Molecular alterations suggesting new treatment strategies in uterine carcinomas

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Scientific environment

Professor Camilla Krakstad, Professor Jone Trovik and the late professor Helga Salvesen supervised this PhD project performed within the Gynecologic Cancer Research Group at the Department of Clinical Science, University of Bergen. The group is closely linked to the Department of Obstetrics and Gynecology, Haukeland University Hospital both located at Kvinneklinikken. The research group consists of more than 20 members including PhD students, post-docs, senior researchers, technicians research nurses and medical students.

In 2001, a systematic prospective collection of material from gynecological malignancies was initiated. The collection is still ongoing consisting of blood samples and fresh frozen tumors from more than 4000 individuals treated for gynecological malignancies at the Department of Obstetrics and Gynecology and in a multicenter setting (MoMaTEC). Clinical, pathological and follow-up data have concomitantly been collected from patients with a gynecological cancer diagnosis.

Bergen Gynecologic Research Group is embedded in the Centre for Cancer Biomarkers (CCBIO), which, in 2013 was awarded a 'Norwegian Centre of Excellency'. CCBIO focuses on translational cancer research, predominantly by searching for new biomarkers with the aim to improve and individualize cancer treatment.

The cell line studies were performed at the Centre for Molecular Medicine Norway (NCMM) at the University of Oslo by Siv Gilfillan, Sachin Sign and Madhumohan R. Katika led by Dr. Antoni Hurtado.

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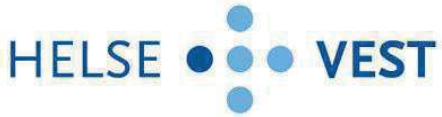

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Abbreviations

AC	Adenocarcinoma
ADC	Antibody-drug conjugate
AI	Aromatase inhibitor
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ARID1A	AT-rich interaction domain 1A
ASCO	American Society of Clinical Oncology
BAX	BCL2 Associated X Protein
BCAR4	Breast cancer anti-estrogen resistance 4
BCL-2	B-cell lymphoma 2
BCL-X_L	B-cell lymphoma-extra large
BIM	BCL-2-like protein 11
CAF	Cancer-associated fibroblast
CAP	College of American Pathologists
CHI3L1	Chitinase-3-like protein 1
ChIP	Chromatin Immunoprecipitation
CISH	Chromogen in situ hybridization
COX-2	Cycloxygenase 2
CNV	Copy-number variations
CSC	Cancer stem cell
CXCL8	C-X-C motif chemokine ligand 8
DAB+	Diaminobenzidine chromogen
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ERα	Estrogene receptor- alpha
EBRT	External beam radiation therapy
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EIC	Endometrial intraepithelial carcinoma
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
FBS	Fetal bovine serum
FFPE	Formalin Fixed Paraffin Embedded
FIGO	International Federation of Gynecology and Obstetrics
FOXA1	Forkhead box A1
GSEA	Gene Set Enrichment Analysis

GOG	Gynecologic Oncology Group
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor
HIF-1α	Hypoxia-inducible factor 1-alpha
HIV	Human immunodeficiency virus
HOXB13	Homeobox B13
HPV	Human papillomavirus
HRP	Horseradish peroxidase
IARC	International Agency of Research on Cancer
IBM	International Business Machines Corporation
IFG	Insulin-like growth factors
IHC	Immunohistochemistry
KRAS	KRAS proto-oncogene, GTPase
L1CAM	Neural cell adhesion molecule L1
LCR	Long control region
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSigDB	The Molecular signature database
MSS	Microsatellite stable
mTOR	Mammalian target of rapamycin
NET	Neuroendocrine tumors
NGS	Next-generation sequencing
PARP	Poly [ADP-ribose] polymerase
PCR	Polymerase Chain Reaction
PBS	Phosphate-buffered saline
PET	Positron emission tomography
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
POLE	DNA polymerase epsilon, catalytic subunit
PPP2R1A	Protein phosphatase 2 scaffold subunit A
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
REK	Regionale komiteer for medisinsk og helsefaglig forskningsetikk
RB	Retinoblastoma-associated protein
RPPA	Reverse phase protein array

SCC	Squamous cell carcinoma
SCJ	Squamocolumnar junction
SERM	Selective estrogen modulators
SI	Staining index
SLN	Sentinel lymph node
SNP	Single nucleotide polymorphism
SPHK1	Sphingosine kinase 1
SPOP	speckle type BTB/POZ protein
STR	Short tandem repeat
TAF1	TATA-box binding protein associated factor 1
TAM	Tumor-associated macrophage
TCGA	The Cancer Genome Atlas
TDM-1	Trastuzumab emtansine
TIL	Tumor-infiltrating lymphocytes
TMA	Tissue microarray
TP53	Tumor protein 53
TSP-1	Thrombospondin-1
TSR	Tumor-stroma ratio
VB	vaginal brachytherapy
VEGF-A	Vascular endothelial growth factor-A
WHO	World Health Organization
ZEB	Zinc finger E-box-binding homeobox

Abstract

Background: The two main uterine cancer subtypes, endometrial and cervical carcinoma, represent a major worldwide female health problem. Although prognosis is generally good with low stage uterine carcinoma, survival is poor and treatment options few for patients with advanced and recurrent disease. Efforts in enhancing treatment schedules for these patients are lagging behind and precise prognostic and predictive biomarkers are vital to optimize treatment effects. Emerging next-generation sequencing (NGS) studies using available patient cohorts are currently unraveling molecular subtypes with potential clinical implications, however the clinical utility of such studies is dependent on patient cohorts that truly reflect clinical settings.

Aim: First, we aimed to determine if an inclusion criterion of high tumor cell content in fresh tissue for molecular profiling studies could introduce a selection bias towards inclusion of more aggressive uterine carcinomas. Secondly, we aimed to identify novel prognostic biomarkers and to validate biomarkers already clinically implemented. Finally, we aimed to investigate HER2 and p53 as potential biomarkers in uterine carcinoma, and to identify potential resistance mechanisms in HER-directed treatment.

Material and methods: The prospectively collected patient cohorts investigated in this study are endometrial hyperplasia, primary tumors and metastases (**Paper I, IV and V**) and cervical primary tumors (**Paper II-III**). Tumor cell content was determined by light microscopy of hematoxylin stained sections (**Paper I-II**). Protein levels of HER2 and p53 were evaluated by immunohistochemistry (**Paper III-IV**). To assess copy number and mRNA expression data, whole exome sequencing (**Paper III**), RNA sequencing (**Paper III**) and DNA oligonucleotide microarray (**Paper IV-V**) data were applied, respectively. The cell line studies included chromatin immunoprecipitation (ChiP) sequencing, cell proliferation and migration assays, western blots and chromatin fractionation (**Paper V**).

Results: High tumor cell content is associated with aggressive phenotype and poor survival in uterine adenocarcinomas (**Paper I-II**). However, such associations were not found in cervical squamous cell carcinomas (**Paper II**). Vascular space invasion,

histological type, tumor size and p53 levels are strong independent prognostic markers in cervical carcinoma. High HER2 protein levels were identified in 21% of the patients. Amplified *ERBB2* significantly linked to poor prognosis, HercepTest did not (**Paper III**). In endometrial carcinoma, high HER2 protein levels correlate significantly with aggressive disease and poor survival. A substantial reduction in HER2 expression was observed from primary to metastatic disease (**Paper IV**). In endometrial carcinoma, HER2-high/FOXA1-low protein levels significantly and independently associates with poor prognosis and poorly differentiated tumors. In the *in vitro* experiments, FOXA1 inhibits proliferation triggered by EGFR-HER2 (**Paper V**).

Conclusions: A selection criterion of ≥80% tumor purity in fresh tissue promotes enrichment of aggressive uterine adenocarcinomas and associates with poorer prognosis (**Paper I and II**). In cervical carcinoma, FIGO III and IV are less likely to be included for molecular profiling studies (**Paper II**).

In cervical carcinoma, vascular space invasion and tumor size 2-4 cm are important determinants for prognosis. p53 is an independent predictor of survival; yet do not reflect *TP53* mutational status. Amplified *ERBB2* significantly links to poor survival; HercepTest do not (**Paper III**).

In endometrial carcinoma, high HER2 protein levels measured by the HercepTest associates significantly with aggressive disease and poor survival. SI identified a subgroup of HercepTest 3+ tumors with significantly poorer survival. Loss of HER2 expression is common in metastatic endometrial carcinoma (**Paper IV**).

FOXA1 expression predicts improved survival in patients with HER2 overexpressing endometrial carcinoma. In endometrial cancer cell lines, FOXA1 attenuates the expression of genes that enable EGFR/HER2 signaling through interaction with polycomb-associated proteins thereby sensitizing the cell to anti-EGFR/HER2 treatment (**Paper V**).

List of publications

This thesis is based upon three publications and two manuscripts submitted for publication, referred to in the text by their respective roman numerals:

I. **Halle MK***, Werner HM*, Krakstad C, Birkeland E, Wik E, Trovik J, Salvesen HB: Stratification based on high tumour cell content in fresh frozen tissue promotes selection of aggressive endometrial carcinomas. ***Histopathology***. *2012;60:516-9.* *Shared first author

II. **Halle MK**, Krakstad C, Engerud H, Bertelsen B, Salvesen H: Molecular profiling in fresh tissue with high tumor cell content promotes enrichment for aggressive adenocarcinomas in cervix. ***Pathol Res Pract.*** *2014;210:774-8.*

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IV. **Halle MK**, Tangen IL, Berg HF, Hoivik EA, Mauland KK, Kusonmano K, Berg A, Hurtado A, Kalland KH, Øyan AM, Stefansson I, Vintermyr OK, Werner HMJ, Haldorsen IS, Trovik J, Salvesen HB and Krakstad C: HER2 expression patterns in paired primary and metastatic endometrial cancer lesions. *Manuscript under revision in* ***Br J Cancer****.*

V. Gilfillan S*, **Halle MK***, Sign S, Tangen IL, Katika MR, Salvesen HB, Krakstad C[#] and Hurtado A[#]: FOXA1 predicts good outcome in HER2+ endometrial cancer patients by inhibiting EGFR/HER2 signaling. *Manuscript under consideration.* *Shared first author [#]Shared last author.

Contents

1. INTRODUCTION

1.1 Cancer

About one fourth of us die from cancer [1]. Cancer cells break the most basic rules of construction and maintenance of multicellular organisms by inducing uncontrolled cell growth, increased cell division, decreased cell death and eventually invading through normal tissue boundaries. In a functioning multicellular organism, each cell acts in a society responsible manner – resting, growing, differentiating, dividing or dying –as needed best for the organism. The human body contains more than 10^{14} cells, and experiences genomic alterations every day, some with the potential to disrupt this social control. The most vigorous genomic alterations may lead to a selective advantage allowing accelerated cell growth, division and the emergence of a mutant cell clone. Over time, the rapid growth and division may facilitate repeated rounds of genomic change, and subsequent natural selection of aggressive cells in a microevolutionary process [2]. This process evolves over years or decades, and the principles of genomic alterations and natural selection applies for emerging mutant clones, precursor lesions, cancer formation and metastatic spread. In the process of carcinogenesis, the tumor becomes more than an insular mass of proliferating cells. Complex tissues composed of numerous distinct cell types interacting with one another emerges [3]. Normal cells are recruited, forming tumor-associated stroma which are active participants in the tumorigenesis [4].

1.1.1 Key aspects of molecular tumor biology

Structure and function of all organisms are ultimately determined by their genomes, comprising the whole set of species-specific heritable information. Cancer is a disease of the genome involving dynamic genomic changes that allow normal cells to transform into neoplastic cells with the ability to invade normal tissue boundaries.

Hallmarks of Cancer

In two landmark papers, Hanahan and Weinberg characterized a conceptual framework ‘the hallmarks of cancer’, categorizing multiple sets of processes governing the

malignant transformation into cancer [4, 5]. They defined eight hallmarks that describe the characteristics of tumor biology and two enabling characteristics (each accentuated in bold below).

Genome instability, which generates random mutations including chromosomal rearrangements, is described as one of two enabling characteristics of cancer development. Higher rates of mutations can be achieved through increased sensitivity to mutagenic agents, through breakdown of one or several components of the genomic maintenance system (e.g. p53) or by compromising the surveillance system normally monitoring genome instability forcing genetically damaged cells into either senescence or apoptosis. Normally cells repair DNA damages through base or nucleotide excision repair, and replication errors through mismatch repair (MMR). Mutations in these genes speed up the mutation rate in cancer [6]. In addition, in many tumors, loss of telomeric DNA generates karyotypic instability. Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telemetric DNA, is almost absent in non-immortalized cells, but is expressed at functionally significant levels in most spontaneously immortalized cells (90%), including human cancer cells. Expression of telomerase enables **infinite replicative potential**, and thereby contributes to accumulation of genetic alterations. The output of genome instability is intratumoral heterogeneity, which generates diverse genetic and epigenetic material upon which selection and Darwinian evolution can act [2].

Whereas the cell cycle in normal cells is tightly controlled to ensure cellular homeostasis and maintenance of tissue architecture and function, cancer cells have the ability to become **self-sufficient in growth signals**. Growth factors bind to the cell surface receptors such as tyrosine kinases. Signals are emitted via their intracellular domain through branched intracellular signaling pathways, resulting in cell growth and division [5]. Cancer cells can achieve this capability in multiple ways: By producing their own growth factors, and express cognate receptors resulting in autocrine proliferation signaling; by sending stimulating signals to supporting tumor-associated stromal cells enabling them in return to release growth signals; or by increasing the amount of cells surface receptors rendering hyper-responsiveness to growth factor

signaling. Growth factor independence may also derive from structural alterations in receptors or downstream components, which may enable constitutively active signaling. Hyper-activation by genetic alterations of downstream signal mediations such as in the catalytic subunit of phosphoinositide 3-kinase (PI3K) isoforms, have been detected in a wide range of cancers, including uterine cancers [7-9]. Conversely, loss-of-function mutations in negative regulators such PTEN, amplify PI3K signaling and promote carcinogenesis. Loss of PTEN function has been detected in numerous cancer types, particularly endometrial cancer [10].

In addition to sustain positively acting growth signals, cancer cells also have to avoid the massive negative proliferation signals by becoming **insensitive to anti-growth signals** [5]. Several such programs involve tumor suppressor genes, retinoblastoma-associated protein (RB) and p53 being the two prototypes. They function as central control points for the cell by determining whether the cell should proliferate or activate senescence or apoptosis, based on growth-inhibitory signals from outside the cell (RB) or from within (p53), such as cellular stress or genomic damage [11, 12]. Additionally, growth control can involve cell-to-cell contact inhibition [13, 14].

The concept that cancer cells must **resist cell death** by apoptosis is well established. The most common strategy to limit or circumvent apoptosis is by loss of the p53 tumor suppressor function, which eliminates this critical damage sensor, from the apoptosis-inducing circuit. Alternatively, cells can achieve the same goal by increasing anti-apoptotic signals (Bcl-2, Bcl-X_L) or survival signals (IGF 1/2), by downregulation of pro-apoptotic factors (Bax, Bim, Puma), or by interfering with the extrinsic ligand-induced death pathway [5]. Another cell death program is autophagy [15]. Autophagy suppresses the initiation and development of many tumors, yet it is also linked to major survival pathways in response to cellular stress [16].

