethylation)</td>
<td>53%</td>
<td>83%</td>
</tr>
<tr>
<td>TP53(^{54,124})</td>
<td>Mutation</td>
<td>10-20%</td>
<td>90%</td>
</tr>
<tr>
<td>HER2 (ERBB2)(^{126})</td>
<td>Amplification, overexpression</td>
<td>3-8%</td>
<td>18-31%</td>
</tr>
<tr>
<td>ER/PR(^{57,68})</td>
<td>Loss of expression</td>
<td>13-21%</td>
<td>56-69%</td>
</tr>
</tbody>
</table>

Table 2: Selected molecular alterations and their frequency in Type 1 versus Type 2 tumours. Abbreviations: LOH: Loss of heterozygosity.
Molecular classification of endometrial cancer: a paradigm change?

The Cancer Genome Atlas (TCGA) consortium has performed global characterisation of several cancer types, integrating genomic, transcriptomic and proteomic data. The hallmark endometrial cancer publication from 2013 described four main molecular subtypes: *POLE* ultramutated, MSI hypermutated, copy-number low and copy number high tumours. Each subgroup displayed characteristic patterns of molecular aberrations, and the classification was also linked to prognosis. Recently, a similar description of uterine carcinosarcomas was published, suggesting shared molecular features with high-grade serous ovarian cancers and serous endometrial tumours, as well as frequent activation of the EMT program. However, there is still no consensus on how to incorporate this costly and labour-intensive classification into routine diagnostics. Recently, a selected panel of markers assessed by IHC and sequencing methods was shown to be able to reproduce the classification and survival curves seen in the TCGA paper. A similar approach was tested in subgroups of intermediate-high-risk endometrioid tumours from the PORTEC1 and 2 trials, pointing out that both molecular classification reflecting TCGA subgroups, and additional markers such as L1CAM expression and *CTNNB1* mutation status, may be helpful to further identify patients with high risk for recurrence and death in a less costly and clinically applicable manner. However, the validity of the TCGA classification needs to be confirmed in a population based setting also incorporating standard clinicopathological markers.

1.6 Treatment of endometrial cancer

The two next sections mainly describe the Norwegian situation, which thus may differ from practices in other countries on some aspects.
1.6.1 Surgery

Standard treatment includes total hysterectomy with bilateral salpingo-oophorectomy with or without lymphadenectomy. In advanced disease, debulking surgery is performed.\textsuperscript{6,130} Laparoscopic/robot-assisted surgery is considered safe for early stage disease, and associated with less post-operative complications compared to laparotomy.\textsuperscript{131,132}

Complete surgical staging according to the FIGO 2009 criteria (Table 1) requires sampling of abdominopelvic lymph nodes; a procedure demonstrated to improve the prognostication, but not the survival in randomised trials.\textsuperscript{133,134} It is also associated with adverse effects, including increased operating time and development of lymphoedema and lymphocysts.\textsuperscript{135} The debate whether or not lymphadenectomy should be performed, and in which subsets of patients, remains unfinished,\textsuperscript{136} and practices vary across countries and even among centres. Pelvic lymphadenectomy is recommended for all patients with presumed high-risk tumours based on preoperative investigations (final risk classification is determined postoperatively, shown in the next section), and lymph node sampling is recommended for assumed moderate risk tumours.\textsuperscript{130} Lymphadenectomy rates are however lower in many European countries compared to Norway.

Much research focus has been put on evaluation of preoperative markers to identify patients with low risk of lymph-node disease where the procedure can safely be omitted. A recent prospective multicentre study evaluated preoperative criteria for this; the following 1) endometrioid type with 2) no evidence of deep myometrial infiltration, enlarged lymph nodes or distant metastasis on MRI, and 3) serum CA125 levels < 35 U/mL, resulted in a negative predictive value of 97.1\% for detection of lymph node metastasis.\textsuperscript{137} Also, loss of ER/PR expression in curettage specimens has been shown to independently predict lymph node metastasis (adjusted OR 2.04, 95\% CI 1.12 – 3.70),\textsuperscript{78} further supported by another study showing increased risk of lymph node metastasis with loss of ER\alpha (adjusted RR 2.25, 95\% CI 1.04 – 4.89).\textsuperscript{138} Hormone receptor status is currently implemented in the treatment stratification algorithm in a
prospective multicentre study led from our institution, the Momatec2 trial (NCT02543710).

1.6.2 Adjuvant therapy

The aim of adjuvant therapy is to eliminate microscopically or macroscopically visible cancer cells that may remain after surgery, in order to avoid disease recurrence or metastatic spread. This is offered to all high-risk patients. According to Norwegian guidelines, all patients with FIGO stage ≥II are considered at high risk for recurrence.\textsuperscript{130} For FIGO stage I, risk is assessed by a combination of disease stage and histological subtype.\textsuperscript{58,130}

<table>
<thead>
<tr>
<th></th>
<th>FIGO stage Ia</th>
<th>FIGO stage Ib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrioid type, grade 1-2</td>
<td>Low risk</td>
<td>Medium risk</td>
</tr>
<tr>
<td>Endometrioid type, grade 3</td>
<td>Medium risk</td>
<td>High risk</td>
</tr>
<tr>
<td>Non-endometrioid type</td>
<td>High risk</td>
<td>High risk</td>
</tr>
</tbody>
</table>

Table 3: Classification of FIGO stage 1 tumours in categories of low, medium and high risk as stated in national Norwegian guidelines.\textsuperscript{130}

Patients with low-risk tumours have good prognosis and no further treatment is recommended. For the medium risk category, most patients are treated with surgery alone. Supplementary assessment of ER/PR and DNA ploidy status is recommended.\textsuperscript{130}

The European Society for Medical Oncology (ESMO) uses a refined risk stratification system to decide on adjuvant therapy, also including LVSI, and this system is used across many European centres.\textsuperscript{139}

Chemotherapy

In case of high-risk tumours, adjuvant chemotherapy is recommended, and a combination regimen combining carboplatin and paclitaxel is commonly used.\textsuperscript{130} In a Cochrane review of 9 randomised controlled trials (RCTs), chemotherapy given in the
adjuvant setting was associated with prolonged progression free survival (PFS) compared to no treatment or in addition to radiotherapy (HR 0.75, CI 0.62 – 0.89), likely due to the systemic effects of chemotherapy versus the local effects of radiotherapy. A trend towards higher risk of local recurrence was observed when chemotherapy alone was compared with radiotherapy (RR 1.28, 95% CI 0.20 – 1.18).\textsuperscript{140} In another recent Cochrane review assessing the effect of adjuvant chemotherapy for stage III-IV endometrial cancers, overall survival (OS) was significantly improved (HR 0.75, 95% CI 0.57 – 0.99) for patients receiving chemotherapy compared to patients receiving radiotherapy.\textsuperscript{141} Currently ongoing clinical trials evaluate the effect of adjuvant chemoradiation versus radiotherapy alone in high-risk patients (PORTEC-3 and GOG0258).

Radiation therapy

Adjuvant radiation therapy can be administered as brachytherapy or external beam radiation therapy (EBRT). This was previously used frequently in the treatment of intermediate-high risk patients, but is now essentially replaced by adjuvant chemotherapy.\textsuperscript{142} Large RCTs (PORTEC1, GOG-99, ASTEC/EN.5) have addressed the role of radiotherapy in intermediate-high risk endometrial cancer, and failed to show any overall or disease specific survival benefit.\textsuperscript{143-145} A Cochrane review from 2012 concluded that EBRT in stage 1 disease significantly reduced loco-regional recurrence (HR 0.36, 95% CI 0.25 – 0.52), but did not improve overall or disease specific survival.\textsuperscript{146} However, adjuvant radiotherapy is standard treatment for intermediate-high risk patients in many countries.\textsuperscript{139}

Hormonal therapy

Progesterone-based therapy without surgery may be a treatment option for a small group of patients with low risk endometrial cancers who wish to preserve fertility.\textsuperscript{147,148} However, this is often done in study protocols and requires careful monitoring due to a high risk of treatment failure and relapse. Otherwise, adjuvant hormonal treatment with progestagens has no role in the primary situation, as no survival benefit has been shown.\textsuperscript{149}
1.6.3 Recurrent endometrial cancer

Recurrence rates for endometrial cancer have been reported to be around 15-20%,\textsuperscript{144,150} and often cited numbers indicate that around 50% of recurrences occur in patients with non-endometrioid tumours.\textsuperscript{61} For recurrent endometrial cancer, treatment options have not improved over the last decade, and response rates to adjuvant therapy are generally poor, with one exception: localised vaginal metastasis has the potential for cure by radiotherapy and/or surgery, and 5-year survival rates have been reported to 65% in radiotherapy-naïve patients.\textsuperscript{151} With systemic disease, median survival is reported to range from 7-12 months.\textsuperscript{152} In this setting, treatment is to a large extent individualised, depending on the localisation of the recurrence and previously administered therapies. Surgery, radiation therapy and systemic therapies with chemotherapy and hormonal therapy are the primary treatment options.\textsuperscript{139} A Cochrane review addressing the effect of hormonal therapy (anti-oestrogens or progesterone based therapy) in the setting of advanced or recurrent disease found no evidence for any survival benefit related to administration of hormonal therapy.\textsuperscript{153} However, few of the reported trials incorporated hormone receptor status in the assessment, which may have affected the results. Regimens combining paclitaxel/carboplatin are standard in the first-line treatment of recurrent/metastatic endometrial cancer, but the effect of second-line chemotherapy regimens is particularly limited.\textsuperscript{154} No targeted therapies are currently available, and development of better medications, with biomarker guided selection of patients who are likely to respond, is an urgent need for these patients.

1.6.4 Targeted therapy

Molecularly targeted therapy aims to block the growth of cancer cells by interfering with specific molecules needed for carcinogenesis and tumour growth,\textsuperscript{155} as opposed to conventional therapy regimens that generally attack all rapidly dividing cells. With an increasing understanding of the dysregulated molecular mechanisms in cancer, there have been high, so far unmet expectations for treatments directly targeting suggested oncogenic drivers.\textsuperscript{156} At the moment, no such therapies except hormonal therapy are
available for clinical use in endometrial cancer, although several potential drugs have been tested and are currently undergoing clinical trials.

Many of the evaluated treatments target members of the frequently altered PI3K/Akt/mTOR pathway; mTOR inhibitors, PI3K inhibitors and dual mTOR inhibitors have been evaluated in multiple phase II trials, but have generally shown limited response rates and toxic side effects. Slightly more promising results were reported from a trial combining everolimus (mTOR inhibitor) and letrozole (aromatase inhibitor), with 32% response rate. Other examples of potential therapeutic approaches include antiangiogenic treatment through targeting VEGF as single or combination therapy, as well as targeting growth factor receptors such as HER2 and FGFR2. New targeted therapeutics are also in early phase trials, such as PARP inhibitors for patients with PTEN deficient tumours (two currently ongoing trials; NCT02506816 and NCT02208375), metformin due to its antiproliferative effects in preclinical and window-of-opportunity trials, and therapy with immune blockade inhibitors (NCT02912572, NCT02899793, NCT02549209 and NCT02628067), which may represent new approaches for treating subgroups of endometrial cancer patients with high mutational burden, including POLE mutated and MSI tumours.

There are many suggested reasons for the apparent lack of success for targeted therapies in endometrial cancer. A general problem with many early phase trials is that they have been performed in heavily pre-treated patient groups, without any biomarker restriction in the inclusion criteria. If performed at all, typically, the search for predictive biomarkers is conducted retrospectively on a panel of candidate markers after trial closure. This has mostly been performed in hysterectomy specimens, whereas in most cases the therapy is supposed to act on the metastatic lesions, known to not always have similar mutational profile and genetic aberrations as the primary tumours. Considerable response rates may have been observed in a few patients, but due to the lack of adequate number of patients to stratify for biomarker analyses, as well as representative metastatic tissue to identify the biomarkers in, drugs are often rejected although they may be effective in subgroups.
Targeted therapies are often extremely expensive, and proper selection of the patients who are likely to respond is thus also crucial from a health-economy perspective. In addition, the majority of such treatments comes with (sometimes severe) side effects. Offering a non-effective treatment with substantial side effects to patients who are already severely ill is an ethical concern that should indeed accelerate the research into identifying better predictive markers and treatments.
2. Aims of the study

2.1 Background

Endometrial cancer is the most common gynaecological malignancy in industrialised countries, and the incidence is increasing. Besides the increasing life expectancy of the population, this increase has partly been ascribed to the increasing incidence of obesity, which is a major risk factor. The majority of endometrial cancer patients are elderly, obese, have comorbidities, and are diagnosed at an early stage. Still, due to a recurrence rate of about 15-20%, a large proportion of the patients are routinely subjected to staging lymphadenectomy, and adjuvant chemo- and/or radiotherapy. This is costly, and associated with adverse effects. Improved ability to target the surgical and systemic therapies to biomarker selected patient groups is likely to increase the benefit from these therapies. Also, no targeted therapy options are available for patients with recurrent and/or metastatic disease. Thus, better understanding of endometrial cancer biology, and particularly the relation between the context in which the tumour arises and its molecular characteristics, is important to develop applicable biomarkers for better tailoring of already available therapies, and to identify targets for therapy based on molecular alterations.

2.2 General aim

The overall aim of this study was to increase the understanding about how context-related factors, including obesity (assessed by BMI and imaging methods) and genomic alterations (assessed by DNA ploidy status), are related to molecular tumour markers and outcome in endometrial cancer. By exploring gene and protein expression data from tumours arising in different settings, we aimed to shed light on potential context-related alterations, that may improve prognostication and represent relevant targets for therapy in future clinical trials.
2.3 Specific aims

**Paper I:** To explore the prognostic role of BMI, and its association with clinicopathological factors and hormone receptor expression in endometrial cancer lesions.

**Paper II:** To validate the prognostic impact of DNA ploidy status in a large endometrial cancer cohort, and compare its prognostic value with ER/PR status. In addition, we aimed to describe aneuploidy-associated transcriptional alterations, to further understand the role of aneuploidy in endometrial cancer biology.

**Paper III:** To explore the information content in CT-quantified abdominal fat volumes and fat distribution patterns, and assess their relation to clinicopathological and survival data, as well as molecular tumour markers in endometrial cancer.

**Paper IV:** To investigate single proteins, pathway activation and gene expression patterns in relation to BMI in endometrioid endometrial cancers.
3. Material and methodological considerations

3.1 Patient series

3.1.1 Haukeland University Hospital study cohorts

All the studies in this thesis are based on data and samples from women treated for endometrial cancer at Haukeland University Hospital during a 35-year period, as graphically illustrated in Figure 8. This is the primary/referral hospital for women in Hordaland county, and a referral hospital for the western region of Norway. Hordaland county has a population of approximately 515,000 inhabitants, around 10% of the Norwegian population. The endometrial cancer incidence rate and prognosis in Hordaland County is similar to the rest of Norway. Although also serving as a referral hospital for the western region of Norway, >94% of the patients treated at our institution had a permanent address in Hordaland, and thus our material is considered to reflect a relatively unselected patient group of Norwegian endometrial cancers (i.e. population based). Two patient cohorts have been collected retrospectively, the 1981-1990 cohort, and the 1991-2000 cohort. The third cohort has been prospectively collected from 2001. In Paper I and II, all three cohorts were merged to create one large data set, considered to be a population based series.

For all the cohorts, clinicopathological data were collected from the patients’ medical records, including information regarding age at primary diagnosis, parity, menopausal status, height, body weight, primary and adjuvant treatment, FIGO stage, histological subtype and grade. Data regarding recurrence and survival were also collected from the patient records, and by correspondence with physicians responsible for follow-up controls. Follow-up data for surviving patients were collected for at least five years. In Paper II and IV, all cases previously classified according to the FIGO 1988 criteria were reclassified to the FIGO 2009 criteria. The use of clinical data and patient material in these studies was approved by the regional ethics committee (REK numbers 2001/052, 2009/2315 and 2015/2333). Written informed consent was obtained for all patients included in the prospective study (from 2001).
The 1981-1990 cohort

This cohort consists of 286 retrospectively included patients with verified diagnosis of endometrial cancer and available formalin-fixed paraffin embedded (FFPE) archival material. All histology sections were revised by two experienced pathologists (Lars A. Akslen and Ingunn M. Stefansson). This is a well-described population-based series, that has been extensively studied.\textsuperscript{66,68,73,103,176,177} Data from this cohort are used in \textbf{Paper I} and \textbf{II} (Figure 8).

The 1991-2001 cohort

For this cohort of 333 retrospectively collected patients, routine pathology reports were used to identify patients with a diagnosis of endometrial cancer, and prior to inclusion the diagnoses were cross-checked with data from the Cancer registry of Norway and the Death registry of Norway. This cohort has mainly been used for epidemiological purposes (tissue not available except ploidy analyses), and is used in \textbf{Paper I} and \textbf{II} (Figure 8).

