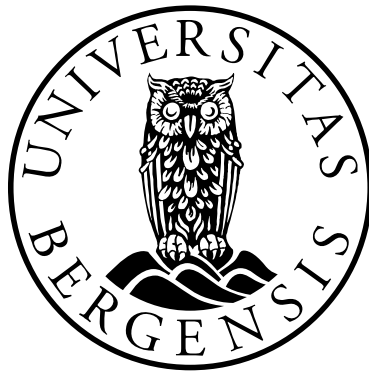# Clinical and molecular markers in endometrial cancer

Studying prognostic and predictive biomarkers that can help to individualise therapeutic decisions

Henrica Maria Johanna Werner

Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

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As I Grew Older

It was a long time ago.
I have almost forgotten my dream.
But it was there then,
In front of me,
Bright like a sun
My dream.
And then the wall rose,
Rose slowly,
Slowly,
Between me and my dream.
Rose until it touched the sky
The wall.
Shadow.
I am black.
I lie down in the shadow.
No longer the light of my dream before me,
Above me.
Only the thick wall.
Only the shadow.
My hands!
My dark hands!
Break through the wall!
Find my dream!
Help me to shatter this darkness,
To smash this night,
To break this shadow
Into a thousand lights of sun,
Into a thousand whirling dreams
Of sun!

James Langston Hughes, 1902-1967

List of original research publications

This thesis is based on the following original research articles (in order of publication):

1. Stratification based on high tumour cell content in snap-frozen tissue promotes selection of aggressive endometrial carcinomas.
Halle MK*, **Werner HM***, Krakstad C, Birkeland E, Wik E, Trovik J, Salvesen HB.
Histopathology. 2012 Feb;60(3):516-9.
*both authors contributed equally to the paper

2. Revision of FIGO surgical staging in 2009 for endometrial cancer validates to improve risk stratification.
Werner HM, Trovik J, Marcickiewicz J, Tingulstad S, Staff AC, Amant F, Salvesen HB; MoMaTEC study group.
Gynecol Oncol. 2012 Apr;125(1):103-8

3. A discordant histological risk classification in preoperative and operative biopsy in endometrial cancer is reflected in metastatic risk and prognosis.
Werner HM, Trovik J, Marcickiewicz J, Tingulstad S, Staff AC, Engh ME, Oddenes K, Rokne JA, Tjugum J, Lode MS, Amant F, Salvesen HB.
Eur J Cancer. 2013 Feb;49(3):625-32.

4. ARID1A loss is prevalent in endometrial hyperplasia with atypia and low-grade endometrioid carcinomas.
Werner HM, Berg A, Wik E, Birkeland E, Krakstad C, Kusonmano K, Petersen K, Kalland KH, Oyan AM, Akslen LA, Trovik J, Salvesen HB.
Mod Pathol. 2013 Mar;26(3):428-34.

5. Stathmin protein level, a potential predictive marker for taxane treatment response in endometrial cancer

Werner HMJ, Trovik J, Halle MK, Wik E, Akslen LA, Birkeland E, Bredholt T, Tangen IL, Krakstad C, Salvesen HB
Plos One, in press

Research environment and mentoring

The setting where I was able to accomplish my PhD is The 'Bergen gynecologic oncology group', headed by prof HB Salvesen. It is embedded in the Centre for Cancer Biomarkers (CCBIO) at the University of Bergen, which, in 2013, was awarded the prestigious title 'Norwegian Centre of Excellence'. CCBIO is focused on translational research, predominantly biomarkers and personalised cancer treatment. Within this context, the activities of prof. Salvesens group are centered around prognostic and predictive molecular markers in endometrial cancer, with many articles in high ranked journals in the recent past, including PNAS and NEJM. Her multi-national research group is diverse and counts clinicians in various disciplines, molecular biologists, biostatisticians, laboratory technicians and a research nurse. Prof Salvesens mentoring, past and present, includes 'forskerlinje' students (medical students on a dual clinical-research trajectory, n=4), PhD students (n=11) and post-doctoral fellows (n=8). She started a gynaecological cancer biobank at Kvinneklinikken in Haukeland University Hospital in 2001, which currently includes over 3000 samples. From all consecutively consented endometrial cancer patients, snap-frozen tissue, blood and urine are prospectively collected in parallel to clinical data, including (at least 5-year) follow-up. It forms the foundation for many of the research projects, using techniques as different as IHC, RNA arrays and cell lines or xenografts, in the group. It is also the foundation for an international study called Molecular Markers in the Treatment of Endometrial Cancer (MoMaTEC), which investigates clinically well-annotated preoperative specimens and which was initiated by her in 2007. She can boost on a wide collaborative network; locally, nationally and internationally. Through her international contacts, part of this PhD was spent at the department of Systems Biology at the University of Texas MD Anderson Cancer Center, Houston, Texas, USA; headed by prof GB Mills. The aims of his department are broad and include an improved understanding of the molecular mechanisms of cancer progression and cancer drug resistance, ultimately translating this back to the clinic, developing biomarkers and personalised treatments. My interest for the department was specifically generated by their wide experience in reversed phase protein arrays (RPPA).

appreciated, including patients and collecting specimens in spite of often already overfilled workdays.

Dr Mills is highly thanked for allowing me to spend a year in his department at MD Anderson and giving me every opportunity, not only obtain the knowledge and skills I seeked, but also to participate in new and running projects in the department. My colleagues at systems biology at the University of Texas MD Anderson Cancer Center in Houston Texas USA, thank you for making me feel at home, treating me like a true colleague and including me in many scientific and social events. Knowing I cannot mention all, I still want to mention specifically Jane, Patrick, Maria, Shreya, Lorenzo, Jennifer, Pradeep, Wei, Yiling and the RPPA core for all their help, support and collaboration.

Jappe, my dear husband, thank you for allowing me to pursue my own personal goals, for all your listening, troubleshooting, interest, trust and encouragement. Julius, although perhaps you will be frustrated that you actually lived abroad, but cannot remember a single thing, you helped me to keep reasonable working days, even in the USA. My dear parents, papa en mama, you have always encouraged me to try and achieve whatever I wanted and were interested not only in the destination but even much more so in the journey. Thank you raising me with that attitude and your never-ending support. Toine, my brother, you as well thank you for your support and interest in again a new project your little sister undertook.

Last but not least the voluntary participation, with no personal benefit, of all patients is highly appreciated, without them these studies would never have been possible.

Bergen, 15th February 2014

Henrica Maria Johanna (Erica) Werner

Abbreviations

AJCC	American joint commission on cancer
ARID1A	AT rich interactive domain 1A (SWI- Like)
BMI	Body mass index
BRCA	Breast cancer early onset gene
Ca125	Cancer antigen 125
CDH1	Cadherin 1, e-cadherin
CI	Confidence interval
CONSORT	Consolidated standards of reporting trials
CT	Computer tomography
CTNNB1	Beta-catenin
DAB	3,3'-diaminobenzidine
DSS	disease-specific survival
DNA	Deoxyribonucleic acid
EPHA2	EPH receptor A2
ER(A)	Estrogen receptor alpha
ERBB2 (HER2)	v-erb-b2 erythroblastic leukemia viral oncogene homolog2
EQUATOR	Enhancing the quality and transparency of health research
FAS	Fas cell surface death receptor
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FIGO	International federation of gynaecology and obstetrics
FGFR2	fibroblast growth factor receptor
GIPZ	type of lentiviral vector
HE4	Human epididymis protein 4
HIF1A	Hypoxia inducible factor 1α
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Hazard ratio
ICGC	International cancer genome consortium

IHC	Immunohistochemistry
KRAS	v-Ki-ras2 kirsten rat sarcoma viral oncogene homolog
MLH1	MutL Homolog 1
MoMaTEC	Molecular markers in treatment of endometrial cancer
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
MYC	v-*MYC* myelocytomatosis viral oncogene homolog (avian)
N	number
OS	overall survival
PARP	Poly (ADP-ribose) polymerase 1
PASW18	Predictive analysis software, version 18
PIK3CA	Phosphoinositide 3-kinase catalytic subunit α
PI3R1	Phosphoinositide 3-kinase regulatory subunit 1
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
RECIST	Response evaluation criteria in solid tumours
REMARK	Reporting recommendations for tumour marker prognostic studies
RNA	Ribonucleic acid
RPPA	Reversed phase protein arrays
RTK	Receptor thyrosine kinase
SAM	Significance analysis of microarray
SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEER	Surveillance, epidemiology and end results program
shRNAmir	microRNA-adapted small hairpin RNA