Angiogenesis, the formation of new capillary blood vessels ensuring adequate oxygen and nutrient supply and evacuation of metabolic wastes and carbon dioxide, is a prerequisite of tumor growth over a microscopic size [17]. Abnormal regulation of angiogenesis is essential in several pathological conditions, including cancer [18]. In

tumor formation, the ‘angiogenic switch’ is nearly always on. Regulated by the angiogenic inducer vascular endothelial growth factor-A (VEGF-A) and the angiogenic inhibitor thrombospondin-1 (TSP-1), quiescent endothelial cells become activated and enter into a cell-biological program that permits them to create new blood vessels [5].

The end-point of carcinogenesis is **metastatic spread**. Metastasis is the most deadly, but least understood cancer phase contributing to 90% of all cancer-associated deaths [19]. Whereas surgical removal and adjuvant therapy can cure well-defined primary tumors, metastatic disease is largely incurable do to its systemic nature and the resistance of disseminated tumor cells to current therapeutic agents [20]. The tumor invasion-metastasis cascade is a multi-step process whereby tumor cells disseminate from primary sites, and involves local invasion, intravasation, survival in the circulation system, arrest at distant organ sites, extravasation, micrometastatic formation and metastatic colonization [21, 22]. A developmental program, the ‘epithelial-mesenchymal transition’ (EMT) by which transformed epithelial cells acquire the capability to evade, resist apoptosis and disseminate, is highly linked to the invasion-metastatic cascade. A subset of tumor cells within the primary tumor switches off epithelial markers such as E-cadherin and turns on mesenchymal markers such as vimentin, leading to loss of cell polarity, cytoskeletal reorganization and dissolution of adherens and tight junctions. A set of pleiotropically acting transcription factors Snail, Slug, Twist and Zeb1/2, expressed in various combinations in a number of tumor types, coordinates the EMT process [5]. The multifaceted EMT program consists of multiple and dynamic transitional states between the epithelial and mesenchymal phenotypes [23] and many of the underlying molecular processes are still unknown.

Carcinogenesis is reliant on **reprogramming of cellular energy metabolism** as both direct and indirect consequence of oncogenic mutations. A common feature in cancer cell metabolism is the ability to obtain necessary nutrients from a frequently nutrient-poor environment and utilize these nutrients both to maintain viability and to build new biomass [24]. Even in the presence of oxygen, cancer cells can reprogram their glucose metabolism largely towards glycolysis, leading to a state called ‘aerobic glycolysis’ [25]. The glycolysis has relatively poor energy generating efficiency compared to the

mitochondrial oxidative phosphorylation. Still the increased glycolysis found in several cancer cells allows the diversion of glycolytic intermediates into various biosynthetic pathways, including those generating nucleosides and amino acids required for formation of new cells [26, 27]. Aerobic glycolysis is crucial to achieve the ability to overcome nutrient and energy deficiency necessary to survive and form metastases [28].

In order to support continuous cell growth and proliferation, tumors must be able to **avoid immune destruction.** The success of immunotherapies in cancer treatment targeting T-cell immune checkpoint receptors such as Programmed cell death protein 1 (PD-L1) and Programmed death-ligand 1 (PD-L1) has established the position of immunoevasion as a hallmark of cancer [29, 30]. The immune system has a dichotomous role in carcinogenesis, both as an antagonist and enhancer of tumor growth. An important enabling characteristic of carcinogenesis involves **tumor-promoting inflammation.** Inflammation can lead to cancer development by supplying bioactive molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors and extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion and metastasis [5].

Interplay between cancer cells and cells of the tumor microenvironment facilitate the acquired ability to invade and metastasize in multiple ways. Contrasting the early, reductionist view of tumors as an assembly of relatively homogeneous cancer cells, tumors are now regarded as complexed organs with specialized cell types both within the tumor itself and within the tumor-associated stroma [5]. The tumor microenvironment is composed of a variety of cell types including cancer-associated fibroblasts (CAFs), endothelial cells, pericytes and immune inflammatory cells among others [3]. Increasing evidence supports the ability of tumor cells to shape their own advantageous growth environment [2].

There is indisputable evidence supporting the genetically unstable nature of solid cancers and its contribution to the genetic heterogeneity of solid tumors, even if the

tumor originate from specific clones [31]. A new dimension of intratumoral heterogeneity involves the cancer stem cells (CSCs), which are the cells within the tumor that possess capacity of self-renewal. Their resistance to therapy and their ability to regenerate a tumor once therapy has been halted, makes CSCs a double-threat to cancer treatment [32].

1.1.2 Molecular profiling in cancer

Over the last decades, numerous omics based techniques have emerged, including genome sequencing, single nucleotide polymorphism (SNP) profiling, gene expression and epigenetic analyses and proteomic profiling. NGS has emerged as the leading edge of tumor analysis allowing unique advances in the molecular profiling of solid tumors. Massive parallel sequencing has uncovered previously unknown genomic alterations in many malignancies, including gynecological cancers, thus expanding the potential range for the use of targeted therapies. However, issues remain regarding cost and clinical utility.

NGS studies of various tumors have identified extensive tumor heterogeneity and high degrees of genetic alterations, independent of morphological phenotypes and histopathological classification [33, 34]. Such genetic sequencing studies are in most cases performed on bulk tumor specimens, which often fails to identify minor sub-clones. In addition, the degree of stromal invasion in the tumor bulk is essential to determine. Most NGS studies require tumor cell content of 80% or above. Such inclusion criterion could potentially introduce a selection bias when including tumors in NGS studies.

1.2 Endometrial cancer

The uterus consists of three layers: The serosa is a thin tissue layer that envelops the uterus; the myometrium, consisting of smooth muscle cells, constitutes the uterine wall and makes up most of the uterine volume; the endometrium is the inner epithelial layer. The endometrium lines the uterine cavity and consists of glands and stromal tissue. Endometrial cancer is a tumor developing from the endometrial lining of the uterus.

The most prevalent type of endometrial cancer is endometrial carcinoma, a cancer developing from epithelial cells of the endometrial lining.

1.2.1 Epidemiology

Endometrial cancer is the most common gynecological malignancy in developed countries and the incidence is increasing. In 2012, around 320 000 women were diagnosed with this disease worldwide, being the fourth most common cancer type for women in industrialized countries after breast, colorectal and lung cancer [35]. According to the Norwegian Cancer Registry, the endometrial cancer incidence has increased steadily since 1956 (**Figure 1**), with 779 new cases in 2015 [36]. The number of diagnosed cases is predicted to increase steadily reaching between 1,016 and 1,257 in 2025 [37]. The majority of patients presented with endometrial cancer are in the postmenopausal state, still 14% of the women are less than 45 years at diagnosis, and 5% are younger than 40 years [38, 39].

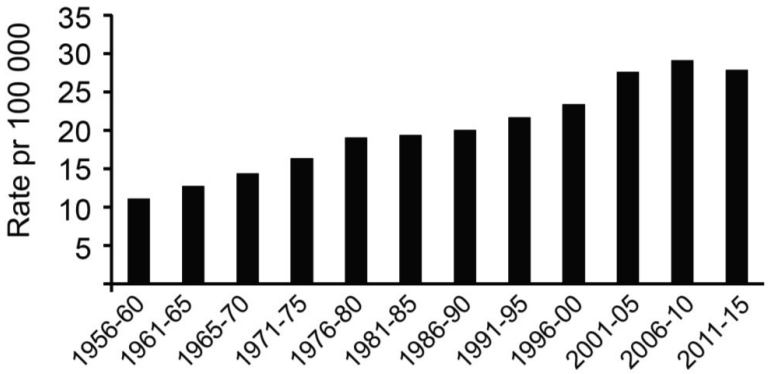

Figure 1 Endometrial cancer age-adjusted incidence rates (Norwegian mid-year population, 2015) in Norway. Numbers are given per 100,000 person-years in five-year time-periods of diagnosis.
Based on data from Cancer Registry of Norway [36].

In term of mortality, endometrial cancer is the 14th cancer with 76 000 worldwide deaths annually (2.1% of cancer deaths in women) [35]. In Norway, the overall survival has increased over the last 40 years, with a 5-year relative survival of 83.4% (2011-

2015) [36]. Although the 5-year relative survival for localized disease is generally good (95.4%), it dramatically declines for patients with regional (59.3%) and distant (36.2%) spread [36].

1.2.2 Risk factors

Most endometrial cancers occur sporadically. Unopposed long-lasting estrogen stimulation not counterbalanced by progesterone, is considered a major contributing risk factor within the most frequently occurring histological type, the endometrioid carcinomas (approx. 80%) [40]. Estrogen stimulation leads to increased proliferation in the endometrium, which may lead to precancerous lesions (hyperplasia) with a subsequent risk of endometrial cancer development [41]. Conditions associated with increased estrogen stimulation include overweight, early age at menarche, late onset of menopause, postmenopausal estrogen therapy (not opposed by progesterone) and previous use of tamoxifen [40]. The same set of risk factors have also been identified within the non-endometrioid endometrial carcinomas, yet the association to disease risk and overweight is stronger within the endometrioid carcinomas [42].

First-degree family history of endometrial or colorectal cancer yields an increased risk of developing endometrial cancer compared to those without a family history [43]. In total, 5-10% of endometrial cancers have hereditary causes, and the main causality is the Lynch syndrome with an associated 40-60% lifetime risk of developing endometrial cancer [44]. Lynch syndrome is linked to a dominantly inherited mutation in mismatch repair (MMR) genes, which may potentially lead to microsatellite instability (MSI) hereby enabling carcinogenesis [45].

1.2.3 Clinical aspects and diagnostics

Clinical presentation

Abnormal vaginal bleeding is an early symptom of endometrial cancer present in more than 90% of the patients [40]. Irregular bleeding is frequent for premenopausal women, but postmenopausal bleeding is a disconcerting symptom urging the majority to seek medical care. Thus, diagnosis is often set at an early stage. Patients with advanced

disease might have symptoms similar to those of advanced ovarian cancer, namely pelvic or abdominal pain and abdominal distension [40].

Diagnosis

The preliminary diagnosis can be set on the basis of office-based endometrial biopsy (Pipelle®) sampling [46] substituted with cervical dilation and curettage (D&C) if the material is sparse or endometrial sampling is infeasible due to cervical stenosis [40]. Endometrial cytology may accurately discriminate cancer from premalignant lesions, but additional biopsies are acquired for optimal histological subtyping and grading. The histopathological diagnosis of the endometrial biopsy is the basis of the diagnostic process commencing the planning of further treatment.

Preoperative staging

The goal of preoperative staging is to establish recurrence risk groups, mainly based on assessment of myometrial invasion and lymph node metastasis for optimal surgical management. Magnetic resonance imaging (MRI) is judged the best imaging technique for preoperative staging, although emerging molecular imaging techniques, such as hybrid Positron Emission Tomography (PET)/MRI could improve diagnostic accuracy [40].

The most widely used classification systems, based on surgical staging is the International Federation of Gynecology and Obstetrics (FIGO) system, last revised in 2009 (**Table 1**) [47]. The FIGO system includes assessment of extent of myometrial invasion, presence of cervical stromal involvement and extent of local and distant metastatic spread. FIGO stage is a strong prognostic marker in endometrial cancer and together with histopathological subtyping and grading, it guides further treatment schedules [48].

Table 1. FIGO staging system for endometrial cancer, 2009.

Stage	Description
I	Tumors confined to the corpus uteri
IA	Tumors limited to endometrium or invading less than 50% of the myometrium
IB	Tumors invading 50% or more of the myometrium
II	Tumors invading cervical stroma
III	Tumors with local or regional extension
IIIA	Tumor involving serosa or adnexa, or both
IIIB	Vaginal involvement or parametrial involvement
IIIC	Regional lymph node metastasis
IIIC1	Regional pelvic lymph node metastasis
IIIC2	Regional para-aortic lymph node metastasis with or without pelvic lymph node metastasis
IV	Tumors invading bladder or bowel mucosa, or distant metastatic disease present (or any combination thereof)
IVA	Tumors invading bladder or bowel, or both
IVB	Distant metastatic disease (includes inguinal lymph node, intraperitoneal disease, lung, bone, or liver)

Adapted from Pecorelli (2009) [47].

1.2.4 Histological classification

Final histopathological diagnosis is obtained after surgical removal of the tumor. In endometrioid carcinomas the histopathological grading is based on their solid growth patterns. Grade 1 tumors are well differentiated with glandular architecture and ≤5% solid growth, grade 2 tumors have less differentiated glands and 6-50% solid growth, and grade 3 tumors are poorly differentiated with barely recognizable glands and >50% solid growth [49]. Non-endometrioid tumors, including serous carcinomas, clear cell carcinomas, carcinosarcomas and undifferentiated tumors are classed as grade 3 by definition [41].

Histological types

Endometrioid carcinomas

Glandular structures are characteristic for this histological type, resembling the original endometrium in varying degrees of differentiation. Areas with more solid growth or with papillary growth can exist [49]. Endometrioid tumors are graded based on their amount of glandular and solid growth patterns [41]. These tumors often ascend from precursor lesions called hyperplasia [49].

Serous carcinomas

First described by Hendrickson et al in 1982, serous papillary carcinoma is an aggressive type of endometrial carcinoma [50]. Presence of papillae, covered by highly pleomorphic tumor cells with frequent mitoses and necrosis is indicative of serous carcinoma [41]. Serous proliferation, either serous carcinoma or the presumed precursor lesion endometrial intraepithelial carcinoma (EIC) have a propensity to arise in endometrial polyps [46]. While representing only 10% of all endometrial carcinomas, it accounts for a disproportionate 40% of deaths from this disease [51] characterized by deep myometrial invasion, lymph vascular space invasion and intra-abdominal as well as distant spread and resistance to therapy [52].

Other non-endometrioid carcinomas

Clear cell carcinomas are characterized by polygonal or hobnail-shaped cells with clear or eosinophilic cytoplasm arranged in papillary, tubulocystic or solid patterns [49]. The overall survival varies greatly from 21-75%, probably reflecting misclassification of histological mimics; serous and secretory endometrial carcinoma [49]. Carcinosarcomas are biphasic tumors composed of high-grade carcinomatous and sarcomatous elements associated with poor prognosis [49]. The monoclonal nature of carcinosarcomas points to an endometrial origin [41]. Undifferentiated carcinoma of the endometrium is an uncommon and highly aggressive malignant epithelial neoplasm without any differentiation [49].

1.2.5 Treatment

Dissimilarities in recommended treatment schedules for endometrial cancer do exist within Europe. Patients included in this study are treated based on current Norwegian guidelines, and hence these will be the focus in this section.