Prospective patient series (Momatec)

From March 2001 until October 2015, all patients treated for endometrial cancer at Haukeland University Hospital, who gave written informed consent to participate, have been prospectively included in the Molecular Markers in Treatment of Endometrial Cancer study (Momatec study, NCT00598845). This is an international multicentre study including patients from 10 centres across Norway and Europe. For the studies in this thesis, only patients treated at Haukeland University Hospital were included. Prior to and during surgery, blood, urine and fresh tumour tissue were routinely sampled in the Momatec biobank. Routine diagnostic FFPE tissue from primary tumour was also available. Fresh tissue from premalignant and metastatic lesions (where possible) was also systematically collected in the biobank. Momatec data were used in \textbf{Paper I-IV} (Figure 8).
Figure 8: Overview of patient series and methods applied in the different projects of this thesis. Abbreviations: FFPE: Formalin fixed paraffin embedded tissue; IHC: Immunohistochemistry; FF: Fresh frozen tissue; qPCR: Quantitative polymerase chain reaction; FEF: Fresh ethanol-fixed tissue; FC: Flow cytometry; CE-CT: Contrast-enhanced Computed Tomography; RPPA: Reverse phase protein arrays. The numbers inside each bar represent the number of patients with available data. A complete overview, including overlapping data between the different methods is given in the respective papers (Paper I-IV).

BMI calculations

Body mass index (Paper I, III and IV) was calculated from measured weight and height (kg/m²) at the time of diagnosis. Measured BMI is considered more reliable than self-reported data applied in some studies, which increases risk of response bias. 178 A
further discussion of BMI and its limitations as obesity estimate is found in the discussion of results.

Treatment changes over time

The patients included in the cohorts used in Paper I and II have been treated over a time-period spanning 35 years (1981-2015). During this time, there has been a shift from clinical to surgical staging with introduction of the FIGO1988 criteria, leading to a gradual increase in pelvic lymphadenectomies performed, from null in the period 1981-1990 to 77% in the period 2001-2010, p<0.001. However, para-aortic lymphadenectomy has not been routinely performed. Thus, our results regarding prediction of lymph node metastasis (Paper II) mainly reflect the risk of pelvic lymph node metastasis, and not necessarily the risk of para-aortic node metastasis.

3.1.2 External validation cohorts

The Cancer Genome Atlas data

TCGA clinical data were downloaded (November 3rd 2014) and used as an external validation set (Paper II). The median follow-up time was 2.15 years (range 0.3-15.9). Data for overall survival (OS) were available, and used in the survival analyses. 81% of the tumours were of endometrioid histological type, and 19% were of non-endometrioid histology. 50% of the tumours were grade 1-2 and 50% were grade 3.

M.D. Anderson Cancer Center data

A cohort of patients treated for endometrioid endometrial cancer at the M.D. Anderson Cancer Center, Houston, TX, USA, and with available reverse phase protein array (RPPA) data (method described in section 3.3.2), was used as external validation set (Paper IV). For this series, data for progression free survival (PFS) were available, and used in survival analyses. 84% of the patients had grade 1-2 tumours, and 16% grade 3 tumours.
3.2 Standard methods applied on patient material

3.2.1 Immunohistochemistry

Immunohistochemistry (IHC) is an antibody-based technique to detect protein expression in tissue.

Tissue microarrays

In the studies included in this thesis, IHC was performed on tissue microarray (TMA) slides, constructed from FFPE tissue of the hysterectomy specimens. On full section haematoxylin and eosin (H&E)-stained slides, areas with representative tumour were identified, and in case of tumour heterogeneity, the least differentiated areas were selected. By a precision instrument (Beecher Instrument, Silver Spring, MD, USA), three tissue cores with a diameter of 0.6 mm were punched out from the selected tumour areas, and mounted in a recipient paraffin block.

The main advantages of the TMA method compared to full sections are that it is both time and cost-effective, reducing analysis time, reagent costs, and use of tissue. It also reduces risk of day-to-day variability, as more samples are stained simultaneously. The method has been demonstrated to yield reproducible results compared to full sections for antibodies targeting both focally expressed and diffusely expressed proteins if three cores or more are used. However, TMAs do not provide the same morphological information as full sections do. Thus, it should be used with caution, and depending on the research question. If for example one would study specifically the infiltrating tumour boarder or tumour heterogeneity, this method would be less suitable.

Immunohistochemical staining

TMA slides of 5 μm thickness were dewaxed in xylene, followed by rehydration in serial diluted ethanol and water. Epitope retrieval was performed by boiling in target retrieval buffer TRS-EDTA pH9 in microwave oven for 20 minutes, and endogenous peroxidase was blocked by a peroxidase blocking reagent (Dako S2023). For all antibodies, incubation was done in room temperature. The EnVision+ visualisation
system was used with an HRP-conjugated secondary antibody and diaminobenzidine (DAB) chromogen. Slides were counterstained with haematoxylin (Dako S2020). For details of the staining protocols of antibodies included in this thesis, see Table 4.

<table>
<thead>
<tr>
<th>Antibody target</th>
<th>Provider, catalogue #</th>
<th>Dilution</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAG2</td>
<td>Santa Cruz, USA, sc-81852</td>
<td>1:500</td>
<td>1 hr</td>
</tr>
<tr>
<td>PPP2R3A</td>
<td>Sigma-Aldrich, USA, HPA035829</td>
<td>1:100</td>
<td>1 hr</td>
</tr>
<tr>
<td>ERα&lt;sup&gt;66,181&lt;/sup&gt;</td>
<td>Dako, Denmark, M7047</td>
<td>1:50</td>
<td>30 min</td>
</tr>
<tr>
<td>PR&lt;sup&gt;66,67&lt;/sup&gt;</td>
<td>DAKO, Denmark, M3569</td>
<td>1:150</td>
<td>30 min</td>
</tr>
<tr>
<td>AR&lt;sup&gt;182&lt;/sup&gt;</td>
<td>Abcam, UK, Ab133273</td>
<td>1:100</td>
<td>1 hr</td>
</tr>
</tbody>
</table>

Table 4: Antibodies and incubation conditions for primary antibodies used for IHC in this thesis.

Staining evaluation and cut-offs applied

After staining, the slides were evaluated using a standard light microscope. First, the cellular localisation of the protein was determined: nuclear, membranous or cytoplasmic. A semi-quantitative ‘staining index’ system, widely used and quality assured in our group, was then used to evaluate staining. The area of stained cancer cells was graded from 0-3 (0: no cells stained positive, 1: <10% of the cells, 2: 10-50% of the cells, 3: >50% of the cells stained positive) as well as the intensity of the staining graded from 0-3 (0: no staining, 3: intense staining).<sup>183</sup> The product of the two variables is the staining index, ranging from 0-9. Each case was given one score for all three tumour cores combined. Cut-off for the nominal scoring data was determined by categorising in quartiles, and supported by visual examination of the Kaplan-Meier curves and ensuring a reasonable number of patients adhering to each group.<sup>184</sup>

By combining two staining measures (area and intensity) in the staining index, one potentially better quantitates the amount of protein epitope bound by the antibody. However, it remains a rather subjective system, particularly the intensity component,
that may lead to reduced reproducibility across different laboratories. Before clinical implementation of any immunohistochemical biomarker, staining evaluation and cut-off determination should be thoroughly evaluated and validated. A less subjectively influenced system may be easier to apply in the clinical setting. For instance, for breast cancer, it has been shown that tamoxifen response may occur when only 1% of the cells are stained positive for ER, which is currently recommended as cut-off.\(^8^3\)

In **Paper II**, ER/PR status was dichotomised (ER and/or PR positive versus ER and PR negative tumours). This was done in accordance with a previous study from our group,\(^7^8\) where double ER/PR loss strongly predicted lymph node metastasis.

### 3.2.2 DNA ploidy analyses

In cancer cells, an abnormal DNA content, aneuploidy, is thought to reflect general chromosomal aberrations.

Fresh tumour tissue was collected during primary surgery, ethanol fixed, and used for DNA ploidy analysis (**Paper II, Paper III**). At Haukeland University Hospital, DNA ploidy analysis by flow cytometry has been performed by the routine pathology laboratory on endometrial cancer specimens since 1992. Prior to this, DNA ploidy was studied in a research setting using a similar protocol. From mechanically and enzymatically disaggregated tumour tissue, DNA is labelled with propidium iodide. Fluorescence emitted from the labelled DNA is detected by flow cytometry, and a DNA histogram is produced. Cell populations with abnormal DNA content may thus be identified.

Another commonly applied method to assess DNA ploidy is image cytometry, by which morphological information is obtained, and heterogeneity on single cell level is better assessed.\(^1^8^5\) However, fewer cells can be analysed per sample by image cytometry, and it is also a more time-consuming method.\(^1^8^5\)
3.3 High throughput analyses applied on patient material

Fresh tumour tissue for gene expression microarray and reverse phase protein array (RPPA) analyses was obtained during primary surgery, and snap frozen in liquid nitrogen. Tumour cell content for each sample was evaluated on a haematoxylin-stained frozen section, and at least 50% tumour purity was required for inclusion (most samples had purity >80%).

In a previous study from our group, it was demonstrated that selection of fresh frozen samples with high tumour purity (>80%) was associated with features of aggressive endometrial cancer and reduced survival compared to tumours with lower purity. Thus, although ensuring high quality specimens with little stromal contamination, a high cut-off for tumour purity may introduce selection bias, and relevant biomarkers identified by methods using fresh tissue (in this study gene expression microarray and RPPA) should be validated in population based cohorts.

3.3.1 Gene expression analyses

Functional genomic alterations that underlie cancer (e.g. gene copy number gain/loss, mutations) are often reflected at the transcriptional level. Characterization of transcriptional alterations has been shown useful in defining molecular cancer subtypes, and may also be relevant to understand mechanisms associated with aggressive disease, predict prognosis, point to targets for therapy or predict response to treatment.

mRNA microarrays

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), reversely transcribed and amplified, before hybridisation to Agilent Whole Human Genome Microarray Kit, 44k (Catalogue number G4 112F) according to the manufacturers’ instructions. Arrays were scanned using the Agilent microarray scanner bundle. The software J-express (www.molmine.com/jexpress) was used to determine the intensity
signal. We used median spot as intensity measure. All the microarray experiments included in this thesis were performed on single-channel microarrays.

One concern with DNA microarrays is the use of pre-formed probes, not accounting for e.g. isoforms, mutations, splice variants and pseudogenes. This issue is to a large extent solved with RNA sequencing (RNAseq) technology, explaining why that method is becoming increasingly popular. Also, studies have also shown variable correlations between gene expression level and protein level. Since proteins are the effectors of most cellular functions, and since most approved targeted therapies interfere with proteins, this is an important notion to keep in mind when interpreting gene expression data.

**qPCR validation**

Data for ERα and PR mRNA levels analysed by qPCR using the TaqMan Low Density Array technique were used to compare mRNA levels of hormone receptors to hormone receptor status estimated by IHC (Paper I).

**TCGA RNAseq validation cohort**

An independent cohort of publically available level 3 TCGA RNAseq data (IlluminaGA_RNAseqV2) was downloaded from [https://tcga-data.nci.nih.gov](https://tcga-data.nci.nih.gov) (Nov 20th, 2014) and used for evaluation the aneuploidy signature (Paper II). The ABSOLUTE algorithm was used to estimate ploidy status for TCGA samples.

**3.3.2 Reverse phase protein arrays (RPPA)**

To obtain high quality, quantitative protein data, RPPA was performed. RPPA is an antibody based, high-throughput functional proteomic method for tumour tissue and cultured cells.

Fresh frozen tissue samples were homogenised in lysis buffer and denatured in sodium dodecyl sulfate. Lysates were five-fold serial diluted before printing on nitrocellulose-
coated slides, to allow for quantitative analyses. Slides were stained with RPPA-validated antibodies for a large selection of phosphorylated and total proteins, and signal captured by a secondary antibody, using DAB colorimetric reaction. Details of available antibodies and staining conditions are available at: https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppa-core/antibody-information-and-protocols.html. The ArrayPro software was used to quantify spot signal intensities. Relative protein levels were determined by fitting each dilution curve with a logistic model, Supercurve (R package, available at http://r-forge.r-project.org/projects/supercurve/).

RPPA allows investigation of a large number of samples simultaneously from a small quantity of initial lysate (40 μg needed for analysis of 350 proteins). Both proteins and phospho-proteins may be detected, thus also yielding functional protein information. The method allows for a more objective quantification of protein amount compared with IHC. However, by RPPA no spatial information is obtained such as tumour versus stromal origin, or cellular localisation(s), of the protein. Also, similar to IHC, RPPA is dependent on the quality and availability of antibodies. Many proteins have multiple sites undergoing posttranslational modifications: e.g. ERα has > 20 known such sites, and only one antibody targeting a specific phospho-ER site was included in our arrays (Paper IV). Nonetheless, the RPPA proteins cover major pathways of relevance to human cancer, many of which have available therapeutic targeting options. Thus, this represents a promising method to screen for clinically important pathway aberrations.

As RPPA is not as widely applied as the other methods used in this thesis, a visualisation of the workflow is presented in Figure 9.
3.3.3 Bioinformatics: normalisation and data analysis

Bioinformatic analyses were performed using the softwares J-express (www.molmine.com/jexpress) and R (https://cran.r-project.org/). A brief overview of normalisation methods and approaches to microarray and RPPA analyses applied in the papers of this thesis is presented in Table 5:
<table>
<thead>
<tr>
<th>Approach</th>
<th>DNA microarray</th>
<th>RPPA</th>
<th>Purpose</th>
<th>Pros/cons (+/-)</th>
</tr>
</thead>
</table>
| Normalisation method           | Quantile normalisation\(^{199}\) (Paper II-IV) | Median centring across antibodies\(^{194}\) (Paper IV) | Adjust individual hybridization intensities, avoid systematic differences related to method/procedure itself (remove batch effects) prior to data analysis\(^{200}\) | +: Reduces risk of false positive findings, removes “noise”  
-: Increases risk of removing biologically interesting differences |
| Unsupervised analyses          | Unsupervised hierarchical clustering\(^{201}\) (Paper II) |                                            | Identify global expression patterns without predefining groups         | +: Unbiased description of data                           
-: Interesting signals in patient subgroups may not be captured |
| Supervised analyses            |                                     |                                            |                                                                        |                                                           |
| Single genes/proteins          | SAM\(^{202}\) (Paper II)           | LIMMA\(^{203}\) (Paper IV)               | Identify differentially expressed genes/proteins between two groups    | +: May point to biologically relevant differences between groups  
-: Does not distinguish drivers from passengers, interpretation requires knowledge about function |
| Multiple genes/proteins        | GSEA\(^{204}\) (Paper II-IV)       |                                            | Identify patterns of differentially expressed genes between two groups  | +: Provides information on pathway level, detection of global changes not visible on single gene level  
-: No identification of single causative drivers, included signatures may be cell/tissue/disease specific, not necessarily relevant for other diseases |
| Supervised machine learning algorithms | Support vector machine with 10-fold cross-validation\(^{205}\) (Paper II) | Akaike information criterion\(^{206}\) (Paper IV) | Identify the best predictors of predefined categories from supervised analyses | +: Unbiased approach for model fitting  
-: Pure mathematical modelling, relevant biological information may be lost |
| Expression signature score     | Calculated from list of differentially expressed genes\(^{207}\) (Paper II) | Calculated from predefined pathway activation signatures\(^{196}\) (Paper IV) | Define “meta-expression value” for multiple genes/proteins of biological relevance within a sample | +: Enables comparison of expression signatures within and across data sets  
-: Numerical values not directly comparable across data sets |

Table 5: Methods for normalisation and data analysis applied for microarray and RPPA data in Paper II-IV. Abbreviations: GSEA: Gene set enrichment analysis; LIMMA: Linear models for microarray and RNAseq data; RPPA: Reverse phase protein arrays; SAM: Significance analysis of microarray.
Publically available signature gene sets from the molecular signatures database, MSigDb (http://broadinstitute.org/gsea/msigdb/) were used in GSEA analyses (Paper II-IV).

We also used Connectivity map (Paper II), a publically available database containing gene expression signatures derived from treatment of cell lines with a panel of around 1300 compounds (http://www.broadinstitute.org/cmap). Gene expression analysis is performed before and after treatment, and the derived drug signatures reflect changes in expression level related to the different treatments. A gene expression signature of interest can be compared to the drug signatures in the database. A ranked list with the drug signatures that are most strongly positively and negatively correlated with the input signature is obtained. Connectivity map is a hypothesis generating tool, that can be applied to associate gene expression data, small molecules and disease states.208

3.4 Computerised tomography (CT) analysis

Abdominal fat can be divided into two main compartments: subcutaneous fat, which is the fat between the skin and the abdominal muscles, and visceral fat, located in the abdominal cavity, mainly composed by mesenteric and omental fat.