STMN1	Stathmin 1
STR	Short tandem repeat
SWI/SNF	Switch/sucrose non fermentable
TCGA	The cancer genome atlas
TMA	Tissue micro array
TRS	Target retrieval solution
TNM	Tumour, node, metastasis (cancer staging system)
TP16	*CDKN2A,* cyclin-dependent kinase inhibitor 2A
TP53	Tumour protein 53
UICC	International union for cancer control
VEGF	Vascular endothelial growth factor
WNT	Wingless/Integrated
2D	2-dimensional
3D	3-dimensional

Synopsis

Background: Endometrial carcinoma is one of the most common cancer types in women, and incidence is increasing globally. Although many cancers are detected at an early stage and will be treated adequately with surgery alone, 15-20% of cancers will recur. After systemic recurrence, median survival approximates 7-12 months, in spite of treatment, with no improvement over the last decades. Our abilities to predict which patients will suffer recurrence, give ample room for improvement and robust prognostic biomarkers are needed to better recognise these high-risk patients. Response rates to medical treatment, both conventional and targeted, do not pass 40%, and are often considerably lower, even more so in the recurrent setting. Contrasting some other frequent cancer types such as breast and colorectal, in endometrial cancer algorithms, predictive biomarkers to support treatment choices are non-existent. Using preclinical models and large prospectively collected population-based patient series, potential biomarkers can be studied and tested at a pre-trial stage, which can accelerate the process of their identification and development, and increase the chance of succesfull trials.

Objectives: We studied clinical and molecular variables for their abilities to function as prognostic or predictive biomarkers, with the ultimate aim to improve and individualise treatment strategies for endometrial cancer patients.
Exploring the behaviour of these biomarkers during cancer progression, followed as a logical consequence.

Materials and Methods: For all studies included in this thesis (studies 1-5) clinical data, including follow-up data, have been retrieved and analysed, either from the Haukeland University Hospital Series or from the significantly larger MoMaTEC series. The hyperplasia cohort has been studied in paper 4 and (paired) primary tumours and metastases in studies 4+5. From the biobank material, FFPE tissue has been used for immunohistochemistry (ARID1A; study 4, stathmin1; study 5), snap-frozen tissue for RNA microarrays (study 4) and haematoxylin stained frozen sections (study 3). For

studies 1 and 2 only clinical data was used. Cell-line studies, including dose response studies, viral transfection techniques and immunoblotting formed a strong basis under study 5.

Results: After restaging all 1268 included patients, we demonstrated an improvement and simplification of the prognostic stratification using the FIGO 2009 version. In stage 1 patients, the myometrial infiltration depth was an independent prognostic factor, only for those patients that did not undergo lymphadenectomy. Cox multivariate survival analysis showed FIGO 2009 to be a stronger, independent prognostic factor than FIGO 1988. (study 1)

The 16% (207) tumours with discordant risk between preoperative and operative specimens, proved to be an interesting group with intermediate prognosis and risk of lymph node metastasis, in the entire dataset (n=1374) and in stage 1 tumours only (n=954). Cox multivariate survival analysis showed the risk classification to have independent prognostic value, and different hazard rates for the concordant high risk (HR 5.1) and discordant groups (HR 2.7 and 2.9). (study 2)

High tumour cell content (n=136, 50%) was in our series associated with more aggressive disease and reduced disease specific survival. (study 3)

Loss of ARID1A was linked to the endometrioid and clear cell subtypes, and associated with less aggressive disease, with the exception of the positive association with deep myometrial infiltration. No relation was found between loss of ARID1A and survival. Loss was noticed in a considerable percentage of the hyperplasias with atypia; this percentage further increased with disease progression. (study 4)

Stathmin1 knockdown in cell lines was associated with increased apoptosis after paclitaxel treatment. Patients with high stathmin1 level showed worse response to paclitaxel containing chemotherapy, but not to other treatments, compared to patients with normal stathmin level using RECIST criteria. In Cox multivariate analysis, stathmin1 was an independent predictor of survival only in the subgroup of patients who received paclitaxel containing chemotherapy. (study 5)

Conclusions: The FIGO 2009 classification system both simplified and improved prognostic stratification abilities compared to the previous system from 1988. (study 1)

Through integration of the preoperative histology with the final or operative histology, prognostic information can be further improved, especially when discordance between both results exists and results in the identification of subgroups with intermediate risk for metastatic spread and disease specific death that currently go unnoticed. (study 2)

The 80% tumour-cell content cutoff, meant to ensure high tumour purity, is, in endometrial cancer, associated with high-risk clinicopathological characteristics and reduced disease specific survival and may thus introduce an unintended selection bias. (study 3)

Loss of ARID1A occurs most in endometrioid and clear cell subtypes and is predominantly linked to clinicopathological parameters of less aggressive disease, but lacks correlation with survival. Loss starts early in endometrioid endometrial cancer carcinogenesis and further increases with tumour progression. (study 4)

Stathmin1 has potential as a predictive biomarker for response to paclitaxel containing chemotherapeutic regimes in endometrial cancer. (study 5)

Biomarker switch is a frequent phenomenon during endometrial cancrinoma disease progression and re-assessment of biomarker status in metastatic disease may be relevant. (study 4 and 5)

Contents

1. INTRODUCTION

Malignancies of the *corpus uteri* can be broadly classified into epithelial malignancies (endometrial carcinomas and precursor lesions), mesenchymal malignancies (uterine sarcomas), mixed epithelial and mesenchymal malignancies of the uterus (malignant mixed müllerian tumours) and trophoblastic malignancies.

Endometrial carcinoma originates in the endometrium. It is the fourth most common cancer in women in the western world and currently[1] the 6th (USA) or 8th (Europe) cause of cancer-related death in women.

1.1. Epidemiology

1.1a. Incidence

Incidence rates vary widely across geographical regions worldwide. Recent publications from the USA, report an age-adjusted endometrial cancer incidence of 24.3/100.000, the highest world-wide[2]. In Western Europe the age-adjusted incidences are somewhat lower; Norway; 16.5[3], UK; 19.5[4], and the Netherlands; 16[5]. Contrasting this, in the less affluent regions of the world, such as larger parts of Africa and South Central Asia, the incidence is as low as 2.1/100.000[6] (Figure 1a). The cumulative risk of endometrial cancer by the age of 75 has been calculated between 1.6 and 2.7% in the western world[2,7,8].

Although endometrial cancers in teenagers has been described[9], endometrial cancer is usually not considered a significant risk below the age of 35. However, in women presenting with anovulatory cycles, up to 14% of will be diagnosed with hyperplasia with atypia or endometrial cancer[2,10]. In general, endometrial cancer remains a disease of the elderly woman, with the highest incidences shown in the 6th and 7th decade of life[2,3,11].

A   B
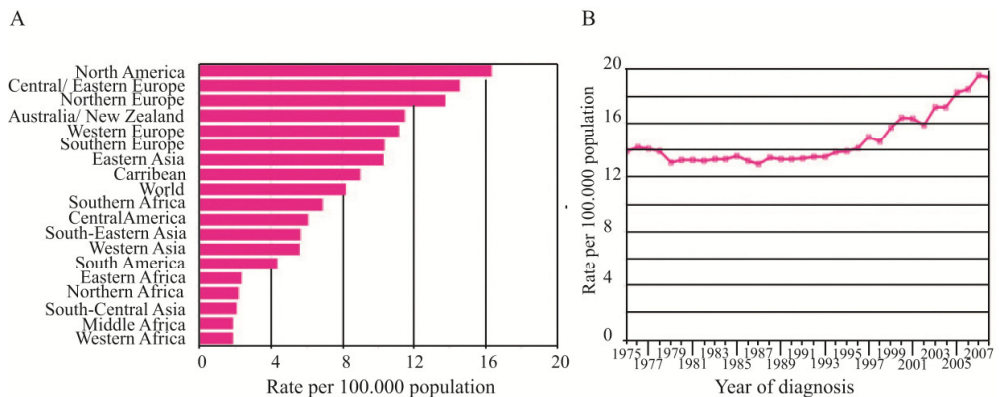
Figure 1. Age-standardised endometrial cancer incidence[12]

1a. World age-standardised incidence per 100.000 population (female), 2008 estimates

1b. UK age-standardised incidence per 100.000 population (female), trends over time 1975-2010

Figures adapted; reproduced with permission

1.1b. Mortality

Overall, the prognosis of endometrial cancer is good, with a mortality rate reported between 2.4/100.000[8] and 4.3/100.000[2]. The 2011 data from the Norwegian cancer registry showed a 5-year survival of 84.4% for all endometrial cancer patients combined[3]. As many women are diagnosed at an early stage, most women with endometrial cancer will not die from their cancer, the leading cause of death (36%) in the entire population is indeed cardiovascular disease[13]. However, in women dying within 5 years of their diagnosis, most women die of disease[13]. Underscoring this, after systemic recurrence of the disease, median survival does not pass beyond 7-12 months, in spite of the currently available treatment options[13-16].