Standard treatment

Total hysterectomy and removal of both tubes and ovaries is the standard treatment for apparent stage I endometrial cancer in Norway [48]. Laparoscopy is a safe option for patients with low stage tumors [53]. For high risk and some of the intermediate risk patients, lymph node sampling is recommended in Norway and may yield valuable

contributions to further adjuvant treatment decisions [48]. Patients with locally advanced disease and/or distant spread may undergo a more extensive surgery, which may include parametrium resection, para-aortic lymph node removal, omentectomy and peritoneal biopsies [42, 48, 54]. For women who want to preserve their fertility, less invasive approaches are possible [55]. Some endometrial cancer patients have a high operative risk where surgery is not recommended due to co-morbidities such as obesity, major cardiovascular disease, diabetes mellitus, and/or old age [56].

Adjuvant treatment

For high-risk endometrial cancer patients with risk of recurrence, chemotherapy is the most frequent form of adjuvant treatment [48]. Current first-line treatment is carboplatin plus paclitaxel [57]. A Cochrane review including 2288 patients enrolled in eleven randomized clinical trials comparing chemotherapy with other interventions concluded that more intense combination chemotherapy significantly improves outcome; however severe side effects such as myelosuppression and gastrointestinal toxicity was increased [58].

Radiation therapy is primarily used as adjuvant treatment aiming to decrease the risk of local and regional recurrence. Radiation therapy can by administered vaginally as brachytherapy (VB), externally as external beam radiation therapy (EBRT) (towards the pelvic area or extended to the para-aortic or whole abdominal area) or as a combination of both [41]. No significant difference in survival is observed between VB and EBRT, and due to less side effects, VB is the radiotherapy of choice [59]. Thus, VB is in many countries offered as standard adjuvant treatment for patients with FIGO 2009 stage 1 endometrial cancer with high-intermediate risk [59]. There has been a steady decline in the use of radiotherapy over the last decades, both internationally and in Norway [48, 54, 60]; initially administered routinely to the vast majority of endometrial cancer patients, whilst currently restricted to intermediate- and high risk groups [54]. Several clinical trials with radiotherapy in combination with chemotherapy are ongoing (such as PROTEC-3, GOG-258).

Adjuvant hormonal treatment may include high-dosage progestogens or anti-estrogen drugs including tamoxifen and aromatase inhibitors (AIs). However, such treatment regimens are infrequently used in Norway [48] and no beneficial effect on survival in endometrial cancer patients has been shown [61, 62].

Recurrent or metastatic treatment

Surgery or other locally directed treatments (such as radiation therapy on previously non-irradiated areas) are options for patients with recurrent or metastatic disease [40, 48]. For patients ineligible for complete cytoreductive surgery, primary chemotherapy followed by surgery is associated with improved survival [40]. For patients not amenable to local therapy, regimens combining carboplatin and paclitaxel are in current clinical use [48], although the benefit to chemotherapy in recurrent or metastatic disease in modest [63]. Hormonal treatment may be an alternative in patients where chemotherapy is not a treatment option do to the patient's health, yet response rates are poor [40, 57].

1.3 Cervical cancer

The uterine cervix is the lower third of the uterus connecting the uterine cavity and the lumen of the vagina. The cervix plays a gatekeeping role in first, preventing pathogens from entering the uterus, and second, allowing the ascent of sperm to the Fallopian tubes [64]. The lower part of the cervix projecting into the vagina is called the ectocervix. It is covered by non-keratinized stratified squamous epithelium. The squamocolumnar junction (SCJ) is the area where the epithelial cells of the endocervix and squamocolumnar cell of the ectocervix meet [65]. Progressively by a process called metaplasia, the ectropion is replaced by metaplastic squamous epithelium. As women age, this ectropion continuously regresses into the cervical canal. The area between the original SCJ and the new SCJ is called the transformation zone. Most cervical cancers arise in the transformation zone of the uterine cervix. The most prevalent type of cervical cancer is cervical carcinoma, which arises from epithelial cells of the uterine cervix.

1.3.1 Epidemiology

Worldwide, cervical cancer is the second leading cause of cancer in the female population in developing countries with annually 265 000 estimated deaths worldwide [35]. The majority of women diagnosed with and dead from cervical cancer live in developing countries (87% and 84%, respectively); while in high-income countries, most women survive the disease [35]. Worldwide, approximately 0.5 million cervical cancer patients are diagnosed each year [35]. According to the Norwegian Cancer Registry, 370 new patients were diagnosed with cervical cancer in 2015 in Norway [36]. The large geographical variation in cervical cancer incidence and mortality rates reflects differences in availability of screening, which enable early detection and removal of precancerous lesions, and prevalence of human papillomavirus (HPV) infection (**Figure 2**). Other factors contributing to the high incidence and mortality rates in developing countries is lack of vaccines, lack of diagnostic personnel (such as gynecologists and pathologists) and lack of treatment options (such as surgery and radiation capacity). In Norway, where screening programs have long been established, cervical cancer age-adjusted incidence rates have decreased from 24.7 per 100,000 in 1971-75 to 12.8 per 100,000 in 2011-15 [36] (**Figure 3**).

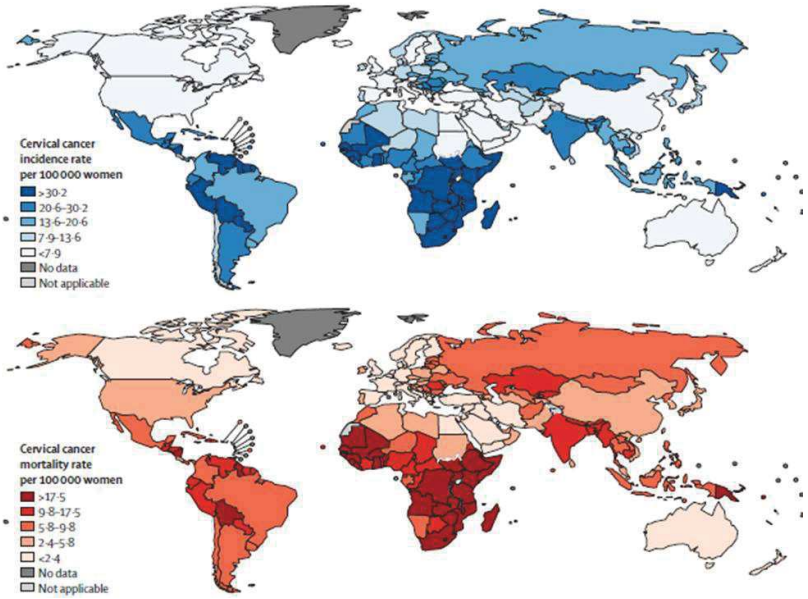

Figure 2 Age-adjusted incidence and mortality rates of cervical cancer in 2012 [66]. (Obtained from http://globocan.iarc.fr with permission to reprint from the International Agency of Research on Cancer (IARC) GLOBOCAN 2012).

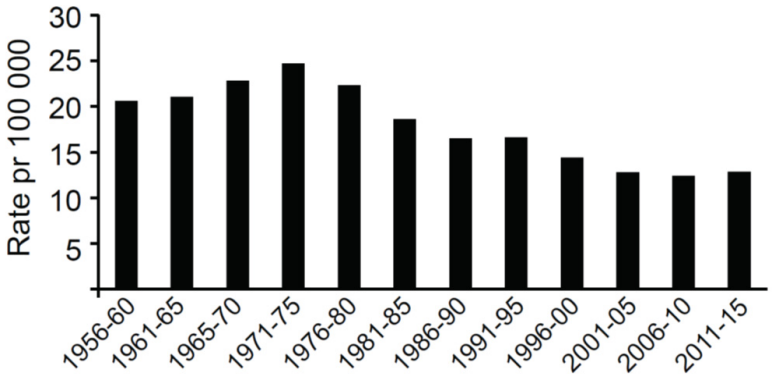

Figure 3 Cervical cancer age-adjusted incidence rates (Norwegian mid-year population, 2015) in Norway. Numbers are given per 100,000 person-years in five-year time-periods of diagnosis.
Based on data from Cancer Registry of Norway [36].

1.3.2 Risk factors

Risk factors

The most important risk factor for invasive cervical cancer is human papilloma virus (HPV), of which several oncogenic subtypes exist (such as HPV 16, 18, 45, 31) [67]. Early onset of sexual activity and multiple sexual partners increases the risk of cervical cancer [68]. Other independent risk factors include high parity, prolonged oral contraceptive use, cigarette smoking and immunosuppression, especially through HIV infection [69-72]

Prevention

In Norway, a quadrivalent vaccine against HPV (Gardasil®) was introduced for girls in the Children's Vaccination Program in 2009 [48]. It protects against HPV 6, 11, 16 and 18. From 2017, a new vaccine, Cervarix® will be introduced in this program. It has an efficacy of 62% in preventing CINII or worse and 93% for preventing CINIII or worse [73]. HPV vaccines are most effective if administered before onset of sexual activity, i.e. before first expose to HPV infection.

In 1995, the Norwegian government introduced a national screening program for detection of premalignant or malignant cervical neoplasia, based on cytological investigation (Papanicolaou test or Pap smear) every 3 year for women between 25 and 69 years. If Pap smears are benign, current Norwegian guidelines recommend a new smear every three years. If smears are inconclusive or of low grade, a HPV test will guide follow-up intervals, 6-12 months if the HPV test is positive and 3 years if negative. Under any suspicion of high grade pre-malignancy (CIN III) or cancer, the woman should be referred for a colposcopy guided biopsy [48]. Recently, an accompanied HPV test in primary screening has been introduced as a pilot in certain Norwegian counties [48]. The fact that progression from low-grade premalignant (CIN I) to invasive cancer evolves slowly over several years and that spontaneous regression is common, makes cervical cancer screening very effective. Accordingly, the incidence rate of cervical cancer in Norway has declined by 22% compared to the period before implementation of the screening program [74].

The UN Secretary General has called for the elimination of cervical cancer as a public health problem by 2030. To achieve this, it is recommended that 70% of girls aged 9-13 should be immunized against the human papilloma virus and 70% of women aged 30-49 should be screened for cervical cancer at least once, followed by appropriate treatment if precancerous- or cancer lesions are detected [75]. Although cervical cancer is largely preventable through public vaccine- and screening programs, such interventions are in many countries, especially in low-income regions, not widely implemented [66].

1.3.3 Clinical aspects and diagnosis

Clinical presentation

Early invasive cancer can be asymptomatic. As the tumor grows and becomes exophytic, common symptoms include vaginal bleeding and discharge, pelvic pain, dyspareunia and postcoital bleeding [48]. If the tumor grows lateral into the parametrium, the ureters may become obstructed [49].

Diagnosis

Gynecological examination and palpation of the cervix, often supplemented by vaginal ultrasound determines the preliminary diagnosis. If the cytological test reveals cancerous cells or a macroscopic tumor is evident, colposcopic guided biopsies will be performed. If histological examination of these biopsies suggests microinvasive cancerous growth, a diagnostic cone biopsy will be performed. Depth of infiltration, horizontal extension and distance from tumor to resection margins determines further treatment. For macroscopic tumors, a gynecological examination including cystoscopy provides information of tumor size and spread. Pelvic MRI gives information regarding local tumor growth, infiltration to the parametrium, lymph node status, urinary tract status and pelvic metastases. Computed tomography (CT) of thorax, abdomen and pelvis gives information regarding urinary tract status, lymph node status and peripheral metastases.

Staging

Cervical cancers are the only gynecological cancers **clinically** staged by physical examination, chest X-ray, intravenous pyelogram, cystoscopy and proctoscopy. In Norway, staging of cervical tumors is performed by the FIGO classification system (**Table 2**) [47].

Table 2. FIGO staging system for cervical cancer (2009).

Stage	Description
I	Tumors confined to the cervix
IA	Microscopic tumor with stromal invasion <5mm in depth and largest extension of <7mm
IA1	Measured stromal invasion of <3 mm in depth and extension of <7mm
IA2	Measured stromal invasion of <3 mm and not >5 mm with an extension of <7 mm
IB	Clinically visible tumors confined to the cervix
IB1	Clinical lesions < 4cm
IB2	Clinical lesions > 4 cm
II	Tumors invading beyond the cervix, but not to the pelvic wall or the lower third of the vagina
IIA	Without obvious parametrial invasion
IIB	With obvious parametrial invasion
III	Tumors extending the pelvic wall and/or involves lower third of the vagina and/or causes hydronephrosis or non-functioning kidney
IIIA	Tumor involving lower third of the vagina, with no extension into the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or non-functioning kidney
IV	Tumor spread outside the true pelvis or has clinically involved the mucosa of the bladder or rectum.
IVA	Spread to adjacent pelvic organs
IVB	Spread to distant organs

Adapted from Pecorelli (2009) [47].

1.3.4 Histological classification

Cervical cancers are histologically classified according to the WHO classification.

Squamous cell carconoma (SCC)

SCCs comprise around 80% of the carcinomas in the cervix and are associated with HPV. Hence, HPV screening is effective in reducing the incidence of SCC. In Norway, introduction of HPV screening has led to an estimated 74% reduction in SCC and 68%

in total for cervical cancer incidence [76]. Prognostic factors for SCC include FIGO stage and age at diagnosis. Histological grade have been shown to have little prognostic value [49]. Invasive SCCs vary in their pattern of growth, cell type and degree of differentiation, and several subtypes exist including keratinizing, non-keratinizing, basaloid, papillary and verrucous types [49]. Most SCCs exhibit sheet-like growth and infiltrate as networks of anastomosing bands or single cells with an intervening desmoplastic or inflammatory stroma.

Adenocarcinoma (AC)

ACs comprise around 10% of cervical cancers globally and up to 25% in western countries, and are in most cases associated with HPV [49, 77]. While the incidence of squamous cell carcinomas is decreasing, adenocarcinomas are increasing [77]. Cytology based cervical screening has been less effective in detecting ACs compared to SCCs [76]. ACs arise from glandular precancerous lesions (adenocarcinoma *in situ*) that often develop inside the endocervical canal, and is considered harder to sample than squamous precancerous lesions (cervical intraepithelial neoplasia) [77, 78]. Although cytology screening is inefficient at preventing ACs, invasive ACs are detected earlier than they would be without screening, substantially preventing stage II and worse [79]. There is evidence suggesting that HPV negative cervical carcinomas exist, and these are more likely to be adenocarcinomas [80]. Yet, some of the HPV negative cervical ACs could be misjudged endometrial carcinomas. This distinction is important, because adenocarcinoma from the cervix versus from the endometrium have different treatment modalities.

Adenosquamous carcinoma

Adenosquamous carcinomas comprise both adenocarcinoma and squamous cell carcinoma components. There should be adequate differentiation of the adenocarcinomatous component to include histologically recognizable glands [49]. Glassy cell carcinoma is a poorly differentiated variant of adenosquamous carcinoma characterized by cells with sharp cytoplasmic margins [49]. There is some evidence suggesting that effect of screening of adenosquamous carcinomas can be of similar magnitude as seen for SCCs [81].