Routine diagnostic abdominal CE-CT scans were used for evaluation of abdominal fat compartments, waist circumference and hepatic steatosis (Paper III). To quantify the abdominal visceral and subcutaneous fat compartments, cross-sectional images were analysed consecutively from the upper right diaphragm to vertebral corpus L5/S1 level, using the software iNtuition (TeraRecon inc., San Mateo, CA, USA). This semi-automated method is based on segmentation of pixels with Hounsfield units (HU) values corresponding to adipose tissue (-195 to -45 HU).209 The correct segmentation of the fat compartments was visually verified by the operator and manually adjusted if necessary. The visceral and subcutaneous abdominal fat volumes (VAV and SAV, mL) were estimated separately, and the sum of the two volumes comprised the total abdominal fat volume (TAV, mL). The visceral fat percentage (VAV%) was calculated
using the following formula: $VAV\% = \frac{VAV}{TAV} \times 100$. Waist circumference (WC; cm) was measured at the level of vertebral corpus L3/L4.

Liver steatosis, as a surrogate marker of obesity, was estimated by measuring attenuation values in HU on contrast-enhanced images in portal-venous contrast phase by the software ImageJ.\textsuperscript{210,211} The mean attenuation value, based on the mean value from three regions of interest, was calculated.

A previous study compared the intra- and interobserver variability for this semi-automated method for CT based quantification of subcutaneous and visceral fat compartments, as well as WC. This study showed excellent reproducibility, with intraclass correlation coefficients (ICC) 0.99 for both intra- and interobserver variability, for all three measurements.\textsuperscript{209} Volumetric measurements had similar reproducibility as simpler, two-dimensional measurements, suggesting that the semiautomatic segmentation to identify the visceral and subcutaneous fat compartments is accurate and effective.\textsuperscript{209}

### 3.5 Statistics

Statistical analyses were performed using the software package SPSS versions 18 and 23 (IBM SPSS statistics, Armonk, NY, USA). Associations between categorical variables were assessed by the Pearson Chi-square test (Fisher’s exact test when appropriate). To compare the distribution of a continuous variable between two or more groups, the Mann-Whitney U and Kruskal-Wallis test were applied, respectively. If the multiple categories were ordered, Jonckheere-Terpstra trend test was used. Binary logistic regression was used to evaluate odds ratios (OR). Univariable survival analyses were performed by the Kaplan-Meier method, assessing survival differences by the log-rank test (Mantel-Cox). If multiple categories were ordered, linear trend test was used. Multivariable survival analyses were performed by Cox proportional hazards regression model, with visual examination of all included variables by a log-log plot to test the assumption of proportional hazards prior to inclusion in the final models. In
DSS analyses, the date of primary surgery was the starting point of observation, and death from endometrial cancer was considered the endpoint. Patients who died from other causes or were alive at the last day of follow-up were censored. For OS, all causes of death were considered as endpoints, otherwise patients were censored as described for DSS. All statistical tests were two-sided, and a p-value below 0.05 was considered significant.

In any study evaluating outcome, selection of the appropriate end-points is important. In our studies, we have mainly studied DSS, where death from endometrial cancer is registered as an event (Paper I, II, III and IV). This was chosen to detect survival differences presumably directly related to endometrial cancer, as opposed to OS, where all causes of death are registered as an event. This may be particularly important in biomarker studies in endometrial cancer, as comorbidities are common in this elderly, often obese, patient group.
4. Main results

The following is a recapitulation of the main findings within Paper I-IV:

**Paper I:**

BMI was evaluated in relation to clinicopathological characteristics, hormone receptor status and outcome in a series of 949 endometrial cancer patients. High BMI was significantly associated with low FIGO stage (p<0.001) and endometrioid histology (p=0.030). High level of PR was associated with higher BMI, confirmed on mRNA level and protein level using qPCR (p=0.02) and IHC (p=0.008). No relation was found between ERα expression and BMI, neither on mRNA nor protein level. Women with BMI ≥ 25 had significantly better DSS compared to women with BMI<25 in univariable analysis, with a 6% difference in 5-year DSS (p=0.035). This effect was not significant in a multivariable model adjusting for age, FIGO stage, histological subtype and grade. Applying OS instead as outcome measure, increasing BMI predicted worse survival adjusted for the same variables (HR 1.02, 95% CI 1.00-1.04, p=0.035). Median BMI increased significantly during the study period spanning over 29 years, from 25.3 (1981-1990) through 26.7 (1991-2000) to 26.9 (2001-2009, p=0.002).

**Paper II:**

In this study, the prognostic value of DNA ploidy was evaluated in a series of 825 patients with comprehensive clinicopathological characterisation. Gene expression data were available to explore aneuploidy-related transcriptional alterations for 144 patients. Aneuploidy was significantly associated with high age, high FIGO stage and high grade, non-endometrioid histology, ER/PR negativity and reduced survival (all p-values <0.001). The frequency of aneuploidy was higher in patients with metastatic disease (42%) or later recurrence (38%) compared to patients without signs of systemic or recurrent disease (17%, p<0.001). Aneuploidy independently predicted poor prognosis adjusted for age, FIGO stage, histological subtype and grade, with HR 1.62
(95% CI 1.11 – 2.37, p=0.013). Due to a significant interaction between hormone receptor status and ploidy status in Cox models, the two were not tested simultaneously. Replacing ploidy with ER/PR in the Cox model, a similar HR of 1.63 (95% CI 1.16 – 2.29, p=0.005) for ER/PR negativity was observed. The prognostic impact of DNA ploidy status was tested in models stratified for ER/PR status. Only in the subset of ER/PR negative tumours, aneuploidy independently predicted poor survival (HR 2.11, 95% CI 1.08 – 4.15, p=0.029), recurrence (OR 4.67, 95% CI 1.78 – 12.27, p=0.02) and lymph node metastasis (OR 5.47, 95% CI 1.58 – 18.99, p=0.007). A nine-gene prognostic ‘aneuploidy signature’, linked to low expression of chromosome 15q genes, was identified and validated in TCGA data. Transcriptional analyses pointed at various dysregulated pathways in aneuploid endometrial carcinomas, underlining a complex biology with consequently a diverse panel of potential drug targets. A previously suggested aneuploidy marker, STAG2, was not differentially expressed between diploid and aneuploid tumours by IHC; nor was the PPP2R3A protein that was significantly upregulated in the aneuploidy gene signature.

**Paper III:**

We quantified abdominal fat volumes (VAV, SAV, TAV), fat distribution pattern (VAV%), liver density (LD) and waist circumference (WC) as markers of obesity using preoperative CT scans, and studied these markers in relation to BMI, molecular tumour markers and disease outcome in a series of 227 endometrial cancer patients. All estimated fat volumes were strongly positively correlated with BMI and WC, and inversely correlated with LD. High fat volumes were associated with low grade endometrioid tumours and PR and AR positivity, but not with ERα positivity. A similar pattern was seen in subset analysis of endometrioid tumours only. VAV% was not correlated with BMI, WC or TAV, however, high VAV% was associated with high age (p<0.001) and aneuploidy (p=0.007), and independently predicted reduced disease-specific survival adjusted for FIGO stage, age, histological subtype and grade (HR=1.05, 95% CI 1.00 – 1.11, p=0.041). Patients with low VAV%, i.e. with a
relatively higher proportion of subcutaneous fat, showed enrichment of gene signatures related to increased inflammatory and immunogenic signalling in tumours.

**Paper IV:**

To identify obesity-related protein expression patterns, we studied three independent data sets with available reverse phase protein array (RPPA) data from patients treated for endometrioid endometrial cancer (n=272, n=68, n=178). All data sets coincided on expression level for 163 proteins and phospho-proteins, and enabled calculation of 12 predefined pathway activation signatures. Global gene expression profiling data and IHC for selected proteins were used for cross-platform validation. BMI was significantly correlated with PR expression, as well as a signature of hormone receptor expression (including ERα, phospho-ERα (S118), PR and AR), across all data sets. BMI was negatively correlated with RTK- and MAPK-pathway activation, and particularly with phospho-MAPK (T202 Y204) level. Using machine learning, a protein signature including phospho-ERα (S118) and phospho-MAPK (T202 Y204) was identified that characterised BMI groups with area under the curve 0.76 and 0.74 in two of the cohorts. In the subset of FIGO stage 1, grade 1-2 tumours, obese patients (BMI≥30) had significantly better DSS compared to non-obese patients (98% versus 78% 10-year survival, p=0.04). The non-obese patients had higher levels of phospho-MAPK (T202 Y204) in all cohorts. The obese patients had higher phospho-ERα (S118) levels, and showed enrichment of gene signatures related to oestrogen signalling, inflammation, immune signalling and hypoxia. In a subgroup analysis of non-obese patients with stage 1 tumours, patients with low PI3K-activation had significantly reduced survival.
5. Discussion of results

The work within this thesis (Paper I-IV) mainly focuses on context-related biomarkers in endometrial cancer. Context refers to the circumstances, or the environment, surrounding a particular event or situation. Obesity and genomic alterations can be considered contextual, or facilitating, factors in endometrial cancer development.\(^{90,119}\) Context is complex to study, for multiple reasons. First, you cannot control for all factors influencing it. Neither is it necessarily the context that directly leads to observed changes (i.e. the context is the driving factor); the context may predominantly act as a facilitator, or a break. Further, a setting such as obesity, may influence multiple factors and signalling pathways, so that the effect you were initially looking for becomes diluted by noise, or become partially contradictory because of other parallel effects. Nonetheless, increased understanding of altered signalling pathways involved in endometrial cancer, and their relation to the setting in which the tumours arise, is critical for identification of prognostic biomarkers to improve therapeutic stratification, and is also relevant in the development of targeted therapies.

Through our studies, we describe associations between these contextual factors and clinicopathological characteristics and outcome. In addition, using explorative approaches, we identify specific patterns of gene- and/or protein expression that are associated with aneuploidy and different measures of obesity in endometrial cancer, and suggest possible avenues to further explore for targeted therapy.

5.1.1 Overweight, obesity and endometrial cancer survival

As shown in the introduction (section 1.2), obesity is tightly related to the risk of developing endometrial cancer. It is also related to increased risk for several other cancer types.\(^{16}\) Obesity is one of the largest health concerns of today’s society, and BMI outside the range that is considered normal (defined by WHO as BMI 18.5-24.9 kg/m\(^2\)) is associated with increased all-cause mortality.\(^{212}\) Thus, maintenance of a
weight within the normal range should always be promoted in public health recommendations and in cancer prevention strategies.

Contrasting this, a number of isolated studies have observed a reduction in mortality from cardiovascular diseases and several cancer types for overweight and mildly obese patients compared to normal-weight patients; this potential protective effect is called the “obesity paradox”. The biological reasons for this obesity paradox are poorly understood. It has been hypothesised that the excess nutritional status may be advantageous in periods of acute illness, or that patients with low BMI are disproportionately sicker and thus at higher risk of mortality. It has also been argued that these observations may in part be related to confounding and bias, thus not representing real biological effects.

Studies reporting on obesity in relation to endometrial cancer survival are conflicting. Two large prospective cohort studies have shown an association between increasing BMI and a reduction in both DSS and OS. We also found increasing BMI to be associated with reduced OS in multivariable analysis, but better DSS in univariable analysis (Paper I). A meta-analysis by Arem et al. showed that increasing BMI was associated with increased all-cause mortality, however only at a similar magnitude as for the normal population. Other studies examining the effect of BMI on survival (DSS and/or OS) have found no association in multivariable analyses, although several reported trends to improved OS/DSS in univariable analysis with increasing BMI, similar to what we found (Paper I). A recent, large Danish cohort study found no association between obesity and OS for patients with Type 1 tumours, but morbidly obese (BMI ≥ 40) patients with Type 2 tumours had significantly reduced OS in multivariable analysis (HR 2.15, 95% CI 1.12 – 4.11). Comparison of these studies is complicated by many clinical and methodological factors: different patient populations studied (including race related differences), different definitions of Type 1 and Type 2 tumours, lack of adjustment for standard clinicopathological variables in multivariable analyses, use of self-reported versus measured BMI, and BMI cut-offs used (different cut-offs applied for BMI as categorical variable, use of BMI as continuous variable).
Also, adjustment for potential confounders differed between studies, and is also complicated by the fact that we do not necessarily know which factors to correct for (“unknown unknowns”). For example, in our studies, we had no information about weight loss prior to diagnosis. This could be a potential source of confounding, as weight loss prior to diagnosis may be a marker of aggressive cancer, even for patients with early stage disease.\textsuperscript{214} We tried to reduce the risk for this in \textit{Paper IV}, by excluding patients with BMI<20. Analysing the subgroup of patients with FIGO stage 1, grade 1-2 tumours, we found that obesity was significantly associated with better DSS in univariable analysis, suggesting that an “obesity paradox” may exist, at least in subgroups of endometrial cancer.

In spite of all the above-mentioned differences between studies, increasing BMI has repeatedly been associated with characteristics of less aggressive tumours, including lower FIGO stage, lower grade and endometrioid histology,\textsuperscript{219-223} observations that were also confirmed in our studies (\textit{Paper I and III}). This may explain the observed tendencies to improved disease specific survival in univariable analyses in several studies.

The lack of finite conclusions regarding BMI in relation to survival supports that BMI, when compared to other clinicopathological variables, likely is a rather weak prognostic marker. This also underscores an important principle: exposure to a known risk factor (here obesity) is not necessarily associated with inferior survival after treatment for the disease for which it is a risk factor.\textsuperscript{223}

\subsection*{5.1.2 Methods to study obesity}

\textbf{Alternative anthropometric measures}

BMI has been criticised for being an imprecise measure of obesity,\textsuperscript{213} particularly on individual level.\textsuperscript{224} Abdominal obesity is more closely associated with risk of several chronic diseases than gluteofemoral obesity.\textsuperscript{225} Several anthropometric measures have been proposed in addition to BMI, including waist circumference and waist/hip ratio,
suggested to better account for abdominal fat deposition and body composition.\textsuperscript{225} A recent meta-analysis found that increased waist circumference was associated with increased risk for endometrial cancer adjusted for BMI (summary RR 1.26, 95% CI 1.18-1.34), but increased hip/waist ratio did not add additional information adjusted for BMI (summary RR 1.07, 95% CI 0.97-1.17).\textsuperscript{226} Also, adult weight gain and cyclic weight changes have been associated with increased endometrial cancer risk adjusted for BMI.\textsuperscript{227} We had no such information available to adjust for in our analyses. However, these measures are mainly used in studies of cancer risk, and to our knowledge few studies have evaluated tumour characteristics and patient outcome in relation to parameters such as waist circumference, waist/hip ratio and weight oscillation in endometrial cancer.

Quantification of abdominal fat compartments

Although BMI and other anthropometric measures are easily determined in the clinic, none of these methods reflect the deposition of adipose tissue into visceral and subcutaneous fat compartments. These are known to have different metabolic properties, with visceral adipose tissue secreting substantial amounts of growth factors, inflammatory markers, free fatty acids and adipokines.\textsuperscript{119,228,229} We found that increasing VAV\% independently predicted increased risk of death from endometrial cancer adjusted for FIGO stage, histological subtype and grade (\textbf{Paper III}). Subgroup analyses of non-obese and obese patients suggested that this effect is independent of BMI. Also, waist circumference was correlated with both VAV and SAV, but not with VAV\%, supporting that waist circumference does not adequately distinguish the two compartments. This is the first study examining the relationship between CT-based fat distribution and survival in endometrial cancer, but our findings are supported by several studies indicating worse outcome with increasing ratio of visceral to subcutaneous fat in other cancer types.\textsuperscript{230-233} What has been shown for endometrial cancer patients though, is a higher frequency of lymph node metastasis and extraterine disease in patients with high proportion of visceral fat, supporting that high visceral fat percentage is associated with more aggressive disease, however survival data were not reported in this study.\textsuperscript{234} Interestingly, we found no association between absolute levels
of VAV (mL) and survival, and in fact, patients with low grade endometrioid tumours had significantly higher visceral fat volumes than patients with tumours of higher grade and non-endometrioid histology. This underlines that the proportion between the two fat compartments may be a more important risk factor/outcome determinant rather than the absolute quantity of fat. A high VAV may both reflect high fat volumes or a propensity to store fat viscerally,\textsuperscript{235} the latter being detected by VAV\%.