Survival (and thus mortality) can be described in multiple ways, such as overall or disease specific. Considering that endometrial carcinoma is predominantly a disease of the elderly woman, with many intercurrent diseases and possibly deaths, and considering that many tumours do not run an aggressive course; it is highly relevant to use disease specific survival. Long term follow-up after treatment, also including deaths potentially related to side effects from treatments (e.g. external radiotherapy), has recently proven relevant[17].

There is a rise in the endometrial cancer incidence globally, across all age cohorts except premenopausal women[2,3,7], which for a larger degree has been ascribed to the obesity epidemic[18-21] and the increasing life expectancy (Figure 1b, 2). The lay public and thus the potential future patients, are however for the bigger part not aware of this increased life style risk[22]. A study by Renehan *et al.*[23] showed that in Europe alone, up to 30% of the endometrial cancers may be attributable to obesity, a significantly stronger association than in most other cancer types. A relative risk increase of 2.89 (2.26-3.18) for every 10 units BMI increase) was reported by the Million Women Study[24]. Whether and how obesity relates to disease specific survival is less clear and an active area of research. In large population based studies, women with high BMI tend to have better survival, although not with independent prognostic value[25,26], and may be related to the stronger association with less aggressive histology[18,25].
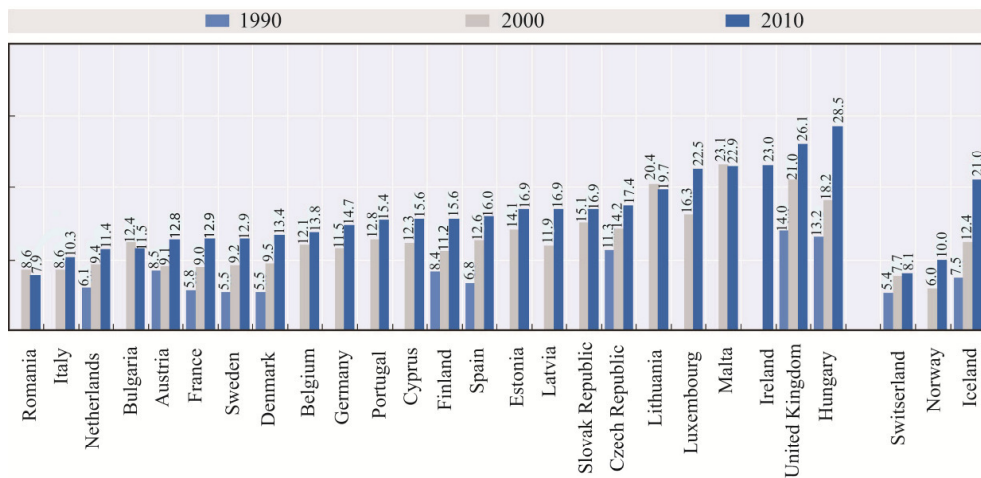
Figure 2. Increasing obesity rates among adults in European countries, 1990, 2000 and 2010 (or nearest years)[27]

Figure adapted; reproduced with permission

There are many confounding factors though, including obesity related comorbidity and life expectancy and the increased risk of complications through surgical and/or adjuvant treatment.

1.2. Etiology

1.2a. Risk factors

Most endometrial carcinomas are sporadic. The majority (roughly 80%) arises on a background of atypical hyperplasia after long-term exposure to unopposed oestrogens and are histologically endometrioid endometrial carcinoomas[28]. Classical risk factors include obesity, chronically anovulatory cycles and diabetes, but also prolonged tamoxifen use and unopposed menopausal hormone treatment; all stimulating growth of the endometrium. In addition to (unopposed) oestrogen production, obesity is associated with insulin resistance, ovarian androgen excess, anovulation and low progesterone levels, leading to changes in adipocyte and inflammatory factor levels[21,28]. As a consequence, proliferation and angiogenesis are stimulated in the endometrium and apoptosis is inhibited[28]. Tamoxifen is a selective estrogen receptor modulator that, while having antagonistic effects in some tissues, like the breast, has agonistic effects in others, including the uterus (estimated relative risk of endometrial cancer $=2$)[29,30].

Additionally, increased risk is associated with nulliparity, positive family history of endometrial cancer[31] and diabetes[20,28].

The remaining 15-20% of endometrial cancers arise on an atrophic endometrium, and are likely preceded by endometrial intraepithelial carcinoma. These are often grouped together as non-endometrioid tumours. In contrast to long term believe, they as well have been associated with obesity in recent publications, although to a lesser extent than the endometrioid variant[18,21,32,33]. A pooled analysis recently concluded even that other riskincreasing or reducing factors such as diabetes on the one hand and parity and oral contraception on the other, and were equally associated with serous as with endometrioid subtypes[33]. A positive history of breast cancer though, specifically increases risk of the

non-endometrioid tumours[34,35]. A small percentage of endometrial carcinomas are related to hereditary factors. HNPPC (Lynch syndrome, affecting 0.1-0.2% of the general population), due to a germ line mutation in one of the DNA mismatch repair genes, puts its carriers at increased risk of endometrial cancer (44%)[36,37] alongside their risk of colorectal cancer and several other cancer types.

Some of the most important negative risk factors or risk reducing factors are life style modifiable, underscoring the need for increased public awareness of endometrial cancer risk factors. These include maintenance of a normal BMI, physical activity and oral contraceptive use (potentially including the Mirena® coil[38,39]). Grand multiparity has been associated with reduced risk, most likely though the high progesterone production during pregnancies. With regards to smoking, literature is conflicting with reports suggesting reduced[20,40] as well as increased risk[41].

1.2b. 'Type 1' and 'Type 2' endometrial cancer

Based on epidemiology and clinical behaviour, but also on light microscopy appearance, Bokhman coined the term 'type 1' and 'type 2' cancers[42], considering low grade (grade 1 and 2) endometrioid tumours as type 1 and high-grade (grade 3) non-endometrioid tumours as type 2 tumours (Table 1). However, the assignment of the high-grade endometrioid tumours remains controversial, often being grouped with the type 2 tumours, although in the original publication[42], being endometrioid tumours, they were considered type I.

1.2c. Tumour biology

From a (tumour) biology point of view, cells function as busy crossroads, constantly receiving large number of signals, including signals for growth and proliferation and apoptosis, and integrating these into responses, whilst they dynamically interact with other cells and tissues. Endometrial cells are no different. Robust homeostatic mechanisms further fine-tune these responses and correct for damage caused by external

factors and by f.e. spontaneous mutations that occurred in the cell. When a cell is damaged, repair mechanisms will try to correct this damage, and if impossible impose a self-destruction pathway of apoptosis[43]. Do such mechanisms fail and the error has occurred in a 'cancer gene', a cell may arise that is less responsive to external signals, and after multiple episodes of non-corrected damage in the same cell, a cancer may arise. Cancer genes are essentially normal genes that confer a growth advantage to the cell, such as growth signal autonomy or invasion potential, when dysregulated. Hanahan and Weinberg defined 'hallmarks of cancer', characteristics that give the cell a growth advantage, indispensable for carcinogenesis[44,45]. Three main types of cancer genes should be highlighted. Oncogenes or tumour inducing genes, arise from normal genes (proto-oncogenes) that regulate cell growth and differentiation through f.e. mutation or overexpression. Examples include *MYC*, *RAS* and different RTK's. Tumour suppressor genes repress cell replication and loss of their proteins through mutation, promotor hypermethylation or other mechanisms may therefore accelerate growth. Classic tumour suppressor genes are *TP53* and the retinoblastoma protein. We have investigated a recently discovered ovarian tumour suppressor gene, *ARID1A*, for its tumour-biological and prognostic value in endometrial carcinoma (this thesis). DNA damage response genes induce apoptosis when cells are damaged beyond repair. Dysfunctional genes may allow damaged cells to survive and proliferate and possibly gain growth benefit; *BRCA* and *FAS* mutations operate in this way.