Undifferentiated tumors

An undifferentiated tumor is an extremely rare carcinoma of the cervix lacking any specific differentiation. Immunohistochemistry may aid to establish a specific tumor type, for instance, p63 immunoreactivity suggesting a squamous carcinoma or with neuroendocrine marker positivity, a neuroendocrine carcinoma [49].

Neuroendocrine tumors (NET)

Neuroendocrine tumors are aggressive diseases developing from neuroendocrine cells. NETs most frequently develop in the gastrointestinal tract and the lungs [82, 83], but can rarely also develop in almost all body tissue including the female genital tract representing 0.9% to 1.5% of cervical carcinomas [84]. Compared to the more abundant SCCs and ACs, NETs comprise higher prevalence of lymph-vascular space invasion, lymph involvement and local and distal relapse [84]. NETs are further divided into low-grade and high-grade tumors. Low grade tumors exhibit neuroendocrine and organoid differentiation. High grade NETs are by far the most common, and both small-cell and large-cell neuroendocrine differentiation exist [49]. No treatment guidelines based on prospective well-designed clinical trials are currently available due to the rarity of this subtype [84]. The Norwegian guidelines recommend to treat these tumors as systemic diseases from stage I with adjuvant cisplatin based chemotherapy [48].

1.3.5 Treatment

Cervical cancer patients are treated based on FIGO stage (with one exception; the NETs which are treated as systemic diseases at FIGO stage I) [48]. For the earliest stages of cervical cancer (FIGO stage 1A to IB) surgery by either cone biopsy (stage 1A if margins are negative) or radical hysterectomy with pelvic lymphadenectomy (stage 1A2-IB1 if cone biopsy margins are positive) is standard treatment. If margins are positive in the cone biopsy and if the patient wishes fertility-sparing treatment, trachelectomy (removal of the cervix and upper vagina) can be considered. To reduce the risk of spontaneous abortion/preterm delivery, a cerclage will be performed at the same time. The risk of recurrence after surgery is imminent for patients with stage IB2

or higher and standard treatment for these patients is radio- and/or chemotherapy. Patients with FIGO stage IIB or higher are treated with external and concomitant intracavitary radiation therapy and chemotherapy [48]. Most standard chemotherapy regimens include a platinum drug (cisplatin or carboplatin) in combination with another drug such as paclitaxel, gemicitabine or topotecan. Bevacizumab is an emerging targeted drug, which may be administered in combination to chemotherapy. It recently gained approval for treatment also including Norwegian cervical cancer patients, however currently not implemented into national guidelines [48]. Recurrent cancer is treated individually and may have curative potential. Previously radiated areas respond poorly to a second a round of radiation, and surgery will be preferred. If the recurrence is in the pelvis only, extensive surgery (pelvic exenteration) may be an option. Radiation and/or chemotherapy can be used to slow tumor growth or to help relieve symptoms. Chemotherapy may be used to treat distant metastases if neither surgery nor radiation is beneficial.

1.4 Tumor biology in uterine cancer

1.4.1 Tumor biology in endometrial cancer

Histopathological characteristics

In 1983, Bokhman *et al* postulated the existence of two distinct types of endometrial carcinoma [85]. The first type (later named Type 1) was characterized by favorable diagnosis, low grade, superficial myometrial invasion and sensitivity to progestogens. Type 1 tumors typically occur in obese patients, and are associated with concurrent hyperplasia and excess estrogen. Estrogen is a ligand of the transcription factor estrogen receptor (ER), and upon estrogen binding, ER modulates cell growth in various tissues [86]. The second cancer type postulated by Bokhman *et al* (later named Type 2) was characterized by absence of metabolic and endocrine disturbances, higher grade, tendency of deeper myometrial invasion, more metastatic spread and poor survival [85]. These tumors are often ER-negative [87]. The exact underlying mechanisms of ER loss are generally unknown. This classification system has later been utilized in the clinic, and is still a major determinant of identifying high-risk

patients [48]. However, the prognostic value of this dualistic system has proved to be limited as 20% of the Type 1 recurs, while 50% of Type 2 cancers do not [88].

Molecular subtypes

Recent integrated genomic characterization of endometrial carcinoma by The Cancer Genome Atlas (TCGA) has identified four separate molecular subgroups: (i) POLE ultra-mutated; (ii) MSI hyper-mutated; (iii) copy-number low/microsatellite stable; and (iv) copy-number high serous-like [89]. MSI was more frequent in endometrioid than in non-endometrioid tumors [89, 90]. The POLE ultra-mutated subgroup is the smallest of the groups characterized by POLE exonuclease domain mutations, ultra-high somatic mutation frequency and excellent prognosis [91]. The POLE mutated tumors are predominantly endometrioid grade 3 [92]. Grade 3 endometrioid tumors do not fit neatly into the dichotomized classification (Type 1 and 2), and the TCGA sequencing data revealed that 25% of grade 3 endometrioid tumors had genetic resemblance to serous tumors [89], while another subset had POLE mutations and associated with low recurrence risk. Uterine serous tumors and the serous-like high-grade endometrioid tumors had extensive copy-number variations (CNVs), few DNA methylation changes, low hormone receptor levels, frequent *TP53* mutations and low Forkhead-box A1 (FOXA1) signaling [89]. The FOXA1 transcription factor is suggested as a tumor suppressor and loss of FOXA1 is associated the poor survival in endometrial cancer [93, 94].

Clear cell (CCs) and carcinosarcomas (CSs) were not included in the TCGA study. Recent integrated molecular characterization of CCs and CSs demonstrated molecular similarities to both serous and endometrioid carcinomas [95, 96]. In the CCs, two of 16 tumors had relatively high mutational loads and MSI, whereas the rest were microsatellite stable (MSS). Frequent somatic mutations in *TP53, PIK3CA, PIKR1*, *ARID1A, PPP2R1A*, *SPOP* and *TAF1* characterized the CCs [95]. Frequently mutated *TP53, PTEN, PP2R1A, FBXW7* and *KRAS* genes and high EMT scores characterized the CSs [96].

1.4.2 Tumor biology in cervical cancer

Emerging molecular profiles

Two major integrated genomic and molecular characterization studies in cervical cancer have recently been published; the Ojesina study [97] and the TCGA study [98]. Several recurrent somatic gene aberrations were identified, among them mutation and amplifications in *ERBB2,* the gene coding for HER2. Both studies confirmed HPV integration sites and increased target gene expression. In the TCGA study, 84% of HPV positive tumors were identified with integration of HPV in host genome. Further, a unique set of endometrial-like cervical cancers was identified. It comprised predominantly of HPV-negative tumors with high frequencies of *KRAS*, *ARID1A* and *PTEN* mutations, also commonly found in endometrial cancers [88]. The TCGA study further discovered an APOBEC mutagenesis pattern. The APOBEC mutation load correlated strongly with the total number of mutations per sample, suggesting APOBEC mutagenesis as a predominant source of mutations in cervical cancers.

HPV-induced cervical carcinogenesis

HPVs are small, non-enveloped DNA viruses that persistently infect stratified squamous epithelia, predominantly in the basal cell layer [99]. Viral lateral expansions through the midzone to the upper epithelial layer occur. In the upper layer of the epithelium, the viral late genes *L1* and *L2* are transcribed. L1 and L2 encapsidate the viral genomes to form progeny virions. Shed viruses can initiate new infections [100]. As HPV-infected lesions progress to cervical cancer, the episomal viral DNA frequently becomes integrated into host-cell DNA [99] (**Figure 4**). The best described interactions between HPV proteins and cellular processes are via the viral proteins E6 and E7. Both interactions lead to rapid degradation of the tumor suppressor proteins p53 and RB [101]. Loss of p53 and RB effectively abrogates apoptosis, allowing genome instability and uncontrolled proliferation, all of which enable carcinogenesis [11, 12]. Oncogenic HPV types have also been found to enhance hypoxia-inducible factor 1α (HIF-1α) protein accumulation and VEGF expression [102] and to invoke cellular immune responses with T cells involved in local immune suppression [103].

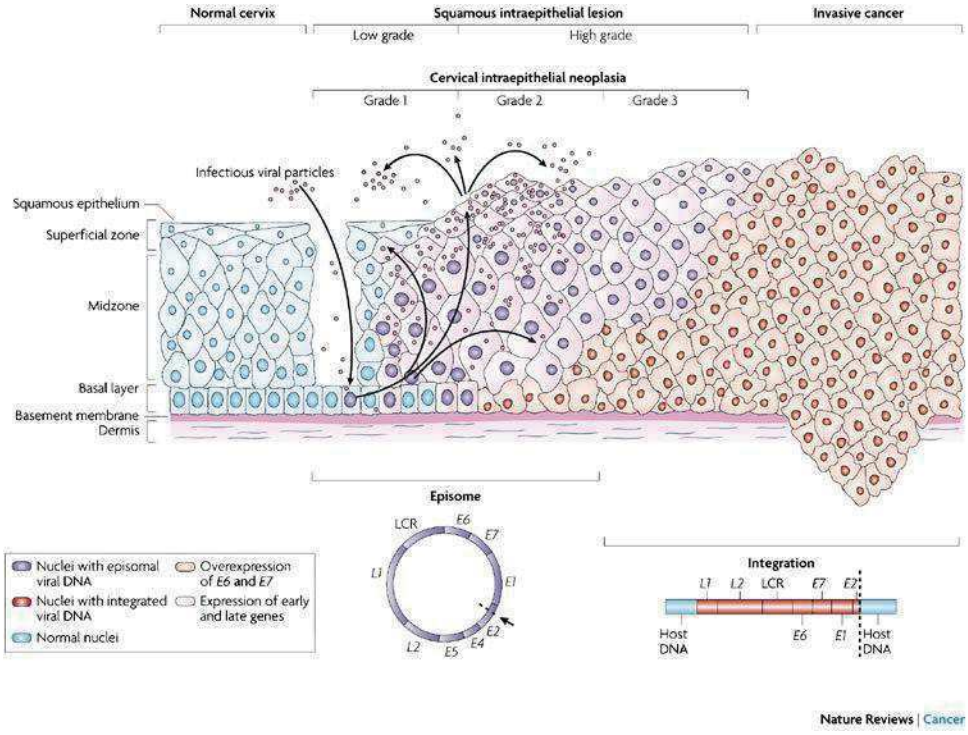

Figure 4 HPV-mediated progression to cervical cancer
Basal cells in the cervical epithelium rest on the basement membrane, which is supported by the dermis. Human papillomavirus (HPV) is thought to access the basal cells through micro-abrasions in the cervical epithelium. Following infection, viral expression in the basal cell layer is limited to the 'early' viral genes (E1, E2, E4, E5, E6 and E7), which results in enhanced proliferation with subsequent lateral expansion. After entry into the midzone, the viral genome is replicated and structural proteins form. In the upper layer of the epithelium, the viral genome replicates further, and the late genes L1 and L2 are expressed. L1 and L2 encapsidate the viral genomes to form progeny virions. Shed viruses can initiate a new infection [99]. As HPV-infected lesions progress to cervical cancer, the episomal viral DNA frequently become integrated into host-cell DNA (red nuclei) [98].
Abbreviations: LCR, long control region.
Figure reprinted with permission from Nature Publishing Group [100].

Ojesina *et al* first reported the association between HPV integration and increased expression of adjacent genes in cervical cancer [97]. The TCGA study further unraveled integration of HPV into the host genome in 83% of HPV-positive tumors

[98]. Distinct molecular pathway activation based on HPV types was identified, emphasizing the biological diversity of HPV [98]. The HPV-negative tumors (5%) displayed a significantly higher EMT mRNA score and a lower frequency of the APOBEC mutagenesis signature compared to the HPV-positive tumors.

The HPV vaccine protects against HPV infection by mimicking the outer shell of an authentic HPV virion forming virus-like particles (VLPs) [104]. VLPs are not infectious as they lack viral DNA. However, they stimulate the body to produce antibodies that in future encounters with HPV, bind to the virus thus preventing it from infecting cells [105].

1.5 Biomarkers in cancer

Biomarkers, defined as characteristics that are objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [106], may assist in unraveling the complexity of each individual tumor and ultimately affect choice of treatment. Biomarkers may be used in several clinical settings, including in screening to detect early disease (such as implemented for detection of cervical cancer and precursor lesions), and to guide surgery (such as preoperative ER/PR status presently investigated in a multicenter study (MOMATEC2/NCT00598845)) [107]. However, prognostic and predictive biomarkers are considered the two main types.

1.5.1 Prognostic biomarkers

Prognostic biomarkers provide information about cancer outcome, regardless of therapy [108]. Such markers may be valuable in stratifying patients in benefit of further treatment or more frequent follow-up. A prognostic biomarker needs to provide additional information in order to be clinically relevant.

In endometrial cancer, prognostic biomarkers in current clinical use include FIGO stage, tumor histological type and grade [48, 88]. Still, based on these biomarkers, up to 20% of the presumed low risk tumors will eventually recur, and around 50% of the

patients with presumed high risk tumors will be fully cured of their disease [85, 109]. Several studies have presented additional histopathological and molecular prognostic markers in endometrial cancer, among them lymph vascular space invasion, which has proven to be of independent prognostic value [110, 111]. A nomogram including all the above-mentioned parameters has been suggested as an even more robust prognostic tool [112]. The best validated molecularly based prognostic biomarkers in endometrial cancer include ER [107], PR [113], p53 [114], L1CAM [115, 116] and DNA ploidy [117]. Still, the clinical use of these markers is limited [118]. The clinical utility and prognostic impact of the four major genomic groups of endometrial described by the TCGA have recently been examined [119].

In cervical cancer, FIGO stage and type, tumor size and vascular space invasion are important clinicopathological markers. Potential prognostic molecular markers include, among others, the proteins VEGF-A, MDV/CD31, CHI3L1, SPHK1, Galectin 1, MMP-9, squamous cell carcinoma antigen, specific cytokines and $p16^{INK4a}$ [120-124].

1.5.2 Predictive biomarkers

Predictive markers identify patients most likely to respond to a given therapeutic intervention [108].

In endometrial cancers, predictive biomarkers are needed to aid optimal selection of patients for conventional therapies, such as chemotherapy and hormonal therapy where response rates currently are low [109]. Yet, no molecularly based biomarkers are in present use to guide adjuvant treatment of endometrial cancer. Hormonal treatment is often initiated without assessing hormone receptor status, despite evidence demonstrating better response and longer survival for patients with positive receptor status [125]. Stathmin, a regulator of microtubule dynamics, is suggested as a potential predictive biomarker for response to taxanes [126, 127]. Another emerging biomarker is MSI status for selection of pembrolizumab treatment [128].

In cervical cancer, no biological marker has yet consistently predicted response to treatment and, therefore, no such marker has been implemented in clinical practice to date [129, 130]. Potential candidates have been suggested, among them expression of the prostaglandin enzyme cyclooxygenase (COX-2) was found to associate with poor response to chemoradiotherapy in stage IIB cervical adenocarcinomas [131].