Studies in cardiovascular diseases support that a high proportion of visceral fat is especially linked to an adverse metabolic risk profile; subcutaneous fat in itself may be protective, particularly in the setting of high visceral fat volume.\textsuperscript{235,236} Abdominal fat quantification represents a promising means of risk stratification that should be further explored for endometrial cancer patients, as this information is quite easily obtained from routine diagnostic images by CT or MRI, without much extra time investment for the radiologist.

The ideal obesity estimate may not exist. On the individual level, BMI is likely not a good enough marker to fully disentangle the complex alterations in systemic and tumour signalling seen in obesity. Studies reporting on panels of biomarkers, including anthropometric estimates, imaging data and/or serological markers, may reveal several levels of information not captured by BMI alone.

### 5.1.3 Obesity, hormone receptor expression and pathway crosstalk

The prognostic impact of hormone receptor expression levels in endometrial cancer is well described,\textsuperscript{67,68,182,237,238} but few studies have focused on whether (and how) obesity influences hormone receptor expression and pathway signalling in this cancer type. As discussed in section 1.5.4, obesity is thought to influence endometrial cancer development through several mechanisms. This includes altered sex hormone signalling, through increased oestrogen and androgen levels, and reduced progesterone levels (Figure 7). Upon hormone binding, the nuclear hormone receptors ER, PR and AR act as transcription factors, and their regulation of gene transcription is dependent...
on co-regulators, that may have promotive or inhibitory effects. A vast range of pathway crosstalk mechanisms have been described for the different nuclear hormone receptors, underscoring that their signalling and regulation are mediated in a complex manner, that may to a large extent also be tissue specific.

Through our studies, we found robust and consistent evidence for an association between different obesity markers and increased expression of PR, measured on mRNA level (qPCR) and protein level (IHC and RPPA, Paper I, III and IV). This is also supported by the study by Westin et al., reporting a positive correlation between PR expression and BMI in RPPA data. In a study of 128 endometrial cancer patients, Gates et al. reported a tendency to higher BMI in patients with intact PR expression (p=0.07), with no such association seen for ER (p=0.77). However, Munstedt et al. reported no association between BMI and ER or PR expression by IHC, but no detailed description of how receptor loss was determined or how this relationship was assessed was provided in their report. A recent study of endometrial cancers showed an increase in both ER and PR expression with increasing BMI, using a scale of different cut-offs to define ER and PR positivity. Also, in asymptomatic patients undergoing bariatric surgery, women with endometrial hyperplasia (n=4) showed reduced expression of both ER, PR and AR expression following substantial weight loss. In our data, we found a similar tendency for AR (by IHC) to be associated with obesity estimates, both when including all histological types and in endometrioid tumours only (Paper III). AR expression was also correlated with BMI in the training set in the RPPA study (Paper IV), where only endometrioid tumours were studied.

Strikingly, ERα status and obesity estimates were not associated (Paper I, III and IV). Since our analyses also included assessment of ERα expression as a continuous variable using RPPA and mRNA data, cut-off selection for ERα by IHC is likely not the explanation for the observed lack of association with BMI and other obesity estimates. As mentioned, oestrogen levels are thought to increase with increasing BMI. The lack of association between ERα and BMI could be due to repressive effects of oestrogen on ESR1 gene expression through a negative feedback loop, or
repressive effects of PR on ER mRNA and protein expression, processes that both have been described in breast cancer cells.

Interestingly, when p-ERα (S118) expression was studied in relation to BMI in endometrioid tumours (Paper IV), a significant correlation with BMI was found. These data underline that obesity does not only influence hormone signalling by regulation of hormone receptor expression on transcriptional level (mRNA) or translational level (protein) in endometrial cancer, but likely also through post-translational modifications of the oestrogen receptor. The main mechanisms for ERα phosphorylation at S118 are: 1) directly by binding of oestradiol to ERα, inducing phosphorylation by cyclin-dependent kinases, and 2) indirectly by growth factors activating RTKs, leading to MAPK activation and thereby ERα phosphorylation.\textsuperscript{250,251} As we found a negative correlation between MAPK-signalling and p-ERα (S118) levels (Paper IV), the direct route of ERα-mediated phosphorylation might be the main determinant of ERα S118 phosphorylation in the setting of obesity.

Our data strongly suggest that PR, and to some extent AR, expression is associated with measures of excess body fat. We also find evidence for differential ERα phosphorylation in tumours from obese versus non-obese patients, supporting the notion that ERα function may be dependent on the metabolic setting. Although our studies were not designed to provide mechanistic answers, these observations suggest that hormone signalling and crosstalk mechanisms may be influenced by the metabolic state of the patient.

5.1.4 Aneuploidy – a clinically useful prognostic biomarker

For a prognostic biomarker to be of clinical interest, it should add independent information to already applied clinicopathological markers. The main markers used in clinical decision-making for endometrial cancer include FIGO stage, histological subtype and grade. Before clinical implementation of a biomarker, several steps of validation are recommended: assay validation, validation in independent cohorts, and
prospective validation of clinical applicability for treatment stratification before application in routine practice. Several prognostic markers have been identified in endometrial cancer, although few are applied in the routine clinical setting for risk stratification. Biomarkers that are to some extent clinically applied include LVSI, DNA ploidy and ER/PR status, depending on local guidelines.

DNA ploidy has a long track record as a promising biomarker in endometrial cancer. It has repeatedly been shown to confer independent prognostic information adjusted for standard applied clinicopathological variables, now also confirmed in our large study (HR 1.62 and 95% CI 1.11-2.37, Paper II) – an important step in the path towards clinical implementation. A previous study from our group found the prognostic information derived from DNA ploidy status assessed in a routine clinical setting to be similar to that of DNA ploidy status assessed in a research setting, thus demonstrating the clinical applicability of DNA ploidy status assessment by flow cytometry. DNA ploidy status has also been shown to predict lymph node metastasis and recurrence in preoperative biopsies, confirming its role as a promising preoperative marker to tailor surgical treatment.

However, until now DNA ploidy has rarely been compared with other molecular markers, such as ER/PR status (Paper II). We showed that, in isolation, the two markers yielded similar prognostic information, with comparable adjusted HRs. Aneuploidy added independent information for the prediction of survival, lymph node metastasis, and recurrence in ER/PR negative tumours. Our results point to a subgroup of particularly aggressive tumours, which may benefit from more extensive surgery and adjuvant treatment.

5.1.5 From hypothesis generating studies towards implementation in clinical trials

We used large scale transcriptomic and proteomic data to assess expression differences related to DNA ploidy, VAV% and BMI (Paper II, III and IV, respectively). By this descriptive, hypothesis-generating approach, we tried to connect observations on very
different levels (molecular tumour characteristics, imaging data, anthropometric data) with global expression changes in the tumours. Such analyses may provide insight in biological characteristics associated with the feature studied, and point to specific genes, proteins or pathways that may be relevant to further explore; both to understand their role in endometrial carcinogenesis, and as potential therapeutic targets.

Based on flow cytometry assessed DNA ploidy status, we identified a nine-gene transcriptional aneuploidy signature (Paper II), which was validated in a TCGA endometrial cancer cohort in spite of different methods used both for ploidy estimation and gene expression analysis. This confirmation in independent data sets and by different methods increase the likelihood that our observations are indeed “real” effects, and not data-set or method specific findings only. The identified signature reflected prognosis in a similar manner as ploidy estimation by flow cytometry, and pointed to low expression of chromosome 15q genes in aneuploid tumours. Loss of 15q regions has previously been reported as a frequently occurring alteration in aneuploid endometrial and colorectal cancers.\(^{255,256}\) TCGA data supported that the observed low gene expression levels could be due to deletions of these chromosomal regions. A high frequency of 15q deletions has also been seen in other cancer types,\(^{257,258}\) speculating on the existence of one or several tumour suppressor genes in these chromosomal regions.

One of the three upregulated genes in our aneuploidy signature was \(PGAM2\), a glycolytic enzyme. Through GSEA, gene sets related to glycolysis were found to be enriched in aneuploid tumours, and increased expression of genes related to glycolysis has been observed in model systems of aneuploidy.\(^{259,260}\) Altered cellular energetics is a hallmark of cancer (Figure 6),\(^90\) and therapeutics interfering with cellular metabolism are currently being developed.\(^{261}\) It is therefore tempting to suggest that aneuploidy should be explored as a predictive marker in future studies with such compounds.

\(PPP2R3A\), encoding a regulatory subunit of the protein phosphatase 2A complex (PP2A), was also upregulated in our aneuploidy signature, although we found no association between \(PPP2R3A\) protein expression and aneuploidy by IHC (Paper II).
Still, this observation merits interest, as alterations in other subunits of the PP2A complex have been described in endometrial cancer lesions: mutations in \textit{PPP2R1A} (a gene encoding another regulatory subunit of the PP2A complex) were frequent in the ‘copy-number high’ tumours (22\%) in the original TCGA endometrial cancer publication,\textsuperscript{93} in carcinosarcomas (28\%) in the very recent TCGA publication,\textsuperscript{127} and in metastatic endometrial cancer lesions,\textsuperscript{94} all of which are related to aneuploidy. A pan-cancer study also showed that PP2A complex alterations are associated with whole-genome doubling.\textsuperscript{262} These data support a role for the PP2A complex in aneuploidy, which is interesting to further explore, as this complex might be targetable.\textsuperscript{263,264}

In \textbf{Paper III}, low VAV\% was associated with better prognosis, and tumours arising in this setting showed enrichment of gene sets related to increased inflammatory and immunogenic signalling. A high visceral fat content has previously been associated with increased systemic inflammation, which is thought to promote carcinogenesis through interaction with the tumour microenvironment, leading to release of growth factors and reactive oxygen species, promoting tumour growth.\textsuperscript{90,265} Thus, this finding was somewhat contrary to what we had expected. However, a recent study showed that visceral adipose tissue may induce an immunosuppressive tumour microenvironment through altering dendritic cell function and suppression of immune surveillance,\textsuperscript{266} supporting our findings of higher immune activation in tumours arising in patients with low visceral fat. Interestingly, a similar tendency was seen when comparing obese and non-obese patients with low stage and low grade endometrioid tumours (\textbf{Paper IV}): obese patients, who had better prognosis, showed enrichment of inflammation and immune related gene sets, as well as gene sets related to hypoxia. Recent evidence support that inflammation and hypoxia may recruit anti-tumour effectors, such as polymorphonuclear neutrophils, in endometrial cancer, and their presence has been linked to improved survival in mice and humans.\textsuperscript{267} Also, high epithelial infiltration of CD8+ T-lymphocytes is reported to be associated with improved endometrial cancer survival.\textsuperscript{268,269} Follow-up studies to further characterise the immune components of the tumour- and the tumour microenvironment in relation to different obesity parameters
seems justified, as several therapies targeting different components of the immune system have generated optimism in the oncological field in recent years.\textsuperscript{270}

In \textbf{Paper IV}, we found that tumours arising in non-obese patients had higher levels of MAPK-activation, and particularly of p-MAPK (T202 Y204). In the subgroup of FIGO stage 1, low-grade tumours, non-obese patients had significantly reduced survival compared to the obese patients. This observation is interesting from a clinical point of view. Recently, the anti-diabetic drug metformin was shown to reduce p-MAPK levels in a window-of-opportunity trial in endometrial cancer,\textsuperscript{271} and could potentially be explored as a therapeutic agent to prevent relapses in this group of low-risk patients. Clinical trials with metformin are ongoing to understand how this drug can be incorporated in the treatment of women with endometrial cancer (NCT01877564, NCT02874430, NCT02065687).
6. Conclusions

1. BMI and imaging based estimates of obesity are associated with clinicopathological markers of less aggressive endometrial cancer (Paper I, III and IV).

2. High BMI is associated with PR and AR but not ERα expression (Paper I, III and IV).

3. Obese patients with endometrioid endometrial cancer have higher levels of phosphorylated ERα. Non-obese patients have higher levels of phosphorylated MAPK (Paper IV).

4. Overweight and obesity are associated with improved DSS in univariable, but not multivariable analysis, and worse OS in multivariable analysis (Paper I). High VAV% independently predicts worse DSS (Paper III). Obesity is associated with improved DSS in patients with assumed excellent prognosis (Paper IV).

5. Gene sets linked to inflammation and immune activation are enriched in tumours from patients with low VAV%, and equally in tumours from obese patients with FIGO stage 1, grade 1-2 tumours (Paper III and IV).

6. DNA ploidy is a robust prognostic marker in endometrial cancer, and aneuploidy independently predicts reduced DSS. In patients with loss of ER/PR, aneuploidy independently predicts increased risk of lymph node metastases and recurrence (Paper II).

7. A nine-gene aneuploidy signature is associated with reduced survival and low expression of chromosome 15q genes (Paper II).
7. **Future perspectives**

This thesis has provided new insights, but also raised several questions that warrant additional investigation in future studies of endometrial cancer.

DNA ploidy status has a long track record as a promising prognostic marker in endometrial cancer, also confirmed in our study (**Paper II**). This marker has also shown predictive value as a marker for lymph node metastasis, and merits further investigation in a prospective randomised multicentre trial, examining its contribution as a marker to individualise surgery (lymphadenectomy or not) and adjuvant therapy. It should also be tested in combination, and thoroughly compared, with other promising markers shown to predict lymph node metastasis.

The differences observed in hormone receptor expression and signalling in relation to obesity (**Paper I, III and IV**) should be further explored. Model systems, including endometrial cancer cell lines, and patient-derived xenograft mouse models (PDX-models), could be used to further examine the mechanistic underpinnings of increased PR expression with overweight and obesity, as well as the differential ER phosphohorylation levels seen according to BMI groups. Increased understanding of the molecular mechanisms underlying obesity-related carcinogenesis is important, in light of the high prevalence of obesity in the population. Also, drugs targeting ER (tamoxifen) and PR (medroxyprogesterone) are at present being clinically used in the setting of advanced/metastatic endometrial cancer, and further knowledge about how obesity influences hormone receptor expression, hormonal signalling and pathway cross-talk may aid in understanding which patients are most likely to respond to therapy, and may also increase our understanding of why some patients develop resistance to therapy.

A study focusing on tumour immune components in endometrial tumours and the tumour microenvironment is also warranted. In two of our studies (**Paper III and IV**), enrichment of immune- and inflammation related gene sets was demonstrated in a subset of the tumours, both from patients who had improved survival. Promising new therapeutics targeting the immune system have emerged in later years, and an increased
understanding of the immune components in endometrial cancer is likely to improve our understanding of how such therapies may best be used.

The prognostic ability of VAV% (demonstrated in Paper III) should be validated in an independent patient cohort. Currently, such a validation study is being planned from our institution, in collaboration with international centres. In addition, exploring panels of serological markers presumably related to obesity in relation to tumour characteristics and outcome, could contribute to increase our understanding of how obesity influences endometrial cancer growth and progression. Interesting markers to study include steroid hormones, inflammatory markers and metabolic profile (e.g. blood lipids, glucose, insulin, IGF1). Importantly, such markers are low-cost, with available, and already clinically used, assays. The suggested markers should be studied in addition to anthropometric and imaging derived obesity measurements, to improve our knowledge on how obesity and altered metabolism influence disease development, tumour characteristics and prognosis. An interesting approach would be to examine serological markers with paired tissue from premalignant lesions, primary tumours and metastases.
8. References


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9. **Errata**

**Paper I: Abstract**

Results section reads: High BMI was significantly associated with low International Federation of Gynaecology and Obstetrics (FIGO) stage, endometrioid histology, low/intermediate grade, and high level of…

This should read: High BMI was significantly associated with low International Federation of Gynaecology and Obstetrics (FIGO) stage, endometrioid histology, age in the middle quartiles, and high level of…

**Paper I:** All p-values listed as p<0.0001 are wrongly typed. The correct value is p<0.0005 / p<0.001.
High BMI is significantly associated with positive progesterone receptor status and clinico-pathological markers for non-aggressive disease in endometrial cancer

**BACKGROUND:** Endometrial cancer incidence is increasing in industrialised countries. High body mass index (BMI, kg m$^{-2}$) is associated with higher risk for disease. We wanted to investigate if BMI is related to clinico-pathological characteristics, hormone receptor status in primary tumour, and disease outcome in endometrial cancer.

**PATIENTS AND METHODS:** In total, 1129 women primarily treated for endometrial carcinoma at Haukeland University Hospital during 1981–2009 were studied. Body mass index was available for 949 patients and related to comprehensive clinical and histopathological data, hormone receptor status in tumour, treatment, and follow-up.