Different cancer types often show a characteristic pattern of aberrations in tumour suppressors and oncogenes or pathways involved.

Endometrioid and non-endometrioid tumours have a largely different set of aberrations, and, in a way, can be considered 2 different diseases[16,46,47], molecularly supporting the type 1 and 2 classification.

Type 1 cancers are characterised by a high mutational frequency and microsatellite instability. The most important aberrant pathway in type 1 endometrial carcinomas is the PI3K pathway, with sometimes multiple aberrations in this pathway (such as

simultaneous loss of PTEN and *PIK3CA* mutations) in one tumour. In addition the RAS-RAF-MAPK pathway is frequently dysregulated.

In contrast the type 2 cancers are characterised by chromosomal instability and TP53 mutation. Also here, the PI3K pathway is frequently dysregulated, however, now charecterised by amplifications in *PIK3CA* or overexpression/amplification of the receptor thyrosine kinases (*HER2*, *FGFR2*, *EGFR*), much more than loss of PTEN and *PIK3CA* mutations. Of note is that whereas in type 1 cancers *PIK3CA* mutations are either in exon 9 (often the low grade tumours) or exon 20, in type 2 mutations appear exclusively in exon 20, highlighting clearly a different molecular basis between the two subtypes[16,46-48]. A number of key molecular aberrations type 1 and type 2 are associated with, are indicated in Table 1. However, as can be observed, no molecular marker or combination of markers is absolute for either type, and classical type 2 characteristics can be found in type 1 cancers and *v.v.*. As such, the division in type 1 and type 2 is not perfect. Challenges include the 'mixed' tumours that have both endometrioid and serous/clear cell components and which are therefore difficult to fit into this classification. Further, tumours as heterogenous as serous, clear cell and carcinosarcomas are all classified as type 2, but are molecularly and behaviourally very different[49,50].

In recent publications it has been argued[50] that the type 1/type 2 division does not reflect biology optimally and that a three-tiered system may more appropriately assign individual tumours to categories and leave less '(morphologically) ambiguous' and likely heterogenous tumours inappropriately and inconsistently assigned[51-54]. This additional, molecularly intermediate type may in addition be intermediate in prognosis.

An optimal (cancer) classification is characterised by high reproducibility and biological relevance, containing prognostic and ideally predictive information[51]. Currently existing classifications are suboptimal for endometrial carcinoma. For, although in general the type I tumours fare better than the type II tumours, still approximately 15% of the type I tumours recur and 50% of the type II do not[16,42,46], underscoring that our current distinction has to be further improved.

Table 1. Clinicopathological variables and molecular markers in type 1 and type 2 endometrial carcinoma[16,47,49,55]

Clinicopathological variable				Type 1	Type 2
Endometrial background				hyperplasia	atrophy
Differentiation				high/moderate	low
Risk for lymphogenic spread				low	high
Prognosis				favourable	unfavourable
Prognostic molecular marker	**Pathway involved**	**Function**	**Alteration**	**Type 1 (%)**	**Type 2 (%)**
PTEN	PI3K	tumour suppressor	mutation, LOH, deletion, hypermethylation	37-61	0-11
ER/PR		transcription factor	loss of expression	27-30	76-81
PIK3CA mutations	PI3K	oncogene	mutation	26-36	26-36
PIK3CA amplification		oncogene	amplification	2-14	46
PIK3R1	PI3K	tumour suppressor	mutation	43	12
MSI[1]	DNA repair	oncogene	promotor hypermethylation	25-30	0-10
TP16 (CDKN2A)	Cell cycle	tumour suppressor	mutation, promoter hypermethylation	10	40
TP53	DNA damage	tumour suppressor	mutation	5-20	80-90
HER2 (ERBB2)	RTK/EGFR signaling	oncogene	amplification, overexpression	rare	30
CDH1	WNT	tumour suppressor	LOH or promoter hypermethylation	22	60-90
FGFR2 mutations	FGF signaling		mutation	16	N/A
KRAS	RAS-RAF-MAPK	oncogene	mutation	10-30	0-10
CTNNB1	WNT	oncogene	mutation	14-44	0-5
ARID1A loss	transcription regulation	tumour suppressor	mutation	29-39	18-26 (clear cell)
chromosomal instability		oncogene	LOH, anaeuploidy	rare	frequent
STMN1	microtubule cytoskeleton	oncoprotein	overexpression	15	64

[1]due to aberrations in MLH1 in sporadic endometrial tumours

N/A: not available

1.2d. Other approaches

Rather than only trying to categorise within a cancer type, tumour types can also be compared to other tumour types for similarities and associations between subtypes based on their molecular features. As an example, TCGA[56] has started to perform, besides integrative analyses on large numbers of tumour samples from one tumour type (such as endometrium, ovary or kidney), cross-tumour type analyses, noting striking molecular similarities between serous endometrial, basal breast and high-grade serous ovarian cancer[57] (Fig 3). Also, similarities were noted between endometrioid endometrial and colorectal cancer[57]. Such molecular resemblance can enhance our understanding of individual diseases and suggest treatment opportunities.
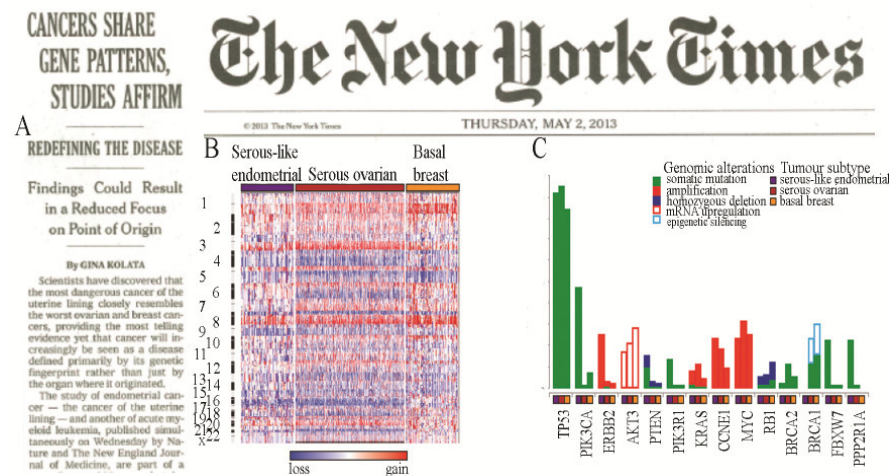
Figure 3. Similarities across different cancers[57]

a. Cover of the New York Times, New England edition, May 2nd 2013, showing lay press attention to an endometrial cancer study[57], which included a comparison between underlying genetic aberrations in serous ovarian, basal breast and serous endometrial cancer.

b. Figure showing the somatic copy number alterations for each tumour type

c. Figure showing the frequency of genomic aberrations, present in at least 10% of one tumour type

b and c, adapted; reproduced with permission

1.3. Clinic

1.3a. Clinical presentation

(Abnormal) vaginal bleeding is an early symptom in up to 90% of patients[58] and most endometrial carcinomas are therefore diagnosed at an early stage. Older women however are in comparison more often diagnosed with cancers at a higher stage and grade[19] (and references therein). The first step in the diagnostic work-up in women with postmenopausal bleeding (cancer risk 1%) is (vaginal) ultrasound scanning assessing the endometrial lining, followed by biopsy taking for histological confirmation, using either office methods or curettage if it appears thickened (>4mm[58]) or difficult to visualise. In the latter case, a 15% risk of cancer has been reported[59]. In pre- or perimenopausal women, the thickness of the endometrial lining is less informative due to cyclical hormonal fluctuations. As such, a suspect anamnesis combined with the presence of risk factors is sufficient to warrant a biopsy in these women.

1.3b. Diagnostical process

The histopathological results, confirming or refuting the diagnosis, allow for a first categorisation into low and high risk disease, looking at subtype and grade. One of the major challenges is to tailor the surgical treatment and limit procedures such as lymphadenectomy or radical hysterectomy to those patients who will benefit from them and not expose patients to potential complications unnecessarily. Both imaging and molecular features of the preoperative specimens are scrutinised for their abilities to better subdivide tumours as low or high risk.

1.3c. Imaging

Preoperative imaging assists in the acquisition of an as optimal as possible understanding of the disease extent, required to plan the surgical therapy and

includes imaging of the thorax (focusing on lung metastases) and pelvis/abdomen (estimating myometrial invasion depth and cervical stromal invasion and/or lymph node metastasis). Whilst X-ray is routinely used for the thorax, the optimal pelvic imaging technique is more controversial and may include (vaginal or abdominal) ultrasound, hysteroscopy, CT or/and MRI and is an area of active research (see biomarker section).