1.5.3 Biomarker driven therapies

The translation of NGS into oncology practice has revealed that the genetic landscape of a tumor may be just as important as the site of origin when considering treatment strategies [132]. Investigating rare mutations in clinical trials can be challenging, and even the most effective targeted therapies fail to impress when evaluated in the wrong patient population. The concept of 'basket trials' is based on the hypothesis that the presence of a molecular marker predicts response to a targeted therapy independent of tumor histology [133]. Based on identified genetic biomarkers, patients are selected into different specific treatment arms [134].

However, a recent review [135] advocate the importance of also considering the tissue-specific signaling of cancer genes, and to take the anatomical site into account ensuring that the right patients are enrolled in the right 'basket'. 'Umbrella trials' stratify tissue-defined cancer populations into molecularly defined subgroups, some of which might be rare [133, 134]. Similar to basket trials, umbrella trials are multi-institutional projects, typically with a common molecular screening platform [133]. A third emerging clinical trial approach is called 'phase 3 enrichment design', in which tumors specified to a single body site or of a single histological type is screened for single mutations. Patients with actionable mutations are identified and randomly assigned to the test drug or control [134].

1.6 Targeted therapy in cancer

The systemic use of chemotherapy relies on cytotoxic agents disrupting mitosis in rapidly dividing cancer cells, with predictable dose-limiting toxicities. Targeted therapy is directed specifically towards molecular mechanisms promoting cancer cell

survival and growth, enabling treatment designed towards specific tumor features. Targeted treatment strategies for uterine cancers are generally limited [88, 136]. Absence of predictive biomarkers and the fact that most trials have been conducted in heavily pretreated patients have likely contributed to the inadequacy.

1.6.1 HER family targeting treatments

One example of targeted treatment successfully implemented for clinical use in several cancer types is through the epidermal growth factor receptor (ErbB) receptor family. This includes four members: EGFR (HER1), HER2, HER3 and HER4. Upon ligand binding, these receptors dimerize, causing activation of intracellular tyrosine kinases by phosphorylation and signal transduction, which subsequently lead to cellular growth. While cetuximab, erlotinib, gefitinib and afatinib are approved drugs directed towards EGFR, trastuzumab and pertuzumab are directed towards HER2 and lapatinib towards both these receptors (**Figure 5**). The antibody-drug conjugate (ADC) trastuzumab emtansine, which delivers the cytotoxic agent emtansine into HER2 expressing cells, has recently shown effect in recurrent or metastatic HER2-positive serous endometrial cancer [137]. TDM-1 binds to HER2, leading to internalization of the complex, thus delivering the cytotoxic agent selectively to HER2-expressing cells [138]. Other drugs, such as ONT-380, neratinib, immunogenic approaches (HER2 vaccines and immune checkpoints inhibitors combined with HER2 targeting agents) and combination therapies with trastuzumab and downstream inhibitors (such as PI3K pathway inhibitors and CDK4/6) are being tested in HER2-positive breast cancer [139, 140] (**Figure 5**).

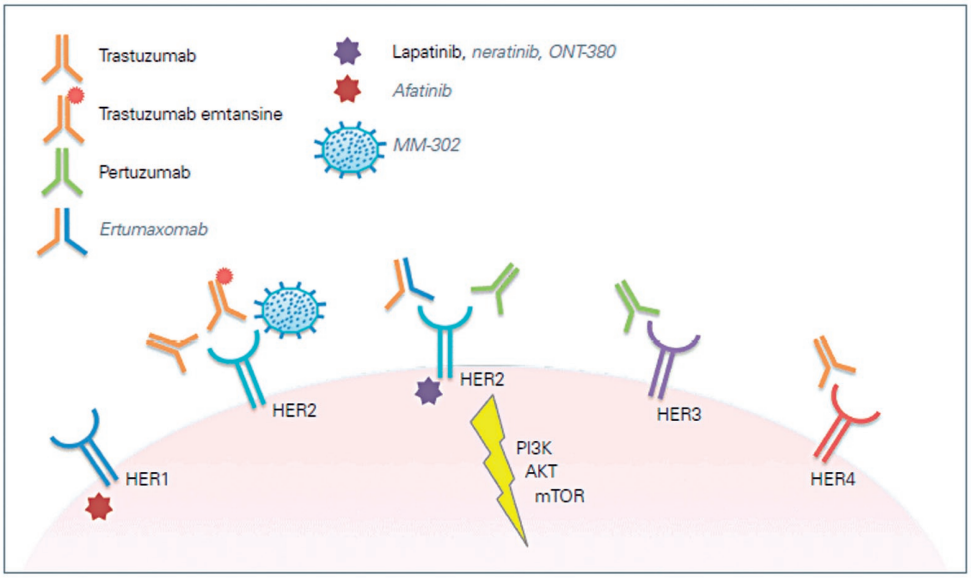

Figure 5 HER family targeting agents.
US FDA-Approved drugs and investigational agents (*in gray*).
Abbreviations: HER: Human epidermal growth factor receptor; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide 3-kinase.
Figure reprinted with permission from Cancer Network/Wright's Media [141].

1.6.2 Targeted therapy in endometrial cancer

A significant proportion of endometrial carcinomas – low-grade endometrioid, in particular – express estrogen receptor and/or progesterone receptor, described to be predictors of favorable survival [142]. Hormonal therapy is an adjuvant treatment opportunity for some of these patients, and include progestogens, selective estrogen receptor modulators (SERM), AIs and GnRH inhibitors [143].

Endometrial carcinoma harbor specific, sometimes overlapping, molecular alterations in significant subsets of tumors, making it eligible for targeted treatment [89]. One of the most altered pathways is the PI3K/PTEN/AKT/mTOR pathway [88, 109]. However, the clinical impact of utilizing deregulation of pathways such as PI3K/AKT, HER and MAPK in a prognostic or predictive manner warrants further investigation [143]. Likewise, the cell cycle and DNA repair pathways constitute potential targets

for future precision therapy [143]. Targeting the microenvironment and more recently the immune infiltration are other promising opportunities [144].

The POLE ultra-mutated and MSI subtypes identified by the TCGA study are associated with high neo-antigen loads and numerous tumor-infiltrating lymphocytes (TILs), which is compensated by overexpression of PD-1 and PD-L1 [145]. Pembrolizumab recently gained US Food and Drug Administration (FDA) approval (https://www.fda.gov/) for treatment of patients with MSI high tumors and progressive disease, also including endometrial cancer MSI tumors [128]. Pembrolizumab targets the cellular pathway of PD-1/PD-L1. By blocking this pathway, pembrolizumab may aid the body's immune system in fighting cancer cells.

1.6.3 Targeted therapies in cervical cancer

Targeted treatment options for women with metastatic, persistent, or recurrent cervical cancer are limited. The VEGF inhibitor, bevacizumab (in combination with paclitaxel and either cisplatin or topotecan) was recently approved by the US FDA for the treatment of persistent, recurrent, or metastatic cervical cancer based on a randomized phase III trial (GOG-240) [146]. Reports from prolonged follow-up of the 452 included patients was recently published showing a sustained treatment benefit, evidenced by overall survival curves remaining separated [147].

The recent cervical cancer TCGA study revealed amplifications in immune targets such as PD-L1/2 and BCAR4lncRNA [98]. BCAR4 is a metastasis-promoting lncRNA that enhances cell proliferation in estrogen-resistant breast cancer by activating the HER2/3 pathway [148]. Lapatinib, the dual EGFR/HER2 inhibitor, counteracts BCAR4-driven tumors growth *in vitro*, and warrants evaluation of effect in BCAR4-positive cervical cancer [149]. *ERBB3* (HER3) was one of the significantly mutated genes identified in the TCGA study with immediately applicable therapeutic targets (**Figure 5**). Furthermore, there was a strong tendency for co-occurrence of *ERBB2* and *ERBB3* alterations within adenocarcinomas, indicating a potential benefit from HER2 and HER3 targeted therapies. The discovery of an endometrial-like cervical cancer subtype characterized with *KRAS*, *ARID1A* and *PTEN* mutations is another intriguing finding

from the TCGA study with PTEN and possibly ARID1A proteins being therapeutic targets.

EGFR immunohistochemical expression has been detected in up to 85% of cervical cancer patients and overexpression has been associated with poor prognosis [129]. Still clinical trials with the EGFR inhibitors gefitinib [150], erlotinib [151], and cetuximab [152] have so far not detected any objective responses. Other ongoing clinical trials are currently testing HDAC [153], mTOR [154] and PARP (NCT01237067) inhibitors.

Immunotherapy is an attractive strategy for cervical cancers because these tumors nearly universally harbor the constitutively expressed oncogenic HPV E6 and E7 antigens. Objective tumor regression in patients with metastatic cervical cancer has been reported after treatment with tumor-infiltrating T cells selected for reactivity against HPV E6 and E7 [155]. Recently, the importance of targeting non-viral antigens in HPV-driven cancers has also been demonstrated [156]. Therapeutic vaccines targeting E6 and E7 are also being tested [103]. Clinical trials testing the checkpoint inhibitors pembrolizumab (NCT02635360, NCT03192059) and nivolumab (NCT02379520) in combination with HPV-specific T cells from the patients' blood are ongoing.

2. AIMS OF THE STUDY

2.1 Background

The two main uterine cancer types, endometrial and cervical carcinoma, represent a major worldwide female health problem. Endometrial cancer is the most common gynecological malignancy in industrialized countries, and the incidence is increasing. Cervical cancer annually accounts for 266,000 deaths, being the fourth leading cancer type among women. Although prognosis is generally good for early-detected disease for both cancer types, patients with metastatic uterine cancer at presentation or recurrent disease have few treatment options and prognosis is poor, despite the use of standard chemo- and/or radiotherapy. Still, no approved targeted treatment exists beyond bevacizumab for cervical cancer and hormonal therapy for some endometrial cancer patients. Thus, identification of novel biomarkers and treatment targets is vital in order to improve risk stratification and treatment for these patients.

2.2 General aims

The overall aims of this study were to identify molecular alterations suggesting new treatment strategies for patients with advanced or recurrent uterine carcinoma.

To ensure that identified molecular alterations are representative of a true patient population, the impact of selection of high tumor cell content when performing molecular profiling needed to be established. Subsequently, the general aim was to identify candidate protein markers and to identify their potential as prognostic and/or predictive markers for uterine carcinoma. Furthermore, we aimed to link the expression of candidate markers with clinical features and transcriptional profiles both in endometrial and cervical carcinomas. Eventually, to assess the clinical utility of such markers, we aimed to identify the protein expression patterns in primary and matched metastatic endometrial cancer lesions and to identify potential mechanisms of resistance to suggested targeted therapies in endometrial cancer.

2.3 Specific aims

Identify potential differences in clinicopathological features and survival between patients with high (≥80%) versus low (<80%) tumor cell content as assessed in fresh tissue of endometrial (**Paper I**) and cervical (**Paper II**) carcinoma.

Validate a set of well-defined clinicopathological markers in a large population-based, prospectively collected cervical cancer cohort with revised histology to evaluate their use in the clinical setting (**Paper III**).

Classify the prognostic impact of CNV, mutational status, mRNA expression and protein levels of *ERBB2*/HER2 and *TP53*/p53 in cervical carcinoma (**Paper III**).

Identify HER2 protein levels and the prognostic value of the HercepTest compared to the staining index in a large endometrial cancer cohort, also including complex atypical hyperplasia and metastatic lesions (**Paper IV**).

Classify HER2 expression patterns between hyperplasia, different histological subtypes of endometrial cancer, and in primary and matched metastatic lesions (**Paper IV**).

Examine the interplay between HER2 and FOXA1 in endometrial cancer by means of using a large cohort of patients and cancer cell lines (**Paper V**).

3. MATERIALS AND METHODS

3.1 Patient series

Tissue from patients treated for endometrial hyperplasia (**Paper IV**), endometrial cancer (**Paper I, IV and V**) (including metastatic lesions in **paper IV**) and cervical cancer (**Paper II and III**) at the Haukeland University Hospital were prospectively collected in a population-based setting from 2001. Haukeland University Hospital is a referral hospital for patients in Hordaland County in Western Norway, with a population of 520 000, representing about 10% of the Norwegian population. The incidence and prognosis in Hordaland is similar to the total Norwegian population (Cancer Registry of Norway, http://kreftregisteret.no). However, the endometrial hyperplasia cohort is not population-based, as it only includes patients that underwent hysterectomy, excluding those conservatively treated. All patients gave informed consent before inclusion and were treated according to standard guidelines.

3.2 Clinicopathological data

For the endometrial and cervical cancer patients, clinical data including age at primary diagnosis, menopausal status, FIGO stage, clinical tumor size, DNA ploidy, recurrence, treatment and follow-up were collected by review of patient hospital records and from correspondence with responsible physician if follow-up was continued outside hospital. All cancer patients were staged according to the FIGO staging system from 2009 (**Table 1** and **2**). In **paper IV**, hysterectomy specimens from 67 patients diagnosed with complex atypical hyperplasia prospectively collected from 2001-2012 was included. Clinical information was collected as for the uterine carcinoma patients, but no follow-up was recorded and data regarding follow-up is thus missing. In **paper III**, an experienced pathologist (BB) performed a histopathological revision on selected formalin fixed paraffin embedded (FFPE) sections from representative areas with invasive tumor for assessment of histological type and grade, depth of invasion, vascular space invasion and inflammatory reaction. Histopathological diagnosis and

myometrial infiltration of endometrial precursor- and cancer lesions were retrieved from routine pathological reports.

3.3 FFPE tissue processing

3.3.1 Tissue sections and tissue microarrays

FFPE tissue was utilized for construction of tissue microarrays (TMAs). Areas with representative tumor or hyperplasia were selected from hematoxylin and eosin stained full sections by an experienced gynecologic oncologist (HS, hyperplasia and endometrial cancer hysterectomy specimens, **Paper IV and V**) and an experienced pathologist (BB, cervical cancer biopsy or primary surgery specimens, **Paper III**). For heterogenic tumors, areas with the least differentiated and highest tumor cell density were selected. By a custom-made precision instrument (Beecher instruments, Silver Spring, USA), three tissue cylinders with a diameter of 0.6 mm were punched out from tumor selected areas (three from primary tumors and hyperplasia and one to three from metastatic lesions) and mounted into a recipient paraffin block.

3.3.2 Immunohistochemistry

Immunohistochemistry (IHC) was performed on TMA sections (5 μm) which were dewaxed in xylene and rehydrated in graded ethanol. Epitope retrieval was performed by boiling in target retrieval buffer Tris-EDTA pH9 (Dako, Denmark) in microwave oven for 15 minutes (p53 and HER2, **Paper III-V**) or 20 minutes (**FOXA1, Paper V**). Endogenous peroxidase was blocked by a peroxidase blocking agent (S2023, Dako, Denmark). For FOXA1 (**Paper V**), sections were additionally also blocked with serum free protein block (X0909, Dako, Denmark). For all antibodies, incubation was performed in room temperature according to optimized protocols (**Table 4**). The EnVision visualization system was used with a horseradish peroxidase (HRP)-conjugated secondary antibody and Diaminobenzidine chromogen (DAB+, K 4010, Dako, Denmark). Counterstaining with hematoxylin (S2020, Dako, Denmark) was performed before dehydration and mounting. Details of each staining protocol are outlined in **Table 4**. All antibodies applied in this study were commercially available.