**RESULTS:** High BMI was significantly associated with low International Federation of Gynaecology and Obstetrics (FIGO) stage, endometrioid histology, low/intermediate grade, and high level of progesterone receptor (PR) mRNA by qPCR ($n = 150$; $P = 0.002$) and protein expression by immunohistochemistry ($n = 433$; $P = 0.003$). In contrast, oestrogen receptor (ERa) status was not associated with BMI. Overweight/obese women had significantly better disease-specific survival (DSS) than normal/underweight women in univariate analysis ($P = 0.035$). In multivariate analysis of DSS adjusting for age, FIGO stage, histological subtype, and grade, BMI showed no independent prognostic impact.

**CONCLUSION:** High BMI was significantly associated with markers of non-aggressive disease and positive PR status in a large population-based study of endometrial carcinoma. Women with high BMI had significantly better prognosis in univariate analysis of DSS, an effect that disappeared in multivariate analysis adjusting for established prognostic markers. The role of PR in endometrial carcinogenesis needs to be further studied.

**Keywords:** body mass index; endometrial carcinoma; prognosis; progesterone receptor
data, hormone receptor status in primary tumours, and disease outcome in a large population-based endometrial carcinoma series.

PATIENTS AND METHODS

Patient series

The patient series include 1129 women primarily treated for endometrial carcinoma at Haukeland University Hospital during the period 1981 through 2009. This is the referral hospital for Hordaland county, with ~475,000 inhabitants, representing about 10% of the Norwegian population (SSB, 2010). The endometrial cancer incidence rate and prognosis in this area are similar to data for the total population (Cancer Registry of Norway, 2009).

Information concerning height, weight, age, menopausal status, International Federation of Gynaecology and Obstetrics (FIGO) stage, histological subtype and grade, treatment, and follow-up was collected by review of the medical records and through correspondence with the primary physicians. In all, 91% of the women underwent hysterectomy with bilateral salpingo-oophorectomy as primary treatment and were classified according to the FIGO 1988 criteria (Mikuta, 1993). If surgical treatment was contraindicated, the staging was based on the available information from curettage results, clinical examination, chest X-ray, and abdomino-pelvic CT.

Follow-up time was defined as the time interval between date of primary diagnosis and date of death or last follow-up. The median follow-up time was 4.9 years (range 0.01–23.2). In all, 223 patients (20%) died from endometrial carcinoma during the follow-up period, while 207 (18%) died from other causes. These data were cross-checked with information from the Cancer Registry of Norway and the Register of Statistics Norway. Last follow-up was 20 December 2009.

Body mass index was calculated as weight (kg) divided by squared height (m²), both measured at the time of diagnosis. These data were available for 949 patients (84%). For the statistical

Figure 1 Distribution of BMI for endometrial carcinoma patients treated in one defined region in Norway (Hordaland county) in the periods 1981–1990, 1991–2000, and 2001–2009. Median BMI and range increase significantly from 25.3 (16.9–44.5) to 26.7 (15.8–50.5) and 26.9 (14.7–73.0) for the time periods studied, P=0.002 (Kruskal–Wallis test). O = minor outliers and ∗= major outliers.

Table 1 Distribution of clinico-pathological factors in 949 patients with endometrial carcinoma according to body mass index (BMI)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total no. of patients</th>
<th>Median BMI</th>
<th>Lean (%)</th>
<th>Normal (%)</th>
<th>Overweight (%)</th>
<th>Obese (%)</th>
<th>P-value*</th>
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<tr>
<td>Age, quartiles&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
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<td></td>
<td>25.6</td>
<td>5 (2)</td>
<td>109 (45)</td>
<td>66 (27)</td>
<td>65 (27)</td>
<td></td>
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<tr>
<td>2 (age 58 – 66)</td>
<td></td>
<td>27.1</td>
<td>8 (3)</td>
<td>76 (32)</td>
<td>82 (34)</td>
<td>73 (31)</td>
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<tr>
<td>3 (age 66 – 74)</td>
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<td>27.3</td>
<td>2 (1)</td>
<td>76 (31)</td>
<td>91 (37)</td>
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<tr>
<td>4 (age 74 – 95)</td>
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<td>25.1</td>
<td>8 (4)</td>
<td>97 (44)</td>
<td>70 (32)</td>
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<td>1 (1)</td>
<td>54 (44)</td>
<td>31 (25)</td>
<td>38 (31)</td>
<td></td>
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<tr>
<td>Post</td>
<td></td>
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<td>22 (3)</td>
<td>304 (37)</td>
<td>278 (34)</td>
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<tr>
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<td></td>
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<td>224 (33)</td>
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<td>30 (29)</td>
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<td>10 (18)</td>
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<td>297 (37)</td>
<td>269 (33)</td>
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<td>61 (44)</td>
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<tr>
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<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td>26.9</td>
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<tr>
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<tr>
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<tr>
<td>Negative</td>
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<td>25.5</td>
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</tbody>
</table>

Abbreviation: FIGO = International Federation of Gynaecology and Obstetrics. *χ²-test when no other specified. Truncated to closest integer. Menopausal status was determined based on the information from the patient records. Data missing for 44 patients. Mann–Whitney U-test.
analyses on BMI we used the quartiles for the data set as cut points, as well as the established WHO classification system; BMI under 18.5 (underweight), between 18.5 and 24.9 (normal), between 25 and 29.9 (overweight), and ≥30 (obese). Height and weight of outliers (BMI < 15 or BMI > 50, n = 7) was double-checked. All analyses were also performed excluding these; this did not affect any of the conclusions.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded tumour specimens were mounted in tissue microarrays (TMA) as previously described (Hoos et al, 2001; Stefansson et al, 2004). Briefly, TMA was constructed by identifying the area of highest tumour grade on HE-stained slides, followed by punching out three tissue cylinders from the selected areas of the donor block and mounting these into a recipient paraffin block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA). Immunohistochemical staining for receptor status was assessed for oestrogen- and progesterone receptors (ERx and PR) and available for 437 and 433 patients for ERx and PR, respectively (38% of study population). The method for immunohistochemical staining was as previously described, using the lower quartile to define receptor loss (Engelsen et al, 2008b).

**qPCR analysis**

From a subset of 150 patients (13%), fresh frozen tumour tissue was collected prospectively and was available for mRNA analysis in parallel with the immunohistochemical staining. Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany), with quality control and method for DNA processing as previously reported (Engelsen et al, 2008a; Salvesen et al, 2009). mRNA expression levels in tumours for ERx and PR were investigated by qPCR using the TaqMan Low Density Array technique (Engelsen et al, 2008a).

**Statistical methods**

Body mass index in WHO categories was applied to assess the distribution of various clinico-pathological variables, using the Pearson’s χ²-test. Hormone receptor status in primary tumour in relation to BMI was assessed by the Mann–Whitney U-test. Univariate survival analyses for disease-specific survival (DSS) and overall survival (OS) were performed using the Kaplan–Meier method (log-rank test). The Cox proportional hazard regression analysis was applied to evaluate the prognostic impact of BMI adjusted for the established prognostic markers in endometrial carcinoma. We compared the distribution of clinico-pathological variables and prognosis for patients with available data for BMI to patients where these data were missing (16%). Women lacking BMI data were older, with median age 69.3 years compared with women with BMI data (65.2 years; Mann–Whitney U-test). No other significant differences were identified. The statistical software PASWStatistics18.0 was used for data analyses (SPSS Inc., Chicago, IL, USA).

The study was approved by the IRB (NSD 15501, REK III nr 052.01).

**RESULTS**

High BMI associates with clinico-pathological markers for non-aggressive disease

The median BMI at diagnosis was 26.4 (range 14.7–73.0), with significantly increasing BMI throughout the study period, P = 0.002 (Figure 1). There was a significant association between BMI and patient age at diagnosis, FIGO stage, and histological subtype, as shown in Table 1. The proportion of patients with BMI < 25 was larger in the lower and upper age quartiles compared with BMI ≥ 25, whereas there was a tendency for the patients of the middle age quartiles to be overweight or obese. The proportion of normal/lean patients was larger for FIGO stages III and IV compared with FIGO stages I and II. High BMI was also associated with endometrioid histology. There was no significant association between BMI and menopausal status nor BMI and grade. Also, there was no significant difference in number of performed analyses on BMI we used the quartiles for the data set as cut points, as well as the established WHO classification system; BMI under 18.5 (underweight), between 18.5 and 24.9 (normal), between 25 and 29.9 (overweight), and ≥30 (obese). Height and weight of outliers (BMI < 15 or BMI > 50, n = 7) was double-checked. All analyses were also performed excluding these; this did not affect any of the conclusions.

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lymphadenectomies related to BMI ($P = 0.99$), but a tendency to
more adjuvant therapy given to patients with BMI < 25 ($P = 0.06$).

High BMI associates with positive PR status in tumour

When investigating biomarkers for receptor status in tumours
related to BMI we found that patients with PR-negative tumours
(by IHC) had lower median BMI compared with the
patients who had PR-positive tumours, median 25.5 vs 26.9,
respectively ($P = 0.003$, Mann–Whitney $U$-test). We did not find
any significant correlation between BMI and ERα status in tumours
($P = 0.08$) (Table 1). To further validate this finding, we examined
a subset of 150 fresh frozen patient samples for
mRNA expression levels for hormone receptors by qPCR. This
confirmed a significantly higher mRNA expression level for
PR in patients with BMI < 25 compared with patients with lower
BMI ($P = 0.02$, Mann–Whitney $U$-test). For ERα, no such
association with BMI was observed for mRNA expression levels
($P = 0.21$).

Loss of ERα and PR (by IHC) was associated with
postmenopausal status ($P = 0.01$ and $P = 0.006$, respectively,
Pearson’s $χ^2$-test).

BMI and prognosis

Univariate analysis  The established clinico-pathological variables
showed, as expected, a highly significant impact on DSS, as listed in
Table 2. There was a trend towards better prognosis for patients with
higher BMI in univariate analysis (Table 2). Patients being
overweight/obese vs normal/underweight as defined by the WHO
had better DSS, with a 5-year survival of 82% for women with
BMI > 25 compared with 76% for BMI < 25 ($P = 0.035$; Figure 2A;
Table 2). For OS, we found that patients with BMI < 25 had a 5-year
survival of 69% compared with 74% for patients with BMI > 25
($P = 0.18$; Figure 2B). In the OS analysis, we also see a pattern of
diminishing survival difference between the two BMI groups > 10 years after diagnosis. This may relate to the higher risk of developing
other diseases for overweight women, being more important than
the risk for cancer-related deaths > 10 years after diagnosis.

Multivariate analysis  The survival effect of BMI observed in
univariate analysis for DSS disappeared when adjustment was
made for age at diagnosis (continuous variable), FIGO stage,
histological subtype, and grade in the Cox multivariate regression
analysis as listed in Table 3. Adjusted HR for BMI < 25 vs > 25 was

![Figure 2](image)

Univariate survival plot by Kaplan–Meier for estimation of DSS (A) and OS (B) in patients with endometrial carcinoma related to BMI. The total number of patients in each group is followed by number of deaths, given in parentheses; $P$-value based on the Mantel–Cox test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients (%)</th>
<th>Unadjusted HR*</th>
<th>95% CI</th>
<th>$P$-value</th>
<th>Adjusted HR</th>
<th>95% CI</th>
<th>$P$-value</th>
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<tbody>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>646 (71)</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt;0.0001</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>II</td>
<td>96 (11)</td>
<td>3.25</td>
<td>1.98–5.31</td>
<td>2.83</td>
<td>1.72–4.65</td>
<td>2.83</td>
<td>1.72–4.65</td>
</tr>
<tr>
<td>III</td>
<td>111 (12)</td>
<td>9.75</td>
<td>6.73–14.12</td>
<td>8.13</td>
<td>5.52–11.97</td>
<td>8.13</td>
<td>5.52–11.97</td>
</tr>
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<td>Histological subtype</td>
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<tr>
<td>Endometrioid</td>
<td>777 (86)</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt;0.0001</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>128 (14)</td>
<td>4.76</td>
<td>3.44–6.57</td>
<td>1.49</td>
<td>0.95–2.32</td>
<td>1.49</td>
<td>0.95–2.32</td>
</tr>
<tr>
<td>Grade</td>
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</tr>
<tr>
<td>1 or 2</td>
<td>668 (74)</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt;0.0001</td>
<td>1.00</td>
<td>1.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1</td>
<td>237 (26)</td>
<td>3.84</td>
<td>2.83–5.19</td>
<td>1.41</td>
<td>0.93–2.13</td>
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<tr>
<td>Ageb</td>
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<td>1.04–1.07</td>
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<td>1.05</td>
<td>1.03–1.06</td>
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</tr>
<tr>
<td>&lt; 25</td>
<td>364 (40)</td>
<td>1.38</td>
<td>1.02–1.86</td>
<td>0.04</td>
<td>0.93</td>
<td>0.68–1.27</td>
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</tr>
<tr>
<td>≥ 25</td>
<td>541 (60)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Abbreviations: BMI = body mass index; CI = confidence interval; FIGO = International Federation of Gynaecology and Obstetrics; HR = hazard ratio. *Analyses based on patients with complete information for all variables ($n = 905$). bAge at primary operation, continuous variable with HR given per year. cWhen including patients with endometrioid histology only: adjusted HR for BMI was 1.07, 95% CI 0.73–1.55, $P = 0.7$.
0.93 (CI 0.68–1.27, P = 0.65). When BMI was applied as a continuous variable in the same Cox model, we found a similar insignificant HR for BMI of 1.01 (CI 0.98–1.04), and pattern for the other variables with independent impact for FIGO stage and age only. In contrast, when using OS as endpoint in the Cox model, we found that BMI had independent impact on prognosis when introduced as a continuous variable with an HR 1.02 (CI 1.00–1.04, P = 0.035). FIGO stage and age were also independent predictors of prognosis (P < 0.0001 for both), while histology was of borderline significance (P = 0.053) and grade was non-significant (P = 0.166).

DISCUSSION

To our knowledge, this is the most comprehensive study of clinico-pathological variables to date. It is also the largest study to date exploring the relationship between BMI and a large panel of markers for tumour phenotype in endometrial carcinoma. The large sample size with careful characterisation of FIGO stage, histological subtype, and grade confers more accuracy to the estimates for the independent prognostic impact of BMI compared with smaller previous studies. Also, the fact that the patient series studied was derived from a well-defined geographic region in Norway, previously shown to be representative for the total Norwegian population (Salvesen et al., 1999), suggests that the findings may be representative for a Caucasian patient population in general.

We found a positive association between high BMI and favourable DSS in univariate analysis but not in multivariate analysis. However, in multivariate analysis of OS, we found an independent unfavourable prognostic impact of increasing BMI.

Previous studies exploring the effect of BMI on survival have reported conflicting results, which may be due to sample sizes, choice of cut point for BMI, outcome variables applied, and the panel of clinico-pathological markers adjusted for in the multivariate analyses. Like the present study, several have reported a trend towards better survival in the overweight compared with the more slender women (Anderson et al., 1996; Temkin et al., 2007; Munstedt et al., 2008). Others have concluded with no difference (Jeong et al., 2010) and even poorer survival for women with higher BMI (von Gruenigen et al., 2006). Disease-specific survival applied in the present study is more likely to be accurate in detecting deaths directly related to the disease studied. Previous studies, mostly applying OS, may have underestimated the positive biological impact of obesity, as obese women have increased risk of dying from intercurrent disease (Anderson et al., 1996; Temkin et al., 2007). Our findings that OS is less favourable for obese women when adjusted for the standard clinico-pathological risk factors may support this.

A limit of our study is that BMI is measured at the time of diagnosis. This may lead to a bias, as aggressive cancers often are associated with weight loss, cachexia, and anorexia (Keller 1993). Hence, we may have underestimated the weight of patients presenting with high stage cancers.

The rise in endometrial carcinoma incidence has been associated with an epidemic of obesity and physical inactivity (Amant et al., 2005). Unopposed oestrogen exposure leads to endometrial hyperplasia, and increased risk of atypical hyperplasia and type I endometrial cancer (Shang, 2006). The significance of progesterone in controlling oestrogen-driven proliferation is underlined by its efficacy in preventing endometrial cancer (Kim and Chapman-Davis, 2010). Still, the molecular basis and cross talk between hormone receptor pathways are poorly understood (Kim and Chapman-Davis, 2010). In previous smaller immunohistochemical studies (Duska et al., 2001; Gates et al., 2006), no significant relationship between hormone receptor status and BMI was identified (n = 41 and n = 165, respectively). We found that BMI was significantly linked to alterations in PR but not ERz status in tumours, confirmed by two different techniques estimating mRNA and protein levels for PR and ERz. The biological function of PR may be altered by genetic variations. Interestingly, recent studies have identified a single-nucleotide polymorphism in the gene coding for the PR, which has been associated with increased risk for endometrial carcinoma (Xu et al., 2009; O’Mara et al., 2010). This supports the complexity in the hormone receptor interactions related to carcinogenesis and tumour development in endometrial cancer, and further studies of these interactions are needed.