1.4. Histopathological diagnosis

1.4a. Histological subtypes

The assignment of endometrial carcinomas to the various histological subtypes[60] is of paramount importance as it impacts both the extent of surgery and need for adjuvant therapy, related to their risk of metastasis and recurrence.

Endometrioid adenocarcinoma

Glandular structures are a prominent feature in this subtype, resembling the original endometrium in varying degrees of differentiation, but it can in addition contain solid areas or areas with more papillary growth. The tumour often arises on a background of endometrial hyperplasia. Endometrioid carcinomas are graded according to their nuclear grade and the glandular and solid (varying percentages) architecture (see 1.4b and Fig. 4).

Serous adenocarcinoma

The most frequent observation in this subtype is a (micro) papillary architecture and individual cells that have detached from the tumour. No obvious precursor lesion is usually present, but the cancer likely progresses from endometrial intraepithelial carcinoma in an atrophic endometrium. The nuclear features are often pleomorphic and per definition high-grade (see Fig. 4).

Clear cell adenocarcinoma

This subtype is characterised by hobnail, glycogen filled, cells, with often very atypical pleomorphic nuclei. Their architecture is usually papillary and/or solid with uniform, high-grade nuclear features (see Fig. 4).

Mixed adenocarcinoma

Mixed adenocarcinomas show components of endometrioid and clear cell/serous subtypes, with at least 10% of either type. They contain characteristics of both subtypes, but are thought to have progressed from an endometrioid tumour based on their molecular profile[47].

Carcinosarcoma

Carcinosarcomas are a major subtype of the malignant mullerian mixed tumours that are histologically characterised by a combination of malignant epithelial (carcinomatous, often serous) and mesenchymal (sarcomatous) components with diffusely high-grade nuclear features. The origin of this tumour has been a matter of debate. However, the currently most accepted theory is that the sarcomatous components evolve through metaplastic dedifferentiation of carcinomatous tissue[61,62] and that the tumour thus originally started as a carcinoma, which is supported by the molecular features of this subtype[61-63]. In line with this, since 2009, the carcinosarcomas are included as an endometrial carcinoma subtype in the FIGO staging (see Fig. 4).
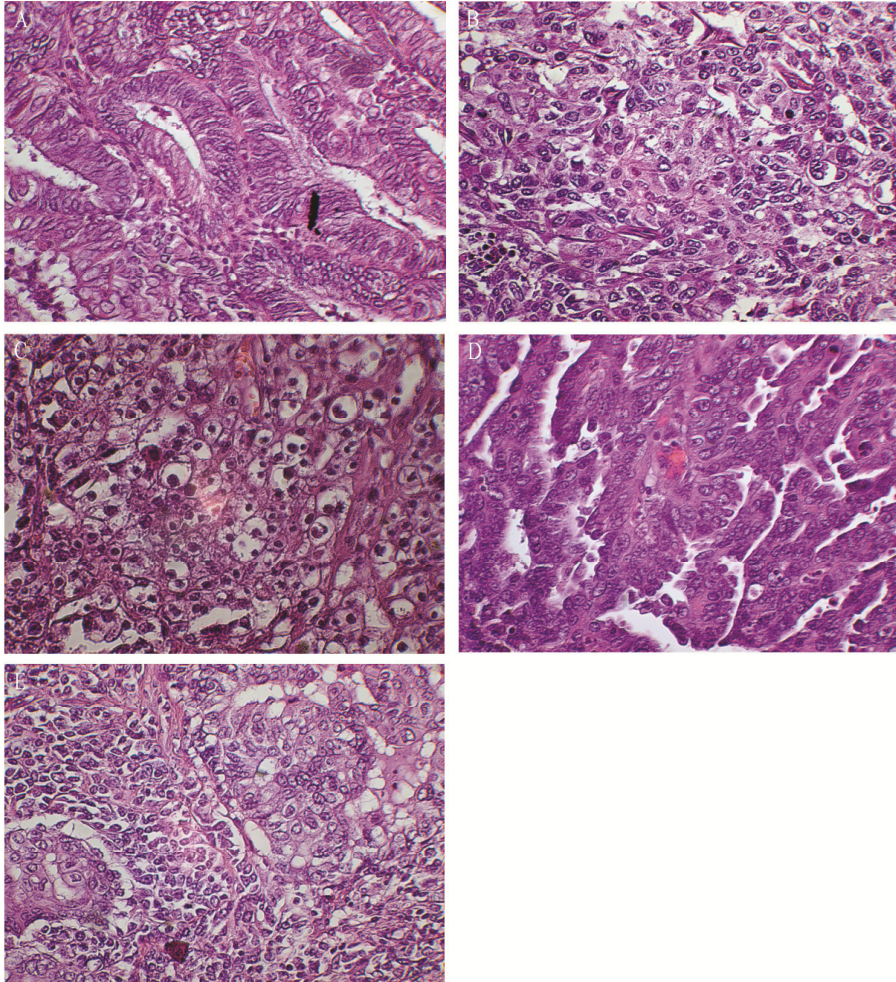
Figure 4. Images of some of the most important endometrial carcinoma subtypes

All images taken with normal light microscope using a 40x lens. A. Grade 1 endometrioid; B. Grade 3 endometrioid; C. Clear cell; D. Serous; E. Carcinosarcoma

Table 2 gives a quick overview of various of the prime characteristics of these subtypes. Further, a few subtypes with low frequency can be distinguished additionally, such as mucinous adenocarcinoma, small cell carcinoma and transitional cell carcinoma[60].

Table 2

Histopathological characteristics of endometrial cancer subtypes[50,60]

Subtype	Architecture	Cell shape/cytoplasm	Nuclear features
endometrioid low grade	complex and glandular structures lined by simple/columnar cells with long axis perpendicular to basement membrane solid components <50%	columnar; metaplastic; cohesive	low grade (somewhat) elongated nuclei, polarised perpendicular to basement membrane
endometrioid high grade	complex glandular and/ or papillary structures solid, nested or trabecular components >50%	columnar; metaplastic; cohesive	high grade
serous	(Micro)papillary, with broad, complex fibrovascular cores; cellular budding cellular budding; cells rounded, no perpendicular orientation; psammoma bodies psammoma bodies	cuboidal and columnar; non-metaplastic	diffusely high grade; pleomorphic; high N/C ratios poorly differentiated; apical; large macronucleoli
clear cell	papillary, tubulary, tubulocystic and/or solid	cuboidal; glycogen rich; clear hobnail cells	high grade; bizarre shape; multinucleated; pleomorphic
undifferentiated	papillary; lacking nested or trabecular architecture	round, dyshesive; lymphoma or plasmacytoma like; non-metaplastic	diffusely high grade; usually lacking pleomorphism

N/C ratio: nucleus:cytoplasma ratio

1.4b. Grading

Grading of tumours of the endometrioid subtype is based on the percentage of solid (non-squamous) growth and is three-tiered (grade 1: <5%, grade 2: ≥5-<50% and grade 3: ≥50%) and measures the degree of anaplasia (lack of differentiation) in the tumour cells, *i.e.* how much the tumour cells still resemble normal (endometrial) cells. When considerable nuclear atypia is present, such as pleomorphic or very prominent nucleoli, the grade is increased by '1'[60]. Approximately 40% of endometrioid tumours will be grade 1, 30% grade 2 and another 30% grade 3. The non-endometrioid subtypes are all considered to be poorly differentiated (grade 3) by virtue of their histology. As the cutoffs have been arbitrarily defined (not data-driven) and are prone to inter-observer variation, especially near the cutoff points, applying grading can be challenging[64]. Similarly, the nuclear atypia is not rigorously defined and therefore open to different interpretations, resulting in different grading of similar cases[64]. Various two-tiered systems have been suggested to be easier to use and more reproducible[65,66]. In this thesis, we have used the FIGO grading system categorised into two groups, *i.e.* grade 1 and 2 combined as low grade and grade 3 as high-grade, as often done in literature. Reproducibility, expressed as κ-value, has been reported to be fair to good for the three-tiered FIGO (0.41-0.65; interobserver and 0.66-0.73 intraobserver) and somewhat higher for the two-tiered FIGO system (0.58-0.71; interobserver and 0.9 intraobserver)[64]. The binarised system also has independent prognostic value for disease specific survival and metastases[64-66].