Table 4 Staining protocols for immunohistochemistry

Protein targeted	Antigen retrieval	Primary antibody	Provider	Dilution	Incubation
p53 (**Paper III**)	Tris EDTA pH9	M-7001	Dako, Denmark	1:1000	1h
HER2 (cervical cancer series, **Paper III**)	Tris EDTA pH9	A 0485	Dako, Denmark	1:250	1h
HER2 (endometrial cancer series, **Paper IV** and **V**)	Tris EDTA pH9	A 0485	Dako, Denmark	1:400	1h
FOXA1 (**Paper V**)	Citrate buffer pH6	Ab23738	Abcam, UK	1:800	30 min

Abbreviations: EDTA: Ethylenediaminetetraacetic acid

For all included biomarkers, the staining protocol was optimized and staining was evaluated against a tissue sample with known expression of the antigen.

3.3.3 Evaluation of staining

For hyperplasia, primary tumors and a subset of the metastatic lesions, three cores per patient were available; these were given one combined score. TMA sections stained for p53, HER2 and FOXA1 were evaluated by the staining index (SI) where both intensity of the staining and portion of positive tumor cells were considered. SI is a semi-quantitative and subjective grading system described in several earlier publications [114, 157, 158], calculating the product of staining intensity (0-3) and area of stained tumor cells (0= no staining, 1=<10%, 2=10-50% and 3=>50%). In addition, sections stained for HER2 were assigned a score from 0 to 3+ in accordance with the HercepTest criteria [159, 160] and cytoplasmic HER2 staining was considered non-specific and excluded from scoring. Samples were classified as HercepTest score 0 when completely negative for staining or membranous staining in less than 10% of tumor cells, a HercepTest Score higher than 0 required 10% of cells having weak/barely visible membrane staining (1+), moderate staining of the entire tumor cell membranes (2+) or strong complete membrane staining (3+).

Cut offs of selected biomarkers

For p53, low IHC score generally reflects wild type p53 due to its very short half-life [161]. In contrast, mutant p53 is often more stable and accumulates to high levels therefore often detected as overexpressed p53 by IHC [162]. Based on this, in **paper III**, SI 0 was considered negative, 1-3 as intermediate and 4-9 as high p53 expression. In **paper III** and **V**, HER2 levels were evaluated solely according to the HercepTest criteria, contrasting paper **IV** were also the SI was applied. Tumors were grouped as low HER2 (HercepTest 0-2+ or high HER2 (HercepTest 3+) according to review of relevant literature and HercepTest guidelines adapted from breast cancer diagnostics [163], and as low HER2 (SI 0-6) or high HER2 (SI 9) based on comparison of survival curves of primary tumors in Kaplan Meier analysis. For FOXA1 (**Paper IV**), data was grouped into tertiles based on the frequency distribution of the marker, size of each subgroup and number of events in each group using a Kaplan Meier survival curve. The cut-offs for all antibodies applied are listed in **Table 5**. For patients with multiple metastatic lesions available, each individual metastasis was scored.

Table 5 Cut off for biomarkers

Protein	SI Loss/low	SI Int	SI High	HercepTest Low	HercepTest High
p53 (Paper III)	0	1-3	4-9		
HER2 (Paper III-IV)	0-6		9	0-2+	3+
FOXA1 (Paper V)	0-4		6-9		

Abbreviations: SI: staining index; exp: expression; int: intermediate

3.3.4 Chromogen in situ hybridization (CISH)

In **paper IV**, *ERBB2* amplification status was analyzed on TMA sections (5 µm) using CISH for 169 metastatic endometrial cancer lesions with overlapping IHC results. The CISH assays were run at the Department of Pathology, University of Bergen following the manufacturer's protocol (BenchMark Ultra, Ventana Medical Systems, USA). The CISH evaluations were performed blinded and without knowledge of the HER2 scoring from IHC. For each cancer lesion, visible gene probe signals were counted in 20 nuclei by standard light microscopy, and mean gene probe signals per cell were calculated and defined as absolute gene copy-number. In line with the American Society of

Clinical Oncology (ASCO)/College of American Pathologists (CAP) guideline recommendations [164], normal copy-number was defined as an absolute *ERBB2* copy-number <4, borderline amplification as 4 to 6 and amplified *ERBB2* as ≥6 gene copies.

3.4 Fresh tissue processing

During surgery, tissue was collected for routine clinical histopathological assessment at the pathology department (FFPE tissue, subsequently used for TMA construction) for all patients. In parallel, and for the majority of patients, biopsies from representative parts of the tumor were collected during surgery, snap-frozen in liquid nitrogen and stored in the local biobank. Whenever possible, FFPE tissue and/or snap-frozen tissue were collected from recurrent lesions. These were paired with the related primary tumor samples for analysis in **paper IV**. The frozen specimens were mounted in 'Mounting Medium for Cryotome' (VWR Chemicals, Radnor, USA) and sectioned using a Cryotome (ThermoFisher Scientific, Waltham, USA) at -20°C. Sections were subsequently stained with hematoxylin and a gynecologic oncologist (HS) estimated the proportion of neoplastic cells by light microscopy for all samples utilized for further molecular profiling techniques. Inclusion criteria for such analysis were at least 50% tumor cell content, and preferably 80%.

3.4.1 Genomic analysis

DNA from cervical cancer tissue (**Paper III**) was extracted from the snap-frozen biopsies. DNA was isolated by digestion over night at 65°C in lysis buffer containing proteinase K, followed by standard ethanol precipitation. DNA from blood was extracted using a standard Qiagen DNA extraction kit according to the manufacturer's protocol (**Paper III**).

The copy-number and mutational data utilized in **paper III** were retrieved from our already published whole exome sequencing (WES) data [97]. Briefly, DNA from 100 tumor/normal paired samples was subjected to Agilent Sure-Select Human All Exon v2.0 based hybrid selection followed by exome library construction for Illumina sequencing.

HPV typing was done by 2 multiplex HPV DNA PCR methods: the fluorescent F-HPV assay [165] and the mass spectrometry-based HPV PCR-Mass Array [166, 167], also formerly published [97].

3.4.2 Transcriptomic analysis

According to above mentioned tumor cell content criteria, fresh frozen tissue was selected for RNA extraction (**Paper III-V**). Total RNA was extracted using the RNeasy kit (Qiagen, Germany) according to the manufacturers' protocol including DNase treatment. To avoid buffer contaminants, one extra wash step with 70% ethanol was introduced before eluation in 50 µl RNase-free H_2O. Quality and yield of total RNA was assessed by spectrophotometry (Nanodrop 1000, Thermo Scientific). Bioanalyzer 2100 (Agilent Tech, California, USA) and agarose gel electrophoresis was applied to ensure RNA integrity and sufficient quality.

The RNA sequencing data utilized in **paper III** were retrieved from our previously published paper [97]. Briefly, cDNA transcriptome libraries were constructed according to standard methods and sequencing was performed by Illumina HiSeq 2000 instruments (Illumina, San Diego, US).

For the endometrial cancer series (**Paper IV** and **V**), cy3-labeled cRNA was constructed and hybridized on Agilent Whole Human Genome Microarray 44k (Cat. No G4112F) according to the manufacturers' instructions. The arrays were scanned using the Agilent Microarray Scanner Bundle. Medium spot signals were used as intensity measures. Expression data were quantile normalized and analysis of differentially expressed genes were performed by using the software JExpress (Molmine, Bergen, Norway). The data set utilized in **paper IV** and **V** included expression values for 20-22 000 genes represented by 41,001 probes.

In **paper V**, Gene Set Enrichment Analysis (GSEA) was utilized to identify gene sets differentially expressed between tumors with high HER2 levels (HercepTest 3+) and high or low FOXA1 levels. The Molecular signature database (MSigDB) v4.0 C2 Curated gene set collection (http://software.broadinstitute.org/gsea/msigdb) was

applied. These gene sets are generated from PubMed publications and online pathway databases, thus providing valuable insights of underlying molecular processes.

3.5 Cell line studies

The two endometrial cancer cell lines used in **paper V** were Ishikawa (Sigma-Aldrich, St. Louis, USA) and Hec1B (American Type Culture Collection, ATCC, Manassas, USA). These lines were obtained in 2009 and authenticity was verified by short tandem repeat (STR) profiling in 2012 [168]. FOXA1 genomic regions were identified by the cross-linking (X)-ChIP protocol, as previously described [169]. Library was constructed following the TruSeq DNA sample preparation kit instructions from Illumina. Total RNA was obtained by using the total RNA isolation kit according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, USA). Cell proliferation and migration assays were performed by live cell imaging Incucyte (Essen BioScience, Ann Arbor, USA). For westers blotting, cells were lysed and protein levels quantified by BSA assay (BSA Assay, Thermo fisher). Protein lysate was resolved using precast SDS-PAGE gels and blots were blocked and incubated overnight at 4°C with the following primary antibodies: β-Actin (4970S, Cell signaling, Danvers, USA), FOXA1 (ab55178, Abcam, Cambridge, UK) and histone H3 (ab1791, Abcam, Cambridge, UK). Chromatin fractionation was performed according to standard methods. (Siv Gilfillan, Sachin Sign and Madhumohan R. Katika led by Dr. Antoni Hurtado performed the cell line studies at the Centre for Molecular Medicine Norway (NCMM), University of Oslo.)

3.6 Statistical methods

The data were analyzed using the software package SPSS (Statistical Package of Social Science) (IBM, Armonk, USA). All p-values were two-sided and considered statistically significant if <0.05. Kappa (κ) value was calculated to assess the inter observer agreement; weighted kappa (κ_w) were used for ordinal categories. The Student t-test was applied when comparing means. Categorical associations were assessed by

Person χ^2 test, substituted by Fisher's exact test if estimated expected counts were less than five. Continuous variables were compared using the Mann-Whitney U Test, replaced with the Kruskal-Wallis test when more than two independent samples were tested. Univariate survival analysis was performed using the Kaplan-Meier (product-limit) method, and differences in survival between groups were calculated by the log-rank test (Mantel-Cox). The prognostic impact of histo- and clinicopathological variables was compared using the Cox proportional hazard regression model.

3.7 Approvals

The Norwegian Data Inspectorate (961478-2), the Norwegian Social Science Data Service (15501) local ethics committee (REK 052.01, Paper I-II; REK 2009/2315, Paper III-V) approved the research. Participants gave informed written consent.

4. MAIN RESULTS

Paper I

Comparing clinicopathological features from women treated for endometrial cancer with high tumor cell content (≥80%) and low (<80%) in snap-frozen samples revealed significant differences. High tumor purity linked to high patient age, and a mean age at diagnosis of 67 years as compared with 63 years for patient with <80% tumor cell content (p=0.01). In addition, ≥80% tumor cell content was significantly associated with high-grade, non-endometrioid histology and peri- or postmenopausal status. No significant association with FIGO stage or presence of metastatic lymph nodes was found. Patients with ≥80% tumor cell content had poorer disease specific 5-year survival rate (76%) than patients with <80% tumor cell content (86%) (p=0.02). The 5-year disease specific survival rate for all endometrial cancer patients from the same region and period was 82%.

Paper II

Within cervical cancer patients, high tumor cell content (≥80%) was significantly more frequent in squamous cell carcinomas (SCCs) compared to adenocarcinomas (ACs) (p=0.03). Within the ACs (n=40), there was a significant association between high tumor cell content in the fresh frozen samples and later occurrence of recurrent disease (p=0.04). In the SCCs, no significant associations between tumor purity and disease stage, grade or outcome were found. In line with this, grade significantly affected prognosis in ACs, but not in SCCs. Recurrent disease (p=0.02) and FIGO stage IB and II (p<0.001) were significantly more frequent in the patient cohort with fresh tissue available for potential molecular profiling as compared to the cohort where only FFPE tissue were available.

Paper III

In the large population-based prospectively collected cervical cancer patient cohort with revised histology, vascular space invasion, histological type and tumor size verified as strong independent prognostic markers. Increasingly larger clinical tumor

size correlated with poorer survival. Notably, we identified a subgroup of patients with intermediate tumor size (tumors 2-4 cm) with worse prognosis than tumors of 0-2 cm size ($p<0.001$). Histological type other than SCC and AC (HR 12.0, $p<0.001$) and vascular space invasion (HR 6.60, $p<0.001$), were the strongest independent prognostic markers followed by FIGO stage (HR 3.87, $p=0.002$). Interestingly, presence of vascular space invasion could identify poor survivors also within subgroups of general good survival including SSCs ($p=0.004$), ACs ($p<0.001$) and tumors smaller than 4 cm ($p=0.01$). High p53 protein levels were significantly associated with markers for aggressive phenotype and survival in uni- and multivariate survival analysis, but did not reflect *TP53* mutational status. High HER2 protein levels were identified in 21% of all tumors. Amplified *ERBB2* significantly linked to poor survival, while HercepTest did not.

Paper IV

Expression levels of HER2 were investigated in a large cohort of endometrial cancer lesions, also including complex atypical hyperplasia and metastatic lesions. High HER2 protein levels assessed by the HercepTest and SI criteria were significantly associated with higher age, stage, histological type and grade, as well as poor outcome. mRNA levels of *ERBB2* were positively correlated to protein levels of HER2, as measured by the HercepTest criteria ($p=0.01$). HER2 expression defined by SI criteria proved to be a better predictor of survival in endometrial cancer compared to HercepTest, also within subgroups of ER and/or PR negative patients. High HER2 expression was more abundant in lesions with aggressive histology, with peak level in serous carcinomas (49%). A heterogenic HER2 expression pattern between matched primary and metastatic lesions was revealed, with a substantial reduction in HER2 expression from primary to metastatic disease.

Paper V

In this study, we investigated the interplay between FOXA1 and HER2 in relation to prognosis and therapeutic implications by utilizing endometrial cancer tumors and cell lines. Within HER2 overexpressing tumors, FOXA1 expression was significantly and independently associated with improved prognosis. The comparison of genes differentially expressed between FOXA1-high *vs.* FOXA1-low within the HER2-high subgroup of endometrial carcinoma patients revealed a gene signature associated with anti-EGFR treatment response. Several of the genes included in this signature were identified as important for receptor tyrosine kinase (RTK) signaling. In the endometrial cancer cell lines, FOXA1 bound to a significant number of these genes. Increased FOXA1 expression attenuated the expression of these genes through recruitment of polycomb-associated proteins to FOXA1 binding sites. The FOXA1 induced attenuation of these genes led to improved response to anti-EGFR/HER2 therapies. This study suggests that HER2-high tumors with low levels of FOXA1 possess innate resistance against HER therapies.