ACKNOWLEDGEMENTS

We acknowledge Helse Vest, The University of Bergen, The Norwegian Cancer Society (Harald Andersens legat), The Research Council of Norway. We thank Britt Edvardsen, Ingrid Bergo, Erlend S Njølstad, Pål-Christiansen S Njølstad, Gerd Lillian Hallseth, and Bendik Nordanger for technical assistance.

REFERENCES

Aneuploidy related transcriptional changes in endometrial cancer link low expression of chromosome 15q genes to poor survival

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⁺⁺Deceased  

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Keywords: endometrial cancer, DNA ploidy, prognosis, transcriptional alterations, chromosome 15q  
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ABSTRACT

Aneuploidy is a widely studied prognostic marker in endometrial cancer (EC), however, not implemented in clinical decision-making. It lacks validation in large prospective patient cohorts adjusted for currently standard applied prognostic markers, including estrogen/progesterone receptor status (ER/PR). Also, little is known about aneuploidy-related transcriptional alterations, relevant for understanding its role in EC biology, and as therapeutic target.

We included 825 EC patients with available ploidy status and comprehensive clinicopathologic characterization to analyze ploidy as a prognostic marker. For 144 patients, gene expression data were available to explore aneuploidy-related transcriptional alterations.

Aneuploidy was associated with high age, FIGO stage and grade, non-endometrioid histology, ER/PR negativity, and poor survival (p-values<0.001). In patients with ER/PR negative tumors, aneuploidy independently predicted poor survival (p=0.03), lymph node metastasis (p=0.007) and recurrence (p=0.002). A prognostic ‘aneuploidy signature’, linked to low expression of chromosome 15q genes, was identified and validated in TCGA data.

In conclusion, aneuploidy adds prognostic information in ER/PR negative EC, identifying high-risk patients that could benefit from more aggressive therapies. The ‘aneuploidy signature’ equally identifies these aggressive tumors and suggests a link between aneuploidy and low expression of 15q genes. Integrated analyses point at various dysregulated pathways in aneuploid EC, underlining a complex biology.
INTRODUCTION

Aneuploidy, defined as an aberrant number of chromosomes, is a commonly observed feature in human cancers [1], including endometrial cancer (EC) [2]. Aneuploidy is often evaluated in tumors as an indirect measure of chromosomal instability, and used as an indicator of poor outcome [3]. It has been among the most widely studied biomarkers in EC since it was first introduced in the eighties [4–7], and is of particular interest since it can be measured preoperatively and hence used to guide treatment decisions [8–10]. Ploidy status estimated by cytometric methods [11] has added prognostic information in several retrospective studies of EC, but has never been fully implemented in clinical treatment algorithms [12]. This is at least in part due to the lack of one common standardized method for measuring ploidy status in tumors [3]. In addition, its clinical usefulness as prognostic marker, adjusted for clinical and histopathologic variables, lacks validation in large prospective patient cohorts [13].

Aneuploidy is suggested to arise through a few major mechanisms, including mitotic checkpoint defects, centrosome over-duplication and defect sister chromatid cohesion [14], for instance by mutational loss of the cohesin subunit STAG2 [15]. However, the exact role of aneuploidy in tumor development and progression remains incompletely understood, and no single causative driver has been identified [16]. For EC, the prognostic impact of aneuploidy has been studied to a large extent, but associated transcriptional alterations have been much less explored. This is relevant for identifying shared molecular traits among aneuploid endometrial tumors, and hence to understand more about underlying biologic mechanisms in aggressive EC with possible relevance for new targeted therapies.

We therefore evaluated flow cytometry assessed DNA ploidy status in a large well-annotated EC patient cohort with long and complete follow-up, and demonstrated clear associations between aneuploidy and markers of poor outcome. Further, we examined transcriptional alterations reflecting ploidy status in primary EC lesions, revealing a prognostic ‘aneuploidy signature’ linked to low expression of chromosome 15q genes, and shedding light on biologic mechanisms accompanying aneuploidy in EC.

RESULTS

Aneuploidy associates with markers for aggressive endometrial cancer

Of the 825 tumor samples with flow cytometry estimated ploidy status available, 638 were diploid (77%) and 187 aneuploid (23%). Example DNA histograms are shown in Supplementary Figure 1. Aneuploidy was significantly associated with well-established prognostic variables, including high age, FIGO stage and grade, non-endometrioid histology, and estrogen receptor and progesterone receptor (ER/PR) negativity (Table 1). The proportion of diploid and aneuploid tumors according to histologic subtype is shown in Figure 1. The frequency of aneuploid tumors was 38% among patients who later suffered recurrence and 42% in patients with metastasis at primary diagnosis, compared to 17% for patients without signs of systemic or recurrent disease (p<0.001).

Aneuploidy associates with reduced survival

In univariate survival analysis (Kaplan-Meier), the 5-year disease specific survival (DSS) for patients with diploid tumors was 89%, versus 68% for patients with aneuploid tumors (p<0.001, Figure 2A). Subgroup analyses confirmed aneuploidy as a significant marker for shorter survival in patients with FIGO stage 1 (Figure 2B), as well as endometrioid and non-endometrioid tumors separately (Figure 2C and 2D). We also explored to what extent ploidy adds prognostic information to endometrioid and non-endometrioid subgroups with known ER/PR status. In non-endometrioid tumors with positive ER/PR status (n=52), ploidy did not show any prognostic impact in univariate analysis (p=0.800, data not shown). However, ploidy significantly affected outcome in non-endometrioid hormone receptor negative tumors (n=62), with 5-year DSS of 63% for patients with diploid versus 35% for aneuploid tumors (p=0.010, Figure 2D).

Lymphadenectomy is advocated as staging procedure, and is documented to improve prognostication, however without effect on survival in randomized trials [17, 18]. We therefore explored the prognostic impact of ploidy compared to ER/PR for three subgroups: patients with lymph node metastases, without lymph node metastases, and where lymphadenectomy was not performed. For patients with lymph node metastasis, ER/PR performed better than ploidy status (Supplementary Figure 2A and 2B). In the other two groups, ER/PR status and ploidy added similar prognostic information in univariate survival analyses (Supplementary Figure 2C-2F).

In multivariate analysis, ploidy maintained independent prognostic impact on DSS, adjusting for the commonly applied standard prognostic markers: age, FIGO stage, histologic subtype and grade; with a hazard ratio (HR) of 1.62 for aneuploid tumors (95% confidence interval (CI) 1.11 – 2.37, p=0.013 (Supplementary Table 1A). Due to a detected significant interaction between ER/PR and ploidy status (HR 3.03, 95% CI 1.23 – 7.42, p=0.016), the two variables were not included simultaneously in the multivariate model. Replacing ploidy with ER/PR status in the Cox model, a similar HR of 1.63 (95% CI 1.16 – 2.29, p=0.005) for ER/PR negativity was observed (Supplementary Table 1B). In subsequent Cox analyses stratified for ER/PR status, aneuploidy independently predicted poor outcome in the
Table 1: Associations between clinicopathologic factors and DNA ploidy status by flow cytometry in 825 endometrial carcinomas

<table>
<thead>
<tr>
<th></th>
<th>Diploid, n (%)</th>
<th>Aneuploid, n (%)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, quartiles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;58</td>
<td>178 (91)</td>
<td>18 (9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>58 – 66</td>
<td>171 (83)</td>
<td>35 (17)</td>
<td></td>
</tr>
<tr>
<td>66 – 75</td>
<td>145 (69)</td>
<td>66 (31)</td>
<td></td>
</tr>
<tr>
<td>≥ 75</td>
<td>144 (68)</td>
<td>68 (32)</td>
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<td><strong>Histologic type and grade</strong></td>
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<td>&lt;0.001</td>
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<tr>
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<td>467 (87)</td>
<td>71 (13)</td>
<td></td>
</tr>
<tr>
<td>Endometrioid, grade 3</td>
<td>75 (63)</td>
<td>44 (37)</td>
<td></td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>76 (51)</td>
<td>72 (49)</td>
<td></td>
</tr>
<tr>
<td><strong>FIGO stage</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage I</td>
<td>502 (81)</td>
<td>121 (19)</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>54 (74)</td>
<td>19 (26)</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>62 (66)</td>
<td>32 (34)</td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>20 (57)</td>
<td>15 (43)</td>
<td></td>
</tr>
<tr>
<td><strong>ER/PR status</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ER and/or PR positive</td>
<td>389 (82)</td>
<td>84 (18)</td>
<td></td>
</tr>
<tr>
<td>ER and PR negative</td>
<td>72 (61)</td>
<td>46 (39)</td>
<td></td>
</tr>
</tbody>
</table>

n=number of patients in each category; *: Pearson χ²-test; ⊵: Data missing for 20 patients; ⊵: Data missing for 234 patients

receptor negative group only, with HR 2.11 (95% CI 1.08 – 4.15, p=0.029) (Table 2A and 2B).

Aneuploidy independently predicts lymph node metastasis and recurrence

Ploidy status was analyzed for its ability to predict lymph node metastasis and recurrence in binary logistic regression models adjusting for histologic subtype and grade. Due to the previously detected interaction, analyses were stratified for ER/PR status. In the group with ER/PR negative status, aneuploidy independently predicted recurrence (n=96), with odds ratio (OR) 4.67 (95% CI 1.78 – 12.27, p=0.002; Supplementary Table 2A), and lymph node metastasis (n=76) with OR 5.47 (95% CI 1.58 – 18.99, p=0.007, Supplementary Table 2B). Thus, ploidy assessment may be a useful additional biomarker especially in ER/PR negative tumors, identifying high-risk patients that could benefit from further systemic treatment.

Aneuploidy and its phenotype is reflected in a nine-gene ‘aneuploidy signature’

Significance analysis of microarray (SAM) was applied to assess aneuploidy related transcriptional alterations. Further, the machine learning algorithm support vector machine (SVM) was applied on the ranked SAM-list in order to identify the genes best discriminating between diploid and aneuploid samples. The nine top ranked genes from SAM were identified as the best discriminators. Of these, six were down- and three were upregulated (Supplementary Table 3). Unsupervised hierarchical clustering of the nine genes identified two patient clusters (Figure 3A). As expected, the ‘aneuploid cluster’ (n=46) reflected more aggressive clinical behavior, comprising the majority (83%) of flow cytometry aneuploid tumors, whereas the ‘diploid cluster’ (n=98) reflected less aggressive clinical features, dominated by flow cytometry diploid tumors (95%). Patients in the ‘aneuploid cluster’ had significantly worse survival than patients in the ‘diploid cluster’ (p=0.006, Figure 3B). An ‘aneuploidy score’ was calculated from expression values of the nine genes [19]. Similar to flow cytometry-assessed aneuploidy, a high score was significantly associated with all markers of aggressive disease (Supplementary Table 4), and reduced survival (p=0.001, Figure 3C). Patients with flow cytometry aneuploid tumors had higher ‘aneuploidy scores’ than patients with flow cytometry diploid tumors (p=0.001, Supplementary Figure 3A). Interestingly, patients with flow-cytometry diploid/
gene cluster aneuploid tumors had significantly higher ‘aneuploidy scores’ than patients with flow cytometry-assessed aneuploid/gene cluster diploid tumors (p<0.001, Supplementary Figure 3B). The score increased from premalignant through low grade malignant to high-grade malignant lesions. However, it did not increase further in metastatic lesions (Supplementary Figure 3C).

To further validate the link between the ‘aneuploidy score’, tumor aneuploidy and survival, the score was calculated from TCGA RNAseq data for 338 EC patients with ploidy status estimated by the ABSOLUTE algorithm [20]. We found a similar pattern for cluster formation based on the nine genes: patients segregated into two distinct clusters, one ‘aneuploid cluster’ including 41% of the tumors, associated with a more aggressive phenotype (high FIGO stage, p=0.04, high histologic grade and high stage, both p<0.001), and one ‘diploid cluster’ including tumors with less aggressive features. High ‘aneuploidy score’ also reflected reduced survival in this validation series (Supplementary Figure 4). The larger proportion of tumors in the TCGA ‘aneuploid cluster’ compared to our data set may reflect the TCGA strategy to enrich for more aggressive subtypes in the endometrial cancer series.

Since the proportion of aneuploid tumors differs significantly between histologic subtypes (Figure 1), we also explored the ‘aneuploidy signature’ in endometrioid and non-endometrioid tumors separately. The results for the endometrioid group were similar to those of the whole series, but more difficult to interpret in the non-endometrioid group with small sample size (n=28) (Supplementary Figure 5A-5B).

**Figure 1:** Ploidy status in histologic subtypes of endometrial cancer. Schematic overview of ploidy status (by flow cytometry) according to histologic subtype for 825 patients. n=number of patients in each category (percent).

**Figure 2:** Survival according to ploidy status. Kaplan-Meier curves showing DSS according to ploidy status, for all histologic subtypes A., FIGO stage 1 tumors B., endometrioid histologic type C., non-endometrioid histologic type D., and ER/PR negative non-endometrioid histologic type E. For each category the number of cases is given, followed by number of deaths.

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Oncotarget
Table 2: Prognostic impact of ploidy status by flow cytometry adjusted for standard clinicopathologic variables, stratified for ER/PR expression (Cox regression model)

A. Survival analysis of patients with ER and/or PR positive tumors (n=470)

<table>
<thead>
<tr>
<th></th>
<th>Patients, n (%)</th>
<th>Unadjusted HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>Adjusted HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td><strong>Age</strong></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>470 (100)</td>
<td>1.05</td>
<td>1.03 - 1.08</td>
<td>&lt;0.001</td>
<td>1.04</td>
<td>1.02 - 1.07</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Histologic type and grade</strong></td>
<td></td>
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</tr>
<tr>
<td>Endometrioid grade 1-2</td>
<td>356 (76)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid grade 3</td>
<td>62 (13)</td>
<td>4.57</td>
<td>2.41 - 8.66</td>
<td>2.81</td>
<td>1.44 - 5.47</td>
<td></td>
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</tr>
<tr>
<td>Non-endometrioid</td>
<td>52 (11)</td>
<td>9.08</td>
<td>4.75 - 17.34</td>
<td>4.51</td>
<td>2.16 - 9.43</td>
<td></td>
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<tr>
<td><strong>FIGO stage</strong></td>
<td></td>
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<tr>
<td>Stage I – II</td>
<td>409 (87)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Stage III – IV</td>
<td>61 (13)</td>
<td>11.53</td>
<td>6.80 - 19.56</td>
<td>8.97</td>
<td>5.12 - 15.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ploidy status</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>386 (82)</td>
<td></td>
<td></td>
<td>0.053</td>
<td>0.666</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneuploid</td>
<td>84 (18)</td>
<td>1.80</td>
<td>0.99 - 3.24</td>
<td>0.87</td>
<td>0.45 - 1.66</td>
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</tr>
</tbody>
</table>

B. Survival analysis of patients with ER and PR negative tumors (n=117)

<table>
<thead>
<tr>
<th></th>
<th>Patients, n (%)</th>
<th>Unadjusted HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>Adjusted HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
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<tr>
<td></td>
<td>117 (100)</td>
<td>1.04</td>
<td>1.01 - 1.08</td>
<td>0.013</td>
<td>1.04</td>
<td>1.00 - 1.08</td>
<td>0.082</td>
</tr>
<tr>
<td><strong>Histologic type and grade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid grade 1-2</td>
<td>27 (23)</td>
<td></td>
<td></td>
<td>0.009</td>
<td>0.184</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid grade 3</td>
<td>28 (24)</td>
<td>0.87</td>
<td>0.27 - 2.85</td>
<td>1.02</td>
<td>0.31 - 3.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>62 (53)</td>
<td>2.82</td>
<td>1.16 - 6.84</td>
<td>1.99</td>
<td>0.81 - 4.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FIGO stage</strong></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage I – II</td>
<td>78 (67)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage III – IV</td>
<td>39 (33)</td>
<td>6.66</td>
<td>3.39 - 13.07</td>
<td>5.56</td>
<td>2.77 - 11.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ploidy status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>71 (61)</td>
<td></td>
<td></td>
<td>0.001</td>
<td>0.029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneuploid</td>
<td>46 (39)</td>
<td>3.06</td>
<td>1.61 - 5.82</td>
<td>2.11</td>
<td>1.08 - 4.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n=number of patients; HR: Hazard Ratio; CI: Confidence interval

Aneuploidy is related to low expression of genes on chromosome 15q

Interestingly, all six downregulated genes in the ‘aneuploidy signature’ (i.e. low expression in aneuploid compared to diploid samples) were located on the q-arm of chromosome 15 (Figure 3D, Supplementary Table 3). To explore whether loss of 15q regions is a general feature of endometrial cancers, we assessed copy number data for endometrial cancer by the TCGA copy number portal [21, 22], without accounting for ploidy status. 79 peak regions of deletion were identified, of which three located to chromosome 15q. Two of these regions included four of the aneuploidy signature genes: C13orf57 and NDUFAL1 in one peak region (residual q-value 3.44 x 10^-3) and WVA9 and RPL4 in another peak region (residual q-value 0.0057). Further, we investigated the publicly accessible cBioportal for copy number alterations of the six genes in relation to mRNA expression level. For all six genes, a proportion of patients had deletion, with correspondingly lower mRNA expression levels compared to patients with normal gene copy number (Supplementary Figure 6). Gene Set Enrichment Analysis (GSEA) with MSigDB c1 positional gene sets, where each gene set corresponds to a cytoband on a human chromosome, showed that nine of the top 30 gene sets
enriched in diploid tumors (FDR<25%), were located on chromosome 15q (Supplementary Table 5A). The same analysis was performed in subgroups of endometrioid and non-endometrioid tumors separately, with consistent results (Supplementary Figure 5C). These findings support the argument that aneuploidy is associated with reduced expression of genes on chromosome 15q, possibly due to deletion of chromosomal regions.