1.4c. Staging

Staging in cancer can either be clinical (based on physical examination, imaging results etc) or surgical-pathological (based on surgical findings and pathological examination of any removed tumour) and is cancer type dependent. In endometrial carcinoma staging has been surgical-pathological since 1988[67]. Two alternative surgical-pathological classification systems exist. The majority of solid tumour

types are staged by the TNM classification, which is maintained by the American AJCC and the UICC[68]. Simply put, the T (tumour) stands for the size of the original tumour and spread to nearby tissues, the N (node) describes number, size and localisation of lymph node metastasis and M (metastasis) informs about distant metastases.

However, in cancers of the female genital tract, such as endometrial carcinoma, the FIGO staging system is routinely applied, including in the articles used in this thesis[69]. The 2 systems fortunately show close resemblance as shown in Table 3. In the remainder of the thesis, only the FIGO system will be referred to.

Using FIGO staging, the extent of tumour growth is divided into 4 stages; stage 1: confined to the uterus (72% of tumours is stage 1)[70]; stage 2: cervical (stromal) involvement (12%); stage 3: spread to the vagina, ovaries, parametria or lymph nodes (13%); and stage 4: presence of distant metastasis/growth into adjacent organs (3%). In 2009, a new version of the FIGO staging system for endometrial cancer was introduced, replacing the 1988 classification and incorporating progressive insights on f.e. the importance of myometrial invasion and positive para-aortic nodes after analysing large patient series[69,71] (Table 3). The 2009 version has been shown to be more prognostically informative than the 1988 classification[72,73] (this thesis).

Table 3. Similarities and differences between FIGO 2009, FIGO 1988 and the most recent TNM staging system version [68,69,71]

Stage	FIGO 2009	FIGO 1988	TNM[1]	Interpretation
1				**tumor confined to the corpus uteri**
	1A	*1A[2]*		*no myometrial infiltration (included in FIGO 2009 stage 1A)*
		1B	T1a, N0, M0	<50% myometrial invasion depth
	1B	1C	T1b, N0, M0	≥50% myometrial invasion depth
2				**invasion in cervix uteri**
		2A[2]		*only glandular involvement of the endocervix (included in FIGO 2009 stage 1)*
	2	2B	T2, N0, M0	invasion in cervical stroma, otherwise limited to the uterus
3				**local and/or regional spread of the tumor**
		3A[2]		positive cytology only (in FIGO 2009 included stage 1 or 2; cytology results are reported)
	3A	3A	T3a, N0, M0	serosa of the corpus uteri invaded and/or adnexal metastasis
	3B	3B	T3b, N0, M0	vaginal and/or parametrial involvement
		3C[2]		*metastases to pelvic and/or para-aortic lymph nodes (further divided in FIGO 2009)*
	3C1		T1 T2 or T3, N1, M0	metastases to pelvic lymph nodes
	3C2		T1 T2 or T3, N1, M0	metastases to para-aortic lymph nodes, independent of pelvic lymph node status
4				**bladder and/or bowel mucosa invaded, and/or distant metastasis**
	4A	4A	T4, Any N, M0	bladder and/or bowel mucosa invaded
	4B	4B	any T, any N, M1	distant metastasis

[1]2009 classification

[2]FIGO 1988 (sub)stage categories, no longer existent in FIGO 2009

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That pathology results are not always optimally reproducible[74,75], is reflected in the discordance rates in grade (up to 8%)[75], in cervical stromal infiltration (concordance 0.35 to 0.69 (average 0.49))[76] and histological subtype (reproducibility 0.62-0.86)[75,77] after central pathology review, sometimes with clinical implications, and the discordance between expert gynaecological pathologists[76]. Comparing preoperative biopsies, obtained by office biopsy or curettage, with the corresponding hysterectomy specimen results, focusing on the ability to accurately predict the final histology, discordance rates ranging from 15-32%[78,79] have been reported. This may be related in part to tumour heterogeneity, not captured in the small volume of preoperative specimens, and the suboptimal reproducibility of endometrial cancer pathology reports compared to other gynaecological cancers. However, this discordance between curettings and tumour pathology results can also be exploited and explored for prognostic information (this thesis).

1.5. Biomarkers

A biomarker has been defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenical processes, or pharmacological responses to a therapeutic intervention" by the Biomarkers Definitions Working Group[80]. Biomarkers can be as diverse as histopathological features, blood components, imaging, molecular markers, or in fact anything assessable or quantifiable in a patient. Both individual markers and so-called signatures or panels of markers in combination can be relevant. Various subdivisions in types of biomarkers can be made, of which one is highlighted here; a division in prognostic biomarkers, related to the survival of the patient independent of treatment and often implicated in the oncogenic processes, and predictive biomarkers, related to the expected response of a patient to a treatment. The latter are specifically interesting in the era of personalised medicine, to help tailor treatment to the patient's needs. Importantly, for a biomarker to be of

clinical interest, it should provide incremental information beyond that already obtained from clinicopathological variables or other known predictors[81]. They should therefore be tested in addition to these, rather than independently.

Unfortunately, the effect estimates in 'highly cited studies' of a high percentage (86%) of biomarkers appear (much) inflated when tested with independent datasets or meta-analyses, as recently demonstrated for 35 top-cited biomarkers, indicating that often publications on biomarkers are too optimistic or reach exaggerated associations[81]. It is therefore recommended that biomarkers should be validated in independent datasets or for example on different platforms. Further, the Enhancing the QUAlity and Transparency Of Health Research network (Equator)[82] is an umbrella organisation focused on research paper quality improvement, through guideline development on accurate and complete reporting of key aspects, increasing generalisability of study results in different health research areas, such as PRISMA and CONSORT. The reporting recommendations for tumour marker prognostic studies are abbreviated REMARK[83].

Figure 5 illustrates the process of biomarker development and (possible) future use.

1.5a. Predictive biomarkers

Few predictive biomarkers in cancer medicine have been shown robust and have, as per today, been incorporated into clinical algorithms. Examples include *KRAS* mutational status in colorectal cancer indicating response to cetuximab and panitumumab[84-86] and *HER2/Neu* amplification or overexpression in breast cancer for eligibility for trastuzumab treatment[84,85,87]. In endometrial cancer unfortunately no predictive biomarkers are available in the clinic yet to guide
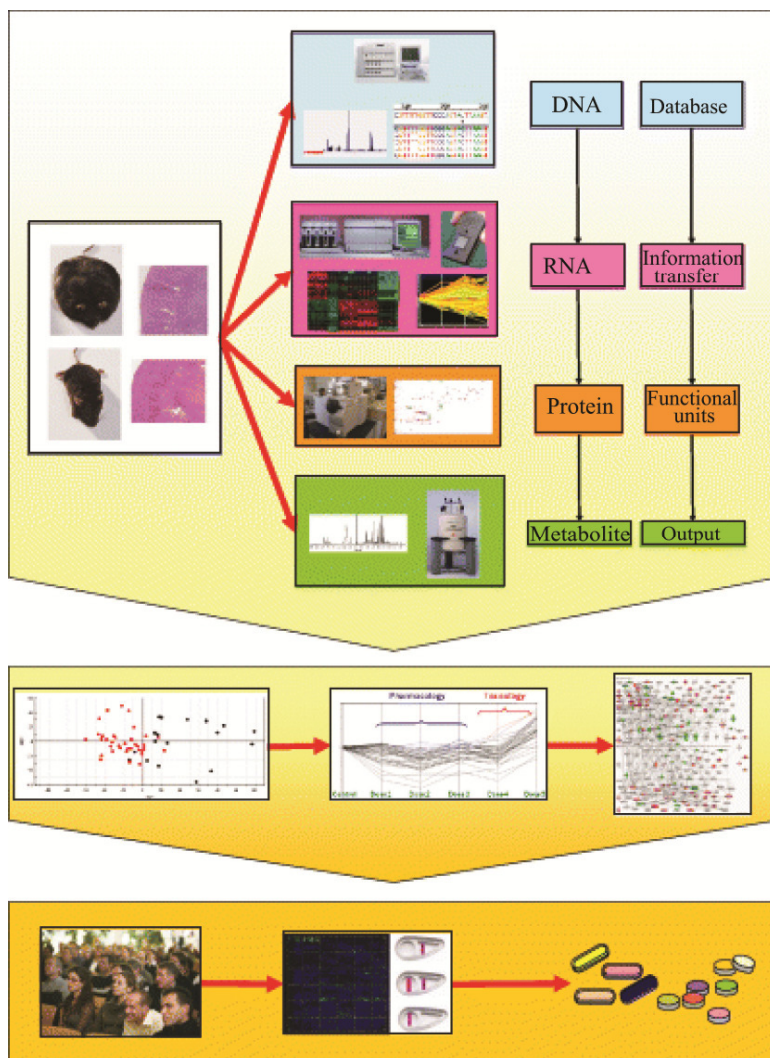
Figure 5. Overview of biomarker development and application in personalised medicine.[88]

The top section shows the biological levels at which biomarkers can be identified with the appropriate technologies. The middle section illustrates the data mining, integration and knowledge building processes. The third box represents the applications of biomarkers in personalised medicine: the *right* medicine at the *right* dose for the *right* patient. Figure adapted; reproduced with permission.

treatment decisions. Although *ERA* may be considered a predictive biomarker for response to hormonal therapy[89,90] and *PIK3CA* mutations are promising in predicting response to PI3K/mTOR inhibition (but not validated in all studies[46,91,92]); they are not used outside clinical trials so far.