5. DISCUSSION

5.1 Methodological concideration

5.1.1 FFPE tissue processing

Tissue microarrays

The TMA method enables high-throughput, cost and time effective analysis and reduces the amount of tissue used. It also reduces the risk of batch effects, as more samples are stained simultaneously ensuring identical conditions. One limitation of this method is that selected areas may not be representative to the entire tumor, as TMAs do not offer the same morphological information as full sections. Thus, sampling from multiple areas of the tumor is recommended. These recommendations were followed in this thesis (**Paper III-V)**. Moreover, several studies have validated the TMA method, and when comparing scoring results from TMA cores with corresponding full tissue sections, high concordance has been demonstrated [170-172]. Although TMA is a very useful tool in a research setting, markers explored by this technique should be validated using full sections before clinical implementation.

Immunohistochemistry

IHC is a frequently applied method for protein detection, both in research and clinical settings. It enables subcellular detection of proteins (nuclear, cytoplasmic, membrane or extracellular), but does not allow precise and objective quantification of protein levels. Reverse phase protein arrays (RPPAs) or mass spectrometry techniques could be applied if a more quantitative measure is required. The validity and robustness of antibodies to detect prognostic impact are debated, as the quality of results is highly dependent on the sensitivity and specificity of the antibody and on the selected tissue. However, when sufficient antibody optimization and validation have been conducted, IHC can be introduced as a robust and clinically applicable tool to guide treatment.

Evaluation of staining

Two different scoring systems were applied in this thesis; the staining index and the HercepTest. Our scorings were performed blinded for the patients' clinical and survival

data. The staining index is a well-established scoring method [157] and has been used for several antibodies and in several tumor types [158, 173, 174]. By combining two staining measures (area and intensity), the SI offers a semi-quantitative measure of protein levels.

In breast cancer treatment, standardized IHC and scoring methods for detection of HER2 levels have been developed alongside FISH or CISH and is currently implemented in routine settings to identify candidates for HER2-directed treatment [175, 176]. Both the intensity and area of staining are considered, resulting in four different categories. Only membrane staining is evaluated, with the argument that HER2 is only active and targetable when located in the membrane. These successfully clinically implemented guidelines for breast cancer treatment were followed in **paper IV** and **V** in order to evaluate their possible clinical utility also within endometrial carcinoma.

In the process of implementing new immunohistochemistry based biomarkers into the clinic, several steps of optimization and standardization of staining protocols and cut-offs are important. In **paper IV**, the established HercepTest was compared against the SI for HER2 protein detection. There was a high concordance between the two methods, yet the SI identified a subgroup of patients with strong staining in ≥50% of the selected tumor area with a particular poor survival. This scoring method had predictive value also within subgroups with poor survival and few treatment options, such as ER/PR negative and non-endometrioid patients. Interestingly, the SI also identified a group with poorer survival within the HercepTest 3+ patients. These findings indicate that the prognostic value of the HercepTest could be improved if cut offs are optimized for endometrial carcinoma.

In **paper III-V,** we used the A0485 antibody. Yet, others do exist, such as 4B5 and SP3. In gastric cancer, where trastuzumab is FDA approved as a HER2-targeting agent, 4B5 was found to be more accurate than the HercepTest and full sections more accurate than TMAs in detecting anti-HER2 responsive tumors [177]. This study indicates that in gastric cancer, the number of cells that respond to anti-HER2 therapies directly

affects the tumor's responsiveness to treatment, and that there is a significant difference between cases that are diffusely positive and those that are only focally positive but still meet the criteria of HER2 positivity. Such studies exploring different antibodies, scoring criteria and TMAs versus full sections should be conducted also for endometrial carcinoma to further optimize clinical utility.

Chromogen in situ hybridization (CISH)

In **paper IV**, the CISH results were scored according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) recommendations. Guidelines of *ERBB2* copy-number scoring are successfully implemented for clinical breast cancer treatment [175, 176], and we wanted to explore their clinical utility also in endometrial carcinoma. In the process of potential implementation of HER2/*ERBB2* testing into clinical settings for endometrial carcinoma, guidelines for identifying patients for HER2-directed therapy based on *ERBB2* copy-number should be refined through clinical trials. Although CISH is used as a standard *ERBB2* detection method in several hospitals, fluorescent in situ hybridization (FISH) is considered the 'gold standard' for copy-number assessment in clinical settings. The clinical utility of both these hybridization techniques needs to be further explored and optimized for endometrial carcinoma. Our results of *ERBB2* amplification status could be further strengthened by inclusion of FISH.

5.1.2 Fresh tissue processing

In **paper I** and **II**, we observed that the inclusion criterion of ≥80% tumor purity introduced a selection bias towards inclusion of more aggressive uterine adenocarcinomas. In **paper III-IV**, the inclusion criterion for DNA and RNA extraction and subsequent molecular profiling techniques (cDNA microarrays, RNA sequencing, WES and HPV typing) was preferably ≥80% and minimally 50% tumor purity. In **paper III**, we performed a comparison of clinicopathological features among the selected cervical carcinoma patient groups, yet no selection biases between groups were found (**Table 3**). Although no significant difference in the patient groups were detected, tumors selected for WES and RNA sequencing are not population-based and

thus findings from these patient cohorts should be validated by other methods and in population-based series to assess clinical relevance. Our findings from **paper I** substantiate that also the inclusion of patient samples to mRNA microarray contain selection biases enriching for aggressive endometrial carcinoma, and that the transcriptional data utilized in **paper IV** and **V** should be validated in population-based settings.

Table 3 Distribution of clinical characteristics for 401 patients treated for primary cervical carcinomas at Haukeland University Hospital, Bergen, Norway, 2001-2014, and within subgroups for additional biomarker studies. No significant differences in distribution of the clinicopathologic features among groups were observed*.

Series (total n)	***Whole cohort (401)***	***IHC (292)***	***SNP/WES[a](100)***	***RNAseq (79)[b]***
Variable	n (%)	n (%)	n (%)	n (%)
Median age at diagnosis	*43*	*42*	*43*	*42*
FIGO stage	*n=400*	*n=292*	*n=100*	*n=79*
I	300 (75)	224 (77)	78 (78)	66 (84)
II-III-IV	100 (25)	68 (23)	22 (22)	13 (16)
Clinical tumor size	*n=252*	*n=208*	*n=86*	*n=69*
< 4 cm	181 (72)	153 (74)	57 (66)	47 (67)
≥ 4 cm	71 (28)	55 (26)	29 (34)	22 (33)
Histological subtype	*n=401*	*n=292*	*n=95*	*n=76*
SCC	282 (70)	198 (68)	61 (64)	46 (60)
AC	90 (23)	70 (24)	24 (25)	21 (28)
Others	29 (7)	24 (8)	10 (11)	9 (12)
Histological grade	*n=390*	*n=286*	*n=92*	*n=74*
Grade 1/2	334 (86)	240 (82)	76 (83)	59 (80)
Grade 3	56 (14)	46 (16)	16 (17)	15 (20)

IHC: immunohistochemistry; *TMA*: tissue microarray; *WES*: whole exome sequencing; *SNP*: single nucleotide polymorphism; *FIGO*: The Féderation Internationale de Gynécologie et d'Obstétrique; *SCC*: squamous cell carcinoma; *AC*: adenocarcinoma

*Pearson's chi-square test (Asymptotic significance 2-sided) all $p<0.05$.

[a]92/93 cases with available SNP/WES data have overlapping IHC data for HER2 and p53, respectively.

[b]75/76 cases with available RNAseq data have overlapping IHC data for HER2 and p53, respectively.

Adapted from Halle *et al [178]*.

At Haukeland University Hospital, we have a large population-based prospectively collected uterine carcinoma study cohort. The combination at our institution of uterine carcinoma study cohorts with population-based prospective inclusion, detailed patient

records and parallel FFPE sections with a large number of overlapping fresh tissue samples available for NGS studies is unique.

Genomic analyses

WES data was utilized to detect copy-number variations (CNVs) in **paper III** WES is targeted to protein coding regions, so reads represent less than 2% of the genome. Whole genome sequencing (WGS) is a more thorough approach investigating single-nucleotide variants (SNVs), indels, structural variants (SVs) as well as CNVs in both the ~1% part of the genome that encodes proteins and the ~99% of remaining non-coding regions. However, WES is more cost-effective than WGS. This enables researchers to increase the sample number, an important factor for large population studies. We used WES data to detect CNV and mutation status, and our main objective was to look at single genes (*TP53* and *ERBB2*). However, CNV from WES sequencing could benefit from validation by FISH or CISH, which are methods currently used in cancer diagnostics. Such methods are quick, less expensive and more sensitive in detecting cluster amplifications. However, WES also allows for detection of gene mutations.

Transcriptomic analyses

For gene expression analyses, we utilized both RNA sequencing (**Paper III**) and microarrays (**Paper IV-V**) techniques. While the microarray technique limits the researcher to detect transcripts that correspond to existing genomic sequencing information, RNA sequencing can detect both known transcripts and explore new ones. Subsequently, in contrast to microarrays, which are limited to the reference information during production, RNA sequencing experiments may be updated as new sequence information is generated. Secondly, RNA sequencing yields low background signals. Hybridization issues seen in microarray experiments, such as cross-hybridization or non-ideal hybridization kinetics, are eliminated in RNA sequencing experiments. Finally, RNA sequencing enables quantification of a large dynamic range of expression levels, with absolute rather than relative values. A disadvantage with RNA sequencing is the cost. Even though the price of RNA sequencing is expected to decrease, it is still more expensive than microarrays. However, high consistency in identified

differentially expressed genes has been found between these two techniques [179], and for the issues addressed in this thesis both techniques are adequate. However, validation of global gene expression analyses is important and expression of crucial genes is often validated by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and/or protein expression arrays.

5.1.3 Cell line studies

In **paper V**, we used the human endometrial cancer-derived cell lines Ishikawa and HEC1A. Human cell lines are fundamental models used in laboratories to study the biological processes in cancer, and to test the therapeutic efficacy of cancer treatments. Cell lines offer several advantages; they are cost effective, easy to use, provide an almost unlimited supply of material and bypass ethical concerns often associated with the use of animal and human tissue [180]. Cell lines also offer a relatively pure population of cells, providing a consistent sample and reproducible results, yet some limitations exist. Cell line misidentification is a typical issue. However, the cell lines used in **paper V** were STR profiled, as is the recommended authenticity verification approach [181]. Still, genetic and phenotypic variations may be introduced with serial passages. The cell lines used in our experiments were kept frozen on liquid nitrogen until start-up of every experiment to minimize the number of passages. Cultured cells are selected for rapid growth, which can promote further differentiation from the primary tumors from which they originated. Moreover, the intratumoral heterogeneity and the microenvironment of primary tumors cannot be comprehended when cell lines are used as preclinical models. Hence, findings of possible drug resistance by the use of cell lines, such as in **paper V** should be verified in more advanced models [181]. Recent developments of organoid human endometrium to study endometrial cancer are encouraging [182, 183]. Such preclinical models can represent valuable platforms for drug development and screening, and might assist in narrowing the gap between cancer genetics and patient trials [184].

5.2 Discussion of results

5.2.1 Potential selection biases in patient inclusion for NGS studies

We have recently reported data from NGS studies that point to potential targets for developing new therapies for uterine carcinomas [97, 185]. Most NGS studies demand fresh frozen tissue with little stromal contamination, and often at least 80% tumor epithelial content is applied as a norm [186, 187]. However, this might introduce a selection bias promoting inclusion of a certain subset of carcinomas. In **paper I,** we find that inclusion of samples with high tumor purity leads to enrichment of aggressive endometrial carcinomas. Similarly, within the cervical adenocarcinomas, we find a significant association between high tumor purity and aggressive phenotype and recurrent disease (**Paper II**). In cervical squamous cell carcinomas, no association between tumor purity and stage, grade or outcome was found. In line with this, grade, a measure of amount of solid tumor growth, was found to influence prognosis in adenocarcinomas, but not squamous cell carcinomas. In **paper II**, we further identified a selection bias already at the operation theatre enriching FIGO stage IB-II tumors for fresh tissue assessment, excluding most of FIGO III and IV for fresh tissue and subsequent NGS study inclusion. Patients with high FIGO stages are presently in a particular need for more targeted and effective treatment options [129] and NGS studies utilizing snap-frozen primary lesions may benefit from addressing logistic obstacles related to inclusions of these patients. Improved access to such samples will be vital to allow studies of the presumably most relevant targets for therapy in a systemic disease setting.

Paper I and **II** demonstrate that molecular profiling studies of uterine carcinoma would benefit from a broader and more population-based patient inclusion. This may be achieved by strategies such as performing multiple biopsies and laser capture microdissection. Moreover, improvements in the ability of sequencing DNA from paraffin sections can potentiate a more unbiased inclusion. In addition, advances in the sensitivity of NGS, such as deep sequencing, the application of algorithms that infer tumor purity and detect subclonal heterogeneity [188, 189] and single cell sequencing

[190] are promising to counteract such potential selection biases. Ultimately, inclusion into NGS studies is unlikely to become totally unbiased. Thus, in order to assess the real clinical utility of potential biomarkers identified in such studies, findings need to be validated in population-based settings with well-annotated patient records and follow-up.

The stromal components of epithelial tumors as drivers for cancer progression is an area of intense research [191]. A recent meta-analysis compared the association between the tumor-stroma ratio (TSR) and prognosis [192]. In a broad range of cancer types, this meta-analysis revealed an opposite pattern compared to what we found in the uterine adenocarcinomas (**Paper I** and **II**). In these studies, low TSR defined as less than 50% tumor cell content, associated significantly with poorer overall survival in all cancer types analyzed, except cervical adenocarcinoma where only a tendency of such association was detected. Endometrial cancer were not included in this meta-analysis. However, a recent study of the TSR and relation to survival in endometrial carcinoma FFPE sections, identified high TSR as significantly associated with poor prognosis [193], in line with our findings from the fresh tissue specimens (**Paper I**). The above-mentioned studies in combination with ours (**Paper I** and **II**), indicate that the biology of tumor-stroma interactions and their prognostic influence are not uniform amongst epithelial tumors. Apparently, better characterization of the uterine cancer-associated microenvironments is needed.

5.2.2 Biomarkers for improved cervical cancer treatment

For the prospectively collected population-based cervical carcinoma series including 401 patients presented in **paper III**, an expert pathologist evaluated and revised the histopathological data. The prognostic impact of selected clinicopathological variables was analyzed in the whole cohort. Vascular space invasion, histological type and tumor size were identified as strong independent prognostic markers. Most cervical cancer patients worldwide receive surgical treatments, yet the current cervical cancer FIGO staging system do not consider surgical-pathological data, contrasting endometrial cancer staging practices [47]. A recent comprehensive study including more than 5000

cervical cancer patients, screened for surgical-pathological risk factors and developed a surgical-pathological staging and scoring system (SPSs) including lymph vascular space invasion, stromal invasion and lymph vascular space invasion. This SPS system outperformed the current FIGO staging system in prediction of tumor severity and disease invasion [194]. Based on these and our results we argue that inclusion of vascular space invasion and tumor size 2-4 cm should be considered in new FIGO guidelines, and that more focus should be aimed at identifying rare histological types in cervical cancer diagnostics. Although we recognize such interventions as more applicable in an industrialized setting with adequate skilled pathology service and modern imaging techniques. Additionally, we identified p53 as an independent predictor of survival, yet p53 protein levels did not reflect *TP53* mutational status. Thus, our study identified patients with poor prognosis that would otherwise go undetected using current clinically applied prognostic biomarkers.