Integrated analyses of aneuploid tumors suggest the involvement of a variety of biological mechanisms and potential drug targets

GSEA identified gene sets related to a wide range of tumorigenic pathways and processes, including cell cycle regulation, cell proliferation and protein transcription as significantly enriched in aneuploid tumors. Notably, several gene sets related to glycolysis were enriched in aneuploid samples. Also, gene sets related to increased expression of known oncogenes including E2F, KRA5 and MYC were frequently upregulated, as well as gene sets related to pluripotency, telomere maintenance and longevity (Supplementary Table 5B). These results suggest that a variety of biological mechanisms may be important in aneuploid tumors, further supported by connectivity map analysis. The 15 top-ranked compounds with negative enrichment score showed a large diversity of drugs potentially relevant for targeting aneuploid tumors (Supplementary Table 6).

Investigation of aneuploidy-related biomarker potential by STAG2 and PPP2R3A

Since mutational STAG2 inactivation may be involved in aneuploidy development [15], we investigated STAG2 protein expression by IHC as a potential marker for aneuploidy. No association was found between nuclear STAG2 expression and ploidy status, other prognostic markers except ER status, or survival (Supplementary Table 7, Supplementary Figure 7A-7C). Further, since PP2A complex alterations have been linked to whole genome doubling [21] and PPP2R3A expression was upregulated in the ‘aneuploidy signature’, we also evaluated PPP2R3A protein expression by IHC as a potential aneuploidy marker. No association between PPP2R3A expression and ploidy status, clinicopathologic variables or survival was observed (Supplementary Table 7, Supplementary Figure 7D-7F).

DISCUSSION

Despite extensive support for ploidy as a prognostic factor in endometrial cancer [4-7, 23-25], ploidy assessment is not implemented in routine clinical practice. The independent prognostic impact of aneuploidy, after adjusting for histopathologic parameters and surgical staging, is still uncertain, as all these parameters were often not included in earlier studies [4-7, 23-25]. Likewise, the prognostic impact

Figure 3: ‘Aneuploidy signature’. Formation of diploid and aneuploid clusters based on the ‘aneuploidy signature’, related to flow cytometry ploidy status, FIGO stage, grade, histologic subtype, and ER/PR status. Unsupervised hierarchical clustering of 144 samples A. DSS for patients segregating within the ‘diploid’ compared to the ‘aneuploidy cluster’ B, and for patients with low versus high ‘aneuploidy scores’ C. Schematic mapping of the six downregulated ‘aneuploidy signature’ genes, located on chromosome 15q D.
of hormone receptor status in endometrial tumor tissue is well documented [26–28], and easily assessed by immunohistochemistry, however not applied in the routine diagnostic setting either. Nevertheless, the prognostic information derived from ploidy assessment has rarely been compared to hormone receptor status [29], a gap we have tried to fill. In multivariate survival models, ploidy status and ER/PR status contributed similar independent prognostic information based on adjusted hazard ratios. Interaction between ploidy status and ER/PR status was observed in multivariate survival and binary logistic regression analysis, and importantly, aneuploidy further improved the prediction of prognosis, lymph node metastasis and recurrence in tumors with loss of both receptors, all novel observations not previously reported. To our knowledge, interaction between ploidy status and hormone receptor status has never been reported before, and the biological underpinnings of this interesting phenomenon should be further explored in future studies. Also, in this large patient series with a substantial proportion subjected to staging lymphadenectomy, we demonstrated clear prognostic impact of ploidy status, especially in the group of patients where staging lymphadenectomy was not performed. These findings could have potential clinical impact, especially for identification of high-risk patients with need for further adjuvant therapy and closer follow-up.

There are likely numerous causes and consequences of aneuploidy [30]. We aimed to describe transcriptional traits characterizing aneuploid tumors to further explore mechanisms involved in aneuploidy development and/or maintenance in EC, and potentially reveal drugs of interest for further study. In this context, we are only aware of one differently designed study of 33 EC patients [31]. By GSEA, we identified gene sets related to cell cycle regulation, proliferation and transcription to be important in aneuploid tumors, similar to Habermann et al.’s findings using ingenuity pathway analysis. GSEA also identified gene sets related to a range of known oncogenes, including E2F, MYC and KRAS, as well as gene sets linked to cell longevity and pluripotency. This supports the argument that diverse pathways are dysregulated in aneuploid tumors. Connectivity Map analysis pointed towards a wide variety of drugs rather than one specific drug class, again supporting a complex biology underlying aneuploidy in EC, that might require diverse targeting approaches.

Previous attempts to characterize aneuploidy related transcriptional alterations in preclinical models have identified genes related to cellular stress response, response to reactive oxygen species and activated glycolysis as commonly upregulated in aneuploid cells [32, 33]. In line with this, gene sets related to glycolysis were identified as enriched in aneuploid tumors by GSEA. The upregulated ‘aneuploidy signature’ gene PGAM2 (phosphoglycerate mutase 2), encodes a glycolytic enzyme whose activity increases in response to oxidative stress [34]. Increased PGAM2 level may indicate a link between oxidative stress, increased glycolysis and aneuploidy in EC, although this association needs to be further elucidated. Also, in a study of colorectal cancer [35], a cancer type sharing several molecular traits with EC [36–38], copy number alterations with correlated expression changes (including 15q loss), affected the activity of the oxidative phosphorylation pathway [35]. Thus, a shift towards anaerobic glycolysis seems tentatively linked to aneuploidy across comparable tumor types. This should be further explored in particular for the development of new therapeutics.

PPP2R3A, encoding a subunit of the protein phosphatase 2A (PP2A) complex [39], was also upregulated in the ‘aneuploidy signature’, although we found no association between PPP2R3A expression level by IHC and ploidy status. PP2A complex subunits seem to be frequent targets in EC. PPP2R1A mutations often co-occur with TP53 mutations, a marker for the serous-like subgroup with increased copy number, and are frequently seen in metastatic EC [40]. In addition, PPP2R1A and PPP2R2A alterations have been associated with whole genome doubling in a pan-cancer study of TCGA data [21]. Further studies are needed to understand the exact role of the PP2A complex in relation to aneuploidy, of particular interest since the PP2A complex might be targetable [39, 41].

Our ‘aneuploidy signature’ based on gene expression data seems to perform equally well as ploidy status estimation by flow cytometry for prediction of aggressive tumors, and its prognostic value was confirmed in an independent TCGA data set (n=338), also in the subgroup of endometrioid tumors. To our knowledge, this is the first study demonstrating a link between aneuploidy related gene signature, clinicopathologic variables and survival in EC. The score was not associated with survival in subgroup analysis of the non-endometrioid cases, possibly because of the small group size, but potentially also due to more heterogeneous expression patterns in non-endometrioid tumors [36].

Further, the signature revealed an association between aneuploidy and low expression level of chromosome 15q genes, a finding that persisted in subgroup analysis of endometrioid and non-endometrioid tumors by GSEA. TCGA data supported that the observed low expression level of 15q signature genes could be related to altered gene dosage, i.e. deletion. In line with this, Habermann et al. identified 15q loss as a frequent phenomenon in aneuploid EC, both within the endometrioid (11% loss) and non-endometrioid (50% loss) subgroups [31]. Also, in the previously mentioned study by Sheller et al., aneuploid colorectal tumors had a high frequency of 15q deletions and correspondingly low 15q gene expression level. Patients with 15q loss also had significantly reduced survival and higher disease
stage [35], in line with our findings. Whether this is an aneuploidy associated feature across different tumor types remains to be investigated.

In summary, we have shown that aneuploidy is associated with markers of aggressive endometrial cancer. Aneuploidy independently predicts poor survival adjusted for the commonly applied standard prognostic variables, and identifies patients with high risk of recurrence, lymph node metastasis and poor survival in hormone receptor negative tumors. Thus, aneuploidy should be further explored as a marker to identify patients who could potentially benefit from more aggressive surgical and adjuvant treatment. Aneuploidy associated transcriptional changes point to a complex underlying biological background, and reflects therapeutic challenges in targeting aneuploidy. However, a link towards increased glycolysis in aneuploid tumors is observed, and should be further explored. Our suggested ‘aneuploidy’ signature, linking aneuploidy with low expression of chromosome 15q genes, equally identifies patients with aggressive disease and poor survival, and could provide an alternative method for ploidy status estimation in future diagnostics.

MATERIALS AND METHODS

Patient series and tumor samples

The patient population consisted of 1621 women treated for EC at Haukeland University Hospital, Norway, between 1981 and 2015, thoroughly described previously [42]. Approximately half of the patients had flow cytometry assessed ploidy status available, and were included for further analyses (n=825). Data from 363 patients have been published previously [7]. For a subset of the patients, fresh frozen tissue (n=144) and tissue microarrays (TMA, n=526) were available for biomarker studies. Fresh frozen tissue was also available for 30 primary tumors, 18 complex atypical hyperplasias and 42 metastatic lesions without estimated ploidy status [27], used for extended evaluation of our ‘aneuploidy signature’. Estrogen and progesterone receptor (ER/PR) data was available for 1038 patients, 591 with overlapping ploidy status. Clinicopathologic and follow-up data were collected by review of the medical records, and if needed by additional correspondence with the primary physicians and referring hospitals. 92% underwent primary surgical treatment (at least hysterectomy with bilateral salpingo-oophorectomy; HBSO). Details regarding lymphadenectomy routines at different time periods have been described previously [42]. Disease stage was classified according to the FIGO 2009 criteria [43]; cases included prior to 2009 were reclassified. If HBSO was contraindicated, staging was based on curettage results, clinical examination, and preoperative imaging. Follow-up time was defined as the interval between the date of primary treatment and the date of last follow-up/death.

Median follow-up time of survivors was 5.02 years (range 0.04 to 23.2); last follow-up was September 1st, 2015. The local Institutional Review Board approved the study (IRB-number 2009-2315).

DNA ploidy analysis

Fresh tissue for DNA ploidy analysis was collected during surgery from an area judged macroscopically representative for the tumor. If HBSO was not performed, ploidy analyses were performed on tissue obtained from palliative surgery (n=7), or curettage (n=6). Tumor tissue was rinsed in phosphate-buffered saline, followed by ethanol fixation. DNA ploidy was analyzed by flow-cytometry, according to previously described protocols [4, 7]. In selected analyses, tetraploid (n=8) and triploid tumors (n=1) were classified together with the aneuploid tumors, as numbers were too low for separate analyses. These nine tumors did not have microarray data and are therefore not included when determining the 9-gene signature described in subsequent sections.

Expression microarray and data analysis

RNA extracted from fresh frozen tumor tissue was hybridized to Agilent Whole Human Genome Microarray Kit, 44k (catalogue number G4 112F), according to the manufacturers instruction (www.agilent.com) and as described previously [28, 44]. Arrays were scanned using the Agilent Microarray Scanner Bundle. The software J-express (www.molmine.com) [45] was used for microarray analyses. Median spot intensity was used to define the intensity signal, and expression data were quantile normalized and log2-transformed. To identify differentially expressed genes between two groups, significance analysis of microarray (SAM) [46] was performed. For unsupervised hierarchical clustering we used additionally mean scaled expression values, with complete linkage and Pearson correlation as similarity measures.

Aneuploidy signature: To identify the minimal set of genes providing the highest discriminatory power between diploid (n=113, 78%) and aneuploid (n=31, 22%) samples, the machine learning method support vector machine (SVM), with 10-fold cross validation, was used on the ranked SAM-list. The method was implemented in R, using the Classification for Microarray package [47]. The genes discriminating between diploid and aneuploid samples with highest accuracy (n=9) were selected. From these genes, a gene signature score was calculated from mean and variance scaled expression values, subtracting the sum of expression values of downregulated genes from the sum of expression values of upregulated genes (in aneuploid compared to diploid samples) [19]. Upper quartile was used as cut-off to separate high and low scores in two groups. One of the identified signature
genes; THC252764 (Agilent probe A_24_P9140887, RPL4-variant Q59GY2) was not present in the TCGA validation dataset. Since the probe also targets the RPL4 gene, data for RPL4 was included in the validation of the signature.

Connectivity map: A connectivity map analysis (www.broadinstitute.org/cmap) [48] was performed to identify compounds generating signatures anti-correlated to the gene list separating aneuploid from diploid tumors. The input signature (SAM, FDR=0 and fold change ±1.5 as cut-off) consisted of 287 differentially expressed genes (204 up- and 83 down-regulated).

GSEA: Gene set enrichment analysis (GSEA) (www.broadinstitute.org/gsea) [49], was performed using the Molecular Signatures Database (MSigDB version 5.0, www.broadinstitute.org/gsea/msigdb) datasets c1 (positional gene sets), c2 (curated gene sets), c6 (oncogenic gene sets) and Hallmark gene sets.

Analyses of the cancer genome atlas (TCGA) data

TCGA endometrial cancer level 3 data for mRNA expression estimated by RNAseq (IlluminaGA_RNAseqV2, downloaded November 20th 2014), and clinical data (downloaded November 3rd 2014, https://tcga-data.nci.nih.gov/tcga) were used for external validation. The ABSOLUTE algorithm was applied to estimate ploidy status for TCGA samples [20]. Cut-off value for diploid tumors was set to 2±0.05; samples with values outside this interval were considered polyploid. The cBioPortal for cancer genomics [50, 51] and the copy number portal TCGA Tumorscape [21, 22] were applied to assess copy number status for 539 TCGA EC samples without accounting for ploidy status.

Immunohistochemistry (IHC)

The staining procedure and evaluation for ER and PR on tissue microarrays (TMA) has been described previously [26, 27, 52]. The TMA method has also been described and validated previously [53, 54]. ER and PR status were dichotomized in two categories for binomial analyses: positive ER and/or PR status versus negative ER and PR status by IHC staining. IHC of candidate aneuploidy markers STAG2 and PPP2R3A was performed on TMAs for a subset of 526 and 281 patients, respectively. TMAs were sectioned (5 μm) for immunohistochemical staining: After 20 minutes boiling at pH 9 and peroxidase blocking (S2001 Dako, Denmark), slides were incubated for 60 minutes with STAG2 mouse monoclonal antibody SA-2 (J-12): sc-81852 (Santa Cruz Biotechnology, USA) at 1:500 dilution, or PPP2R3A rabbit polyclonal antibody HPA035829 (Sigma-Aldrich, USA), at 1:100 dilution. Secondary antibody EnVision mouse (labelled polymer-HRP anti-mouse, K4007 Dako, Denmark) was applied for STAG2, and EnVision rabbit (labelled polymer-HRP anti-rabbit, K4003 Dako, Denmark) for PPP2R3A. Dab+Substrate Chromogen System (K3468 Dako, Denmark) was added, and slides counter-stained with hematoxylin. Staining index (SI) was calculated as the product of the area of staining, graded from 0-3, and the intensity of the staining, graded from 0-3, as previously described [55, 56]. For STAG2, nuclear staining was registered, and lower quartile (SI 0-1) defined as low expression. For PPP2R3A, cytoplasmic staining was registered, and upper quartile (SI 6-9) defined as high expression.