Predictive biomarkers do not have to be limited to targeted therapies, but can also be useful in chemotherapeutic regimens, as response rates even in chemosensitive diseases often do not pass 25-30%[93,94]. In line with this, in this thesis we have investigated whether stathmin1 level can be used as a predictive marker for response to paclitaxel based chemotherapy (this thesis).

1.5b. Prognostic biomarkers

Clinicopathological

The strongest prognostic biomarkers in endometrial cancer are FIGO stage, histological subtype and tumour grade. FIGO stage is associated with increasingly worse survival for the higher stages[2,3,72,73,95]; while the overall 5-year survival rate in endometrial carcinoma is 78-82%[2,3], it drops from 93-97% in stage 1 to 17-42% in stage 4 disease[2,3]. Other histopathological factors, indicative of increased risk of recurrence or lymph node metastasis, are deep myometrial invasion[96,97], lymphovascular space invasion[98,99] and tumour type and grade[96,97]. Tumour subtype is an important prognostic biomarker, with poorer 5-year survival in all FIGO stages for any non-endometrioid tumour subtype compared to the endometrioid subtype[28,100].

Molecular

Molecular biomarkers that have shown independent prognostic value in endometrial cancer, focusing on survival and/or risk of lymph node metastases include *TP53* mutation, loss of *TP16*, aneuploidy, loss of hormone receptors, high HER2 level, stathmin1 level, β-catenin mutation[16,55,101-103] (see also Table 1). (Novel) imaging methods[70,104-107], serum biomarkers[70,108] and tumour biomarkers

in preoperative specimens[109-112] are all studied to assess the risk of lymph node metastasis, deep myometrial infiltration or cervical involvement preoperatively, for their utility in selecting those patients in which the benefits of the procedure outweigh the potential additional morbidity associated with for example complete pelvic and aortic lymphadenectomy[113-115].

Disease progression

Serum markers such as Ca125 or HE4 have been studied to monitor disease progression, but unlike in ovarian cancer where Ca125 is strongly incorporated in the clinical algorithms, for various reasons there is no widespread inclusion for the evaluation of endometrial cancer[108,116,117]

1.5c. Cutoff and heterogeneity

The biomarker cutoff levels applied in different studies vary and as a result comparisons across literature can be difficult. Challenging the comparison of (potential) immunohistochemical biomarkers, is that different antibodies, targeting different epitopes in the same protein, could potentially give different results. Further, biomarkers (or biomarker signatures) are identified against a control population. Depending on whether the control population consists of healthy controls or endometrial cancers with a different subtype or different stage, the signature may change.

1.6. Treatment

1.6a. Surgical approach

The main treatment algorithm for endometrial carcinoma is surgical, curative for most patients with early stage disease[100]. It consists of a total hysterectomy with bilateral salpingo-oophorectomy. A 'radical' hysterectomy (removal of uterus, cervix, upper vagina, parametria and pelvic lymph nodes) is performed in case of (suspected) cervical infiltration. The omentum is routinely removed in clear cell and serous tumours. Historically, the procedure was performed by laparotomy,

which continues to be a perfectly safe procedure, but in an increasingly obese population with existing comorbidities, laparoscopic procedures may show advantageous. Three trials explored the (dis)advantages of laparoscopy, performed by experienced surgeons, on clinical and/or economical outcome variables[118-120], and in spite of considerable variation in inclusion criteria and procedural differences, 2 out of the 3 trials concluded that major surgery-related complications, including deep venous thrombosis and pulmonary embolism, bleeding necessitating surgery, blood transfusions, wound infections and burst abdomen, were reduced in the laparoscopic group but equal to laparotomy in the last[118]. But also this latter trial reported benefits in reduced hospital admission duration and earlier resumption of daily activities[118]. A cost-effectiveness analysis returned positive for the laparoscopic procedure[121]. More recently still, robotics-assisted laparoscopy ('robotic surgery') has been introduced and applied to endometrial carcinoma surgery, with the advantages of a reduced learning curve, 3D-vision and more natural ergonomics and hand movements for the surgeon. Although no trials have been published yet, two large American series, retrospectively comparing robotics-assisted procedures with open abdominal procedures in obese populations[122,123], published favourable complication frequencies in the robotics-assisted group and equal ability to perform lymphadenectomy in both groups. Only one study so far compared all three procedures[124], and concluded that compared to open and routine laparoscopic surgery, robotics-assisted surgery was associated with low blood loss and complication rates, high lymph node yield and that operative time was reduced compared to laparoscopy. What these studies did not address is long term follow-up or an economical comparison between robotics-assisted and normal laparoscopy. One level III evidence study modeled the costs associated with the three different types of surgery, and calculated laparoscopy to be the cheapest option from a hospital perspective[125], strongly related to a reduced duration of hospital admission and the high purchasing costs for a robot. However, when

societal variables such as lost wages were factored in, robotics-assisted surgery also became a very cost-attractive option.

1.6b. Lymphadenectomy

It is known from literature that 6-8% of endometrial cancers will have pelvic lymph node metastasis, 4-6% pelvic and paraaortic and 2% paraaortic only[70] (and references therein). One study reported that in patients preoperatively considered grade 1, stage 1 endometrioid cancer, 4.6% of patients had positive paraaortic lymph nodes[114]. Lymphadenectomy is part of a complete staging procedure and thus useful for risk stratification of patients. However, no survival benefit has been associated with the procedure in randomised clinical trials[126,127]. Morbidity, related to the procedure, includes lymphoedema (2.5-10%), thrombosis (2.6%), small bowel obstruction (2.3-4.4%) and lymphocysts (2.4-7.6%)[114,115,128], additional to prolonged anaesthesia, prolonged hospital stay and increased blood loss[127].

In the US, complete paraaortic and pelvic lymphadenectomy is advised for all patients with endometrial carcinoma[129]; in Europe, this is often limited to high-risk categories, excluding stage 1 endometrioid tumours grade 1 and 2[129-131].

The discussion regarding lymphadenectomy in endometrial cancer suffers from absence of standardisation (sampling *vs*. complete lymphadenectomy, pelvic *vs*. pelvic and paraaortic, number of nodes needed to be removed, optimal candidate groups vs. all). Both trials that looked into the prognostic effects of lymphadenectomy in endometrial cancer[126,127] have later been criticised for their methodology and are as such unable to give a definite answer. To oppose the trend of more aggressive surgery in all patients with its related risks and unclear prognostic benefit, different research groups aim to improve the preoperative identification of patients most likely to have lymph node metastases. A sentinel node procedure may be an alternative to balance morbidity and benefits in patients with a low risk of lymph node metastasis[132,133].

Table 4. Stratification for risk of recurrence and need for adjuvant treatment[96,97,134]

Stage	Low risk	High-intermediate risk *	High risk
FIGO 1	endometrioid low/medium grade age <60 (70)	endometrioid high grade & superficial invasion *OR* endometrioid low/medium grade & deep myometrial invasion age >60 (70) lymphovascular space invasion	non-endometrioid endometrioid high grade

* ≥2 risk factors needed to be high-intermediate risk. In literature slight variability in the risk factors describerd for this category

.