5.2.3 Assessment of metastatic biopsies

Intratumoral heterogeneity, which foster carcinogenesis, is a key challenge in cancer medicine [2]. Molecular profiling studies on single tumor-biopsies may lead to underestimation of the entire tumor's genomic landscapes and may potentially complicate development of personalized medicine and novel biomarkers. Despite the increasing awareness of intratumoral heterogeneity, clonal evolution and the competitive release of metastasis-forming subclones, the consequences for treatment management are to a little extent comprehended. Metastatic cancer is responsible for more than 90% of cancer related deaths [19]. Yet, most NGS studies so far, lack inclusions of metastatic biopsies. In the era of individualized cancer therapy, deep knowledge of the molecular landscape in metastatic lesions is required.

In **paper IV**, we found a discordant HER2 expression pattern between paired primary and metastatic lesions, revealing substantial reduction in HER2 expression from primary to metastatic disease. Although this observed discordant expression between primary and corresponding metastatic lesions may be due to methodological factors, tumor heterogeneity may represent another confounding factor. The TMA method is

confirmed to be a reliable system for high-throughput expression profiling by immunohistochemistry [195]; still, only a small portion of the entire tumor is sampled. Important subclones may thus go undetected. Such unrecognized primary tumor subclones could potentially submerge in the metastatic setting. Conversely, detected subclones in the primary tumor may go undetected in the TMA selected areas from the metastatic biopsies. Several studies have shown concordance between full sections and TMA sections [171, 195]. Still, in the clinical setting, utilization of full sections that cover larger parts of the entire tumor could be beneficial for individualized treatment.

Our recent NGS study of endometrial carcinoma comparing primary and matched metastatic biopsies [185], detected extensive heterogeneity and subclonal genetic tumor evolution. Such factors may explain some of the variation in e.g. HER2 status detected between primary and matched metastatic sites (**Paper IV**). Further, endometrial primary tumors are reported to have a complex repertoire of mutational processes [196], another element likely to contribute to the diversity of subclones submerging in the metastatic setting. In line with these findings, our study revealed discordant HER2 expression also between different metastases obtained from the same patients (intra-individual heterogeneity). Our (**Paper IV**) and the above-mentioned studies suggest that in endometrial carcinoma, tissue-based biomarkers (such as HER2) should be evaluated in biopsies from multiple metastatic sites, preferentially in full sections to optimize treatment effect.

In cervical cancer, little is known regarding clonal evolution from primary to metastatic disease. NGS studies on primary cervical carcinomas have observed frequent somatic mutations [97, 98], indicating molecular heterogeneity and subsequent subclonal tumor evolution also in cervical cancer. Still, no comprehensive integrated genomic analysis has been performed on matched primary and matches metastatic cervical carcinoma lesions.

5.2.4 Novel therapeutic strategies in uterine carcinomas

In **paper III**, 5% of the cervical carcinoma patients were identified with *TP53* mutation with an associated poor prognosis. Until recently, *TP53* mutations were largely

considered ‘undruggable’. However, several agents are presently in development that may improve clinical response. The Wee1 inhibitor, AZD1775, acts on a key regulator of the G2 cell cycle checkpoint, which is crucial for DNA damage repair among tumors with dysfunctional p53 [130]. Additionally, numerous molecules are under evaluation with the potential to reactivate mutant p53 or to restore the activity of wild type p53 [197]. Such molecules potentiate a novel treatment strategy for cervical carcinoma patients with *TP53* mutation.

A large number of the cervical carcinoma patients had elevated HER2 levels, still the HercepTest did not identify poor survival, yet *ERBB2* amplification did. *ERBB2* amplifications are known to be associated with HER2 driven tumors [198]. We found that *ERBB2* amplifications significantly associate with worse cervical cancer prognosis, recently also demonstrated in a paper derived from the TCGA [199]. Altogether, findings in **Paper III**) and from the TGGA imply that patients with potential benefit from trastuzumab or similar HER2-directed treatment should be selected based on *ERBB2* amplification status. Yet the high protein levels of HER2 that we found in many of the cervical and endometrial carcinomas (**Paper III and IV**) could point to a potential for HER2-directed trastuzumab-conjugated treatment regimens, such as TDM-1 and SYD985. Trastuzumab-conjugated drugs have the potential of being effective in HER2 expressing tumors even when HER2 is not a cancer driver [200-203]. In preclinical and clinical testing of recurrent or metastatic endometrial serous carcinoma, T-DM1 and SYD985 have shown activity within strong (3+) and low to moderate (1+/2+) HER2 expressing tumors [137, 204]. In **paper IV**, we identified moderate to high HE2 score (≥2+) in 51%, 31% and 29% of metastatic serous papillary, clear cell and carcinosarcomas, respectively. These patients often have poor prognosis, with few existing treatment options [205] and may thus be candidates for HER2-directed ADCs.

Resistance to trastuzumab and other HER2-directed agents have been thoroughly described in endometrial cancer [206]. High levels of oncogenic alteration in the PI3K pathway [8, 89], the truncated p95HER2 variant lacking the trastuzumab binding site [207, 208] and signaling through alternative pathways including other HER family

members such as EGFR and HER3 [209, 210] are factors eligibly involved in trastuzumab resistance. Such mechanism of resistance combined with our observation of an overall lower expression of HER2 in metastases, may explain the trastuzumab resistance experienced in endometrial carcinomas [206]. Altogether, this knowledge should motivate the set-up of clinical trials combining HER2-inhibitors with other agents such as PI3K or mTOR inhibitors attenuating downstream signaling pathway. Additionally, the use of e.g. lapatinib or afatinib, which inhibits the HER2 receptor even when truncated and targets other HER family members such as EGFR could be beneficial. Suggested approaches to overcome HER2-directed treatment resistance are sparsely explored [211], and combination therapy targeting cancer cells simultaneously at multiple checkpoints may prove successful in treatment of HER2-positive endometrial cancer.

In **paper V**, we introduce an alternative factor that may contribute to HER2-directed treatment resistance in endometrial carcinoma. The pioneer factor FOXA1 belongs to a specific class of transcription factors that can interact with compacted chromatin independently of other proteins and directly modulate chromatin structure to facilitate binding of additional transcription factors [212]. In the HER2 overexpressing tumors, FOXA1 expression predicted improved survival. In our *in vitro* experiments, high FOXA1 levels inhibited the EGFR/HER2 triggered proliferation and sensitized the cells to anti-EGFR/HER2 therapies. These findings suggest that HER2-high/FOXA1-low tumors possess innate resistance against HER therapies. Accordingly, our findings may imply that FOXA1 levels should be assessed before initiation of clinical trials with HER2-positive endometrial carcinoma patients to determine the possible role of FOXA1 in sensitizing the tumor to HER2-directed therapies.

HER2 may have a potential as a predictive marker for HER2-directed treatment of uterine carcinomas, but comprehensive clinical trials preselected for HER2 overexpressing and/or *ERBB2* amplified tumors with known molecular profile are warranted to determine clinical utility. If NGS studies are unfeasible, beside HER2 protein and *ERBB2* copy-number assessment, an investigation of FOXA1, p95HER2, HER3 and EGFR levels and PI3K pathway alteration status as a minimum could be

preferable to optimize the drug combination alongside HER2-directed therapy for effective treatment of endometrial carcinoma patients.

5.2.5 Future role of personalized medicine in uterine carcinomas

As genomic tools have become available to identify driver DNA alterations in tumors, treatment of several cancer types is to an increasing extent based on molecular subtypes. This field has been called personalized or precision medicine [134]. Affordable DNA sequencing has enabled clinical trials with preselection of patient subgroups likely to respond. Recent developments of 'basket', 'umbrella' and 'phase three enrichment' clinical trials on preselected patient cohorts are encouraging. Due to difficulties in detecting significant effects in rare histological subgroups, (such as cervical adenosquamous, neuroendocrine and undifferentiated tumors and endometrial serous carcinomas, clear cell carcinomas and carcinosarcomas) the progress of implementing novel therapeutics is lagging behind. These are all patients identified with poor prognosis (**Paper III-IV**), and thus NGS guided clinical trials across histological types and primary site of origin could be novel approaches to drive the discovery and implementation of tailored drugs in these rare diseases. In **paper IV**, we identified such candidates within the rare endometrial cancer histological subtypes with targetable HER2 levels in the metastatic biopsies. 'Basket' designed clinical trials with HER2-directed therapies, based on the HER2 status and genomic profile of the individual tumor, and preferably also metastatic biopsies, could aid to identify novel and effective treatment strategies for such patients.

Exactly how these therapies interact on a cellular basis needs to be addressed before we can consistently alter cell fate [213]. In **paper V,** we identified loss of FOXA1 as a possible resistance mechanism to HER2-directed therapies. Attention should be aimed at identifying such resistance pathways and subsequently; therapies and trials should be designed to target multiple pathways simultaneously. To develop a comprehensive understanding of the genomic alteration and drug resistance in cancer, integrative network approaches have been proposed [214]. Such methods need to be refined in

order to translate molecular changes into drug targets with potential benefit for the individual patient.

The rapidly evolving new technologies for patient risk classification such as NGS and new clinical trial designs will most certainly lead to better management of advanced uterine cancer. Yet, a factor far less expensive and potentially more effective is the prevention of the disease. Increased public awareness of risk factors for uterine cancers, such as obesity for endometrial cancer and risk reducing vaccination programs as well as the importance of attending screening programs for cervical cancers are other important aspects alongside improved treatment for advanced disease. Still, a call for action towards the growing population already diagnosed with endometrial cancer and the many women still suffering from cervical cancer in the developing world in particular, is warranted [35, 215].

6. CONCLUSIONS

A selection criterion of ≥80% tumor purity in fresh tissue promotes enrichment of aggressive uterine adenocarcinomas and associates with poorer prognosis (**Paper I and II**).

In cervical carcinoma, FIGO III and IV are less likely to be included for molecular profiling studies (**Paper II**).

Vascular space invasion and tumor size 2-4 cm are important determinants for prognosis in cervical carcinoma (**Paper III**).

In cervical carcinoma, p53 is an independent predictor of survival; yet do not reflect *TP53* mutational status. Amplified *ERBB2* significantly links to poor survival; HercepTest do not (**Paper III**).

In endometrial carcinoma, high HER2 protein levels measured by the HercepTest associates significantly with aggressive disease and poor survival. SI identified a subgroup of HercepTest 3+ tumors with significantly poorer survival (**Paper IV**).

Loss of HER2 expression is common in metastatic endometrial carcinoma (**Paper IV**).

FOXA1 expression predicts improved survival in patients with HER2 overexpressing endometrial carcinoma (**Paper V**).

In endometrial cancer cell lines, FOXA1 attenuates the expression of genes that enable EGFR/HER2 signaling through interaction with polycomb-associated proteins (**Paper V**).

7. FUTURE PERSPECTIVES

The recent efforts to profile uterine carcinomas by the TCGA and others have unraveled molecular subtypes with potential clinical implications. In order to translate such findings into clinically relevant information, potential selection biases in available patient cohorts must be established. Factors affecting the representability of such patient cohorts can include the distribution of race, sex, stage, histological grade and type as well as life style factors, such as smoking habits. We found that also the tumor-stroma ratio (TSR) was a confounding factor for aggressive disease within uterine adenocarcinomas (**Paper I-II**). Emerging technologies to overcome TSR as a confounding factor in molecular profiling studies include laser-capture microdissection, performing multiple biopsies, algorithms to infer tumor purity, single cell sequencing and the utilization of FFPE tissue for molecular profiling studies. Within cervical carcinomas, logistic obstacles related to inclusions of patients with FIGO stages IA, III and IV need to be addressed (**Paper II**). However, inclusion into molecular profiling studies is unlikely to become unbiased and findings from such studies need to be validated in population-based settings.

FIGO stage is the most important determinant for current treatment decisions for uterine carcinoma patients [48]. In **paper III**, we validated vascular space invasion and tumor size 2-4 cm as important factors predicting prognosis. Our findings indicate alongside other recent publications [194, 216], that the prognostic value of current FIGO guidelines could be improved by inclusion of vascular space invasion and tumor size 2-4 cm. Careful selection of rare histological types, such as adenosquamous, neuroendocrine and undifferentiated tumors could improve risk stratification of cervical cancer patients. Efforts should be made to identify biomarkers that could enhance the classification of these histological types.

In this thesis, biomarkers such as *TP53*/p53 and *ERBB2*/HER2 have been explored for potential prognostic and predictive value (**Paper III-V**). Additional studies are required to determine the clinical utility of such markers. The tremendous effect of HER2 as a predictor for breast cancer treatment is still sparsely exploited in treatment

of other cancer types. One major limiting factor for optimized HER2-directed therapies outside breast and gastric cancer is the lack of biomarker-guided clinical trials. Another potential pitfall might be the gap of knowledge of possible anti-HER2 drug resistance mechanisms. We discovered resistance mechanisms towards HER-directed treatment in endometrial cancer cell lines (**Paper V**). Such mechanisms should be explored in preclinical *in vivo* models. Simultaneously, for patients enrolled in future HER2-directed clinical trials, FOXA1 levels should, in addition to HER2 and *ERBB2*, be determined.

Advances in clinical trial set-ups, especially the 'basket' design, will certainly accelerate the process of implementing targeted therapies to rare subgroups, also including rare uterine carcinoma subtypes. In addition, the clinical utility of rare mutations, such as *TP53* in cervical carcinoma and *ERBB2* in both uterine carcinomas can be better determined in such trial set-ups.

In uterine cancer, targeted therapies are predominantly applicable for patients with metastatic or recurrent disease. To improve treatment for these patients, novel targeted treatment strategies should be based on findings from the metastatic setting. Within the endometrial carcinoma cohort in **paper IV**, we discovered discrepancies in HER2 staining patterns between primary and matched metastatic biopsies as well as between different metastatic biopsies from the same patient. This implies that patient stratification for future clinical trials testing HER2-directed therapies, should be based on evaluation of HER2 status in the metastatic lesions, and if possible, in multiple metastatic biopsies. In line with this, treatment guidelines for breast cancer have recently been changed to include evaluation of HER2 status, also in metastatic biopsies [163]. Within cervical cancer, comprehensive molecular profiling studies also including metastatic biopsies are warranted. Such studies may aid in suggesting new treatment strategies as well as shedding light on possible resistance obstacles for treatment of advanced and recurrent cervical cancer.

Although NGS have a huge predictive power in certain patient subgroups, additional diagnostic approaches may be necessary to complement NGS studies in the effort to

guide therapy of choice in a greater proportion of the patients [217]. Such approaches may involve *ex vivo* determination of live tumor cell responses to drug therapies, direct cytotoxicity (reduction in tumor cell numbers) or *in vivo* models [217]. Functional screening methods will probably become part of future diagnostic, alongside traditional pathology techniques and genetic sequencing.

8. REFERENCES

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