Statistical analyses

Statistical analyses were performed using SPSS (Statistical Package of Social Sciences), version 23.0 (IBM SPSS Statistics, Armonk, NY, USA: IBM Corp, 2015). Associations between categorical variables were assessed by Pearson Chi-square test (Fisher’s exact test when appropriate). To compare the distribution of a continuous variable between two or more groups, Mann-Whitney U-test and Kruskal-Wallis test were applied, respectively. Univariate survival analyses were performed by the Kaplan-Meier method, assessing survival differences between groups by the two-sided log-rank test (Mantel-Cox). To determine the optimal cut-off levels for high and low ‘aneuploidy scores’, STAG2 expression level and PPP2R3A expression level, the Kaplan-Meier curves for the variables ranked by tertiles, quartiles and quintiles were examined visually, and the categories showing largest survival differences were chosen. For multivariate survival analyses, the Cox Proportional Hazards Regression Model was used, after visual assessment of included variables by a log-minus-log plot to check the proportional hazards assumption. For the survival analyses, disease specific survival (DSS) was defined as primary endpoint, except in TCGA data, where overall survival (OS) was used. Binary logistic regression analysis was performed for prediction of recurrence and lymph node metastasis. A p-value below 0.05 was set as threshold significance level for all the statistical analyses.

ACKNOWLEDGMENTS

We would like to dedicate this manuscript to Helga Birgitte Salvesen, who unexpectedly, and way too early, passed away in January 2016. She was a dedicated physician and cancer researcher, and a warm and inspiring supervisor, colleague and friend. We all miss her.

The authors would like to thank Bendik Nordanger, Britt Edvardsen, Ellen Valen, Gerd Lillian Halseth, Hua My Hoang, Kadri Madisoo and Reidun Kopperud for technical assistance.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.
FUNDING

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Aneuploidy related transcriptional changes in endometrial cancer link low expression of chromosome 15q genes to poor survival

SUPPLEMENTARY FIGURES AND TABLES

Supplementary Figure 1: DNA histograms from diploid and aneuploid tumors. Example histograms from flow cytometric assessment of DNA ploidy status. X-axis: emitted fluorescent light from labeled DNA, Y-axis: cell count. Panels A and B. Histograms from diploid tumors, showing a major peak (P1) representing cells with a diploid DNA content, and a minor peak (P2) representing cells in M-phase of cell cycle. Panels C and D. Histograms from aneuploid tumors, showing multiple peaks representative for cell subpopulations with a non-diploid DNA content.
Supplementary Figure 2: Comparison of prognostic value of ploidy and ER/PR status. Survival according to flow cytometry assessed ploidy status compared to ER/PR status in patients with lymph node metastasis (upper panel), no lymph node metastasis (mid panel) and no lymphadenectomy (uncertain lymph node status, lower panel). A, C and E. Diploid versus aneuploid tumors. B, D and F. ER and/or PR positive versus ER and PR negative status.
Supplementary Figure 3: ‘Aneuploidy signature’ score distribution. ‘Aneuploidy signature’ score for diploid and aneuploid tumors by flow cytometry A, four groups based on ploidy status by flow cytometry and cluster formation: concordant diploid/aneuploid versus discordant diploid/aneuploid status B, and complex atypical hyperplasia (CAH), endometrioid tumors grade 1-2 (EG1-2), endometrioid tumors grade 3 (EG3), non-endometrioid tumors (NE) and metastases (MET) C. n=number of patients in each group. P-values represent Mann Whitney (A) and Kruskall Wallis (B and C) tests for significance of differences between groups.
Supplementary Figure 4: ‘Aneuploidy signature’ validation in TCGA data. Cluster formation based on ‘aneuploidy signature’ related to ploidy status estimated by ABSOLUTE (Carter et al, 2012), FIGO stage, grade and histological subtype in a validation data set of 338 TCGA EC samples. Unsupervised hierarchical clustering reveals a similar pattern as demonstrated in Figure 2 A. OS for patients segregating within the ‘diploid’ compared to the ‘aneuploid cluster’ B, for patients with low compared to high ‘aneuploidy score’ C, and for patients with diploid compared to aneuploid tumors by ABSOLUTE D.
Supplementary Figure 5: Summary of subgroup analysis of ‘aneuploidy signature’ in endometrioid and non-endometrioid tumors. Top panel A. represents heatmap of p-values from assessment of differential distribution of ‘aneuploidy score’ according to clinicopathologic variables in this study data set and TCGA data (Mann-Whitney U test). n=number of patients in each category. P-values are colored according to whether the score shows similar distribution pattern in subgroup analysis as when all histologic subtypes were examined together (green); or opposite distribution pattern (red). Mid panel B. shows survival according to aneuploidy score for endometrioid and non-endometrioid tumors separately, in this study data set and in TCGA data. Lower panel C. shows results from GSEA analyses on this study data set. Chromosome 15q related gene sets with p<0.05 and FDR<25% are represented.
Supplementary Figure 6: mRNA expression level and corresponding copy number for chromosome 15q genes. mRNA expression level in relation to copy number analysis of the six downregulated ‘aneuploidy signature’ genes for 539 TCGA EC samples. ‘Deep deletion’ indicates possibly homozygous deletion, ‘Shallow deletion’ indicates possibly heterozygous deletion. Likewise, ‘Gain’ indicates low level gain, ‘Amplification’ indicates high level amplification, as determined by the GISTIC algorithm (Beroukhim et al, 2007). Figure adapted from www.cBioportal.org (Cerami et al, 2012; Gao et al, 2013).
Supplementary Figure 7: Immunohistochemical assessment of suggested candidate aneuploidy markers STAG2 and PPP2R3A. Top left panel A. illustrates no survival difference according to STAG2 expression by IHC for 526 EC patients. Representative staining for low STAG2 expression (SI 0) B. and high STAG2 expression (SI 9) C. are shown. Top right panel D. illustrates no survival difference according to PPP2R3A expression by IHC for 281 EC patients. Representative staining for low PPP2R3A expression (SI 0) E. and high PPP2R3A expression (SI 9) F. are shown. Scale bar: 40 μm (B-C; E-F).
**Supplementary Table 1: Prognostic impact of ploidy status and ER/PR status adjusted for standard clinicopathologic variables (Cox regression model)**

**A. Survival analysis according to standard clinicopathologic variables and ploidy status by flow cytometry (n=804)**

<table>
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<tr>
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<th>Patients, n (%)</th>
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<th>Adjusted HR</th>
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<td>804 (100)</td>
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<td>1.04 - 1.07</td>
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<td>1.04</td>
<td>1.02 - 1.06</td>
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<tr>
<td>Endometrioid grade 3</td>
<td>119 (15)</td>
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<td>2.17</td>
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<td>Non-endometrioid</td>
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<td>3.06</td>
<td>1.95 - 4.81</td>
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<td><strong>FIGO stage</strong></td>
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<td>&lt;0.001</td>
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<tr>
<td>Stage I + II</td>
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<td>Stage III + IV</td>
<td>127 (16)</td>
<td>9.46</td>
<td>6.67 - 13.42</td>
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<td>7.10</td>
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<td>&lt;0.001</td>
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<td>Diploid</td>
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<tr>
<td>Aneuploid</td>
<td>187 (23)</td>
<td>2.91</td>
<td>2.05 - 4.13</td>
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<td>1.62</td>
<td>1.11 - 2.37</td>
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**B. Survival analysis according to standard clinicopathologic variables and ER/PR status (n=1025)**

<table>
<thead>
<tr>
<th></th>
<th>Patients, n (%)</th>
<th>Unadjusted HR</th>
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<th>p-value</th>
<th>Adjusted HR</th>
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<td></td>
<td>1025 (100)</td>
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<td>1.04 – 1.06</td>
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<td>1.04</td>
<td>1.02 – 1.05</td>
<td>&lt;0.001</td>
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<td>Endometrioid grade 1-2</td>
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<td>1.95 – 4.38</td>
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<td>1.73</td>
<td>1.14 – 2.63</td>
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<tr>
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<td>172 (17)</td>
<td>6.69</td>
<td>4.79 – 9.35</td>
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<td>2.76</td>
<td>1.86 – 4.09</td>
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<td><strong>FIGO stage</strong></td>
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<td>&lt;0.001</td>
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<td>Stage III + IV</td>
<td>169 (16)</td>
<td>9.18</td>
<td>6.82 – 12.38</td>
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<td>6.35</td>
<td>4.63 – 8.72</td>
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<td><strong>ER/PR status</strong></td>
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<td>ER and/or PR positive</td>
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<tr>
<td>ER and PR negative</td>
<td>197 (19)</td>
<td>3.77</td>
<td>2.79 – 5.08</td>
<td></td>
<td>1.63</td>
<td>1.16 – 2.29</td>
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*n=number of patients in each category; HR: Hasard Ratio; CI: Confidence Interval*
Supplementary Table 2: Binary logistic regression models for prediction of recurrence and lymph node metastasis

A. Prediction of recurrence for patients with ER/PR positive versus ER/PR negative tumors (n=542)

<table>
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<th>Adjusted OR</th>
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<th>p-value</th>
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<td>Endometrioid grade 3</td>
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<td>2.43</td>
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<td>2.38</td>
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<td>2.95 – 11.05</td>
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<td>5.51</td>
<td>2.74 – 11.06</td>
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<tr>
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<td>0.53</td>
<td>0.12 – 2.34</td>
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<td>1.78 – 12.27</td>
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B. Prediction of lymph node metastasis for patients with ER/PR positive versus ER/PR negative tumors (n=415)

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<td>1.58 – 18.99</td>
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n=number of patients in each category; OR: Odds Ratio; CI: Confidence Interval
Supplementary Table 3: Significance analysis of microarray (SAM) of 113 diploid versus 31 aneuploid endometrial tumors. Genes with FDR=0 and q-value <0.05 are listed

See Supplementary File 1
Supplementary Table 4: Distribution of ‘aneuploidy score’ for 144 endometrial cancer patients according to standard clinicopathologic variables

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<td>&lt; 66.2 years</td>
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<td>≥ 66.2 years</td>
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</tr>
<tr>
<td><strong>Ploidy status (by flow cytometry)</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diploid</td>
<td>113 (78)</td>
<td>-2.4</td>
<td></td>
</tr>
<tr>
<td>Aneuploid</td>
<td>31 (22)</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td><strong>ER/PR status</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ER and/or PR positive</td>
<td>115 (83)</td>
<td>-2.3</td>
<td></td>
</tr>
<tr>
<td>ER and PR negative</td>
<td>23 (17)</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

n=number of patients in each category; *: Mann-Whitney U-test for two categories, Kruskal-Wallis test for three categories; ^: Data missing for 1 patient; b: Data missing for 6 patients.
Supplementary Table 5: Gene set enrichment analysis for diploid (n=113) versus aneuploid (n=31) tumors
Gene sets with FDR<25% and p-value < 0.05 are listed

See Supplementary File 2
Supplementary Table 6: Drug signatures identified by Connectivity map as negatively correlated with gene list separating diploid and aneuploid samples (SAM, FDR=0, Fold Change ± 1.5)

<table>
<thead>
<tr>
<th>Rank</th>
<th>Drug name</th>
<th>n</th>
<th>Enrichment score</th>
<th>p-value</th>
<th>Drug target/action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adiphenine</td>
<td>5</td>
<td>-0.82</td>
<td>0.00046</td>
<td>Inhibitor of nicotinic receptors</td>
</tr>
<tr>
<td>2</td>
<td>Isoflupredone</td>
<td>3</td>
<td>-0.929</td>
<td>0.00052</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>3</td>
<td>Nadolol</td>
<td>4</td>
<td>-0.866</td>
<td>0.00062</td>
<td>Beta-blocker</td>
</tr>
<tr>
<td>4</td>
<td>Colistin</td>
<td>4</td>
<td>-0.843</td>
<td>0.00105</td>
<td>Polymyxin antibiotic</td>
</tr>
<tr>
<td>5</td>
<td>Haloperidol</td>
<td>32</td>
<td>-0.327</td>
<td>0.00143</td>
<td>Antipsychotic</td>
</tr>
<tr>
<td>6</td>
<td>Geldanamycin</td>
<td>15</td>
<td>-0.464</td>
<td>0.00184</td>
<td>Hsp90 inhibitor</td>
</tr>
<tr>
<td>7</td>
<td>Viomycin</td>
<td>4</td>
<td>-0.821</td>
<td>0.00195</td>
<td>Non-ribosomal peptide antibiotic</td>
</tr>
<tr>
<td>8</td>
<td>Wortmannin</td>
<td>18</td>
<td>-0.413</td>
<td>0.00308</td>
<td>PI3Kinase inhibitor</td>
</tr>
<tr>
<td>9</td>
<td>Genistein</td>
<td>17</td>
<td>-0.417</td>
<td>0.0036</td>
<td>Phytoestrogen, isoflavone</td>
</tr>
<tr>
<td>10</td>
<td>Maprotiline</td>
<td>4</td>
<td>-0.785</td>
<td>0.00426</td>
<td>Tetracyclic antidepressant</td>
</tr>
<tr>
<td>11</td>
<td>Levomepromazine</td>
<td>4</td>
<td>-0.78</td>
<td>0.00487</td>
<td>Phenotiazine, neuroleptic</td>
</tr>
<tr>
<td>12</td>
<td>Midodrine</td>
<td>5</td>
<td>-0.701</td>
<td>0.00519</td>
<td>Alpha1-receptor agonist</td>
</tr>
<tr>
<td>13</td>
<td>Dihydroergocristine</td>
<td>4</td>
<td>-0.769</td>
<td>0.00573</td>
<td>Ergot alkaloid</td>
</tr>
<tr>
<td>14</td>
<td>Carbimazole</td>
<td>3</td>
<td>-0.855</td>
<td>0.00603</td>
<td>Thyroid peroxidase inhibitor</td>
</tr>
<tr>
<td>15</td>
<td>Felbinac</td>
<td>4</td>
<td>-0.751</td>
<td>0.00774</td>
<td>NSAID</td>
</tr>
</tbody>
</table>

n=number of times the compound was tested in the Connectivity Map.
Supplementary Table 7: STAG2 and PPP2R3A expression estimated by immunohistochemistry (staining index, SI) in relation to standard clinicopathologic variables

<table>
<thead>
<tr>
<th></th>
<th>STAG2 expression</th>
<th></th>
<th>PPP2R3A expression</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low, n (%)</td>
<td>High, n (%)</td>
<td>p-value*</td>
<td>Low, n (%)</td>
</tr>
<tr>
<td>Age, quartiles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;58</td>
<td>29 (22)</td>
<td>102 (78)</td>
<td>0.71</td>
<td>45 (63)</td>
</tr>
<tr>
<td>58 – 66</td>
<td>33 (23)</td>
<td>108 (77)</td>
<td></td>
<td>51 (65)</td>
</tr>
<tr>
<td>66 – 75</td>
<td>27 (21)</td>
<td>103 (79)</td>
<td></td>
<td>39 (64)</td>
</tr>
<tr>
<td>≥ 75</td>
<td>22 (18)</td>
<td>102 (82)</td>
<td></td>
<td>51 (73)</td>
</tr>
<tr>
<td>Histologic subtype and grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid grade 1-2</td>
<td>82 (23)</td>
<td>271 (77)</td>
<td>0.15</td>
<td>129 (66)</td>
</tr>
<tr>
<td>Endometrioid grade 3</td>
<td>14 (20)</td>
<td>57 (80)</td>
<td></td>
<td>25 (62)</td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>14 (14)</td>
<td>84 (86)</td>
<td></td>
<td>29 (71)</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>85 (21)</td>
<td>318 (79)</td>
<td></td>
<td>149 (67)</td>
</tr>
<tr>
<td>Stage II</td>
<td>9 (25)</td>
<td>27 (75)</td>
<td></td>
<td>11 (61)</td>
</tr>
<tr>
<td>Stage III</td>
<td>13 (21)</td>
<td>49 (79)</td>
<td></td>
<td>17 (63)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>4 (16)</td>
<td>21 (84)</td>
<td></td>
<td>9 (60)</td>
</tr>
<tr>
<td>Ploidy status (flow cytometry)</td>
<td>0.43</td>
<td></td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>68 (24)</td>
<td>211 (76)</td>
<td></td>
<td>111 (70)</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>15 (20)</td>
<td>60 (80)</td>
<td></td>
<td>23 (70)</td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>ER positive</td>
<td>74 (19)</td>
<td>322 (81)</td>
<td></td>
<td>138 (66)</td>
</tr>
<tr>
<td>ER negative</td>
<td>37 (30)</td>
<td>87 (70)</td>
<td></td>
<td>44 (65)</td>
</tr>
<tr>
<td>PR status</td>
<td>0.08</td>
<td></td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>PR positive</td>
<td>89 (23)</td>
<td>304 (77)</td>
<td></td>
<td>131 (65)</td>
</tr>
<tr>
<td>PR negative</td>
<td>20 (16)</td>
<td>109 (85)</td>
<td></td>
<td>55 (71)</td>
</tr>
</tbody>
</table>

n=number of patients in each category; *: Pearson χ²-test