1.6c. Non-surgical routine treatments

Radio- and chemotherapy

Patients with deep myometrial invasion, higher age (>60 years), grade 3 disease or lymphovascular space invasion are at increased risk for recurrence of disease[96,97] (see Table 4). Therefore, in these circumstances, adjuvant treatment is. Radiotherapy can be offered to reduce the incidence of locoregional recurrence from around 20 to 5%[96,97,134]. However, it does not convey a survival benefit[96,97,135]. Brachytherapy has now, in one randomised trial, been shown to perform equal to external radiotherapy in terms of locoregional control, in intermediate to high risk patients reducing locoregional recurrence similarly from 20 to 5%[75,135]. Brachytherapy is associated with a significant reduction in the long term usually gastro-intestinal side effects (54% of patients with externam beam radiation, including chronic diarrhea and bowel stenosis, vs. 13% with brachytherapy)[96,136]. For high risk cases though, including most cases with non-endometrioid histology and higher stage tumours, external beam radiation therapy is still advised to guarantee optimal pelvic control[137,138]. In single agent chemotherapy, paclitaxel is the most effective with response rates of 36%, combination therapies can reach higher objective response rates, up to 57% (paclitaxel, cisplatin and doxorubicin)

against a worse side effect profile[46,139]. Currently, the most common regimen for chemotherapy in both the adjuvant and recurrent setting in endometrial cancer include platinum derivatives combined with taxanes. Cardiac and gastrointestinal side effects as well as leucopenia are common.

Sequential combinations of radio- and chemotherapy (using different platinum based regimens) were compared to single radiotherapy, in early and late stage endometrial carcinomas[94,140], with only one study[94] showing significant and clinical relevant positive effects on progression-free survival and disease specific survival when combining the two strategies. Single chemotherapy treatment has also been compared with single radiotherapy[141-143] and showed in one trial a significant better effect compared to external beam radiation therapy[142], but also a clearly inferior side effect profile. The trial has been criticised methodologically, allowing patients with substantial residual disease after surgery (<2cm) to be included. Other trials have not shown superiority of chemotherapy over radiotherapy on clinical outcome parameters[141,143]. Trials are currently looking into the added benefit of chemo- in addition to radiotherapy, such as for example[144].

Hormonal treatment

A large meta-analysis[145] did not find evidence for clinical benefit of hormonal therapy in advanced endometrial cancer patients although one of the included studies showed a strong association between higher hormone receptor levels and better patient outcomes[145]. Some authors have reported up to 25% response rate in steroid receptor positive tumours[46,139]. Contrasting breast cancer though, where hormone receptor status has been incorporated in the clinical decision algorithms, in endometrial cancer it has not been rigorously studied in the population where most benefit is expected; *i.e*. the hormone receptor positive population with recurrent or advanced disease. Another review looking into the benefit of hormonal treatment in the adjuvant situation was also negative[146]. Even in 40% of the relevant currently active trials (Oct 23rd, 2013) registered at clinicaltrials.gov[147], hormone status, even in only primary tumour, is not mandatory for inclusion.

Practically seen, there is considerable variation in type and indications of the adjuvant treatments offered, clearly in line with the low level of evidence for clinical benefit of one approach compared to another[130,131,148].

1.6d. Targeted treatment[15,16,46,48,91]

Advances in understanding of the molecular pathogenesis of the disease have allowed the development of drugs specifically targeting these aberrations ('targeted treatments'); with agents targeting the PI3K pathway having advanced furthest in endometrial cancer. Other interesting drugs currently in development or clinical trials are *FGFR2* and *HER2* inhibitors, drugs blocking angiogenesis though VEGF, targeting *EPHA2*, or *HIF1A*[16,46]. In keeping with many other tumour types unfortunately, poor response rates (typically up to 10%) to targeted treatments have been reported. However, in most occasions agents were administered as monotherapy without biomarker-inclusion restriction in a metastatic (and heavily pretreated) population[16,46,91]. In addition, it is becoming clear that combinational treatments of *e.g.* targeted agents in combination with anti-hormonal and/or chemotherapy, are likely to increase the therapeutic efficacy[48,149].

Finding suitable biomarkers, predictive of response to different targeted treatments, will be essential in making the targeted treatments fulfill their promise[16,46,91] (Fig 5). One of the reasons clinical trials do not have any biomarker restriction is accurately this lack of known useful biomarkers; instead, several trials test the patient samples for the levels of a wide panel of potential biomarkers to retrospectively study them for their ability to predict treatment response. Identifying and testing possible predictive biomarker candidates at a pre-trial stage (in preclinical studies and large population-based studies) is highly needed to assign patients to the potentially most useful clinical trials and should be further exploited to accelerate the identification of predictive biomarkers. The evidence for example that patients with *PIK3CA* mutations or possibly PTEN loss or

(phospho)AKT may respond better to PI3K/mTOR inhibition comes from preclinical studies, similar to that mutations in the RAS-RAF-MAPK pathway may convey resistance[91,150]. A recent small phase I study of response to PI3K/AKT inhibition in various cancer types including endometrial cancer, confirmed a substantial increase (from 10 to 33%) in response rates in the (small) subset of patients with *PIK3CA* mutations[91], understriking the importance of predictive biomarkers to improve the clinical benefit from targeted therapies.

Another development is the hybridisation of hormone analogues to anti-neoplastic agents, directing the drugs to the organ of interest through the hybridisation molecule, with the goal to decrease side effects and increase drug efficacy[139].

1.7. Recurrence

Roughly 15-20% of all endometrial cancers recur, of which a disproportionally high percentage belong to the non-endometrioid tumours. Percentages of recurrence close to 50% for the non-endometrioid and about 10-15% for the endometrioid tumours are often cited[16,42]. Three-quarter of the recurrences are diagnosed in the first 3 years but may come as late as 6 years after primary diagnosis[14]. In a radiotherapy-naïve population, 2/3 of the recurrences are local (vagina/pelvis) with a potential for curative treatment, while 1/3 are distant recurrences[14,75,135]. When the disease is systemic, average survival is 7-12 months[15,16,93], without significant improvement over the last decennia. Treatment options are limited and at most poorly effective. The only subgroup with a relatively good prognosis consists of patients with vaginal recurrences only, who can be treated with curative intent, allowing for a 5-year survival of 73%[14]. In case of localised or circumscribed solitary metastases, surgery and radiotherapy can be offered, in all other cases only chemotherapy or potentially hormonal therapy in hormone receptor positive tumours, are potentially useful. Unfortunately, radiotherapy cannot be used in areas that received a full radiation dose previously and in second-line chemotherapy only paclitaxel gives more than 20% response

rate and comes at a cost of considerable side effects[139]. There are therefore high hopes that the addition of more targeted therapies will be able to improve the efficacy of endometrial cancer treatment.

For this overview of endometrial carcinoma, literature was searched until December 2013 for English-language original and overview articles in MEDLINE, and pubmed as well as relevant websites in English, Dutch or Norwegian.

2. AIMS OF THE THESIS

2.1. Background

Most endometrial carcinomas are diagnosed at an early stage and many can be treated adequately by surgery alone[16,42,46]. However, identifying those cases that are at high risk for metastasis or recurrence has proven a major challenge. Our ability to do so will impact prognostic estimates for the individual patients, the extent of surgical procedures, the need for adjuvant treatment and the intensity of follow-up of endometrial cancer patients. Currently, the endometrial carcinoma treatment algorithm is, with few exceptions, 'organ based' and empirical, applying standard adjuvant treatments regimes for higher risk cases and recurrent disease, irrespective of underlying molecular aberrations. Clinical response to adjuvant treatment and even more so treatment for recurrent disease is low, as mentioned previously. The identification of robust prognostic biomarkers will allow a more reliable identification of high risk patients, while predictive biomarkers will hopefully give us the tools to apply therapies in only those patients that are most likely to respond and thus help fulfill the promise of personalised treatment, including in patients with recurrent disease. But instead, surgical treatment paradigms have moved towards more aggressive rather than more risk-based (individualised) approaches and most trials on clinicaltrials.gov currently enrolling patients do so without limiting entry to patients more likely to benefit.

2.2. General aims

This thesis project evolved around the search for prognostic and predictive biomarkers in endometrial cancer, including clinical and molecular biological approaches with the ultimate aim to individualise and improve treatment in women with endometrial carcinoma.

2.3. Background and aims of the individual studies

2.3a. Study 1

Specific background: Staging helps clinicians to plan and evaluate treatments in addition to estimate an individual patient's prognosis[67]. Correct staging is therefore extremely important. Staging systems evolve, incorporating improvements suggested by (multiple) large systematic studies. The latest revision of the (FIGO) classification system was effectuated in 2009 and included important changes from the previous that dates back to 1988[69].

The specific aim was to investigate whether the FIGO 2009 staging system improved prognostic stratification abilities in a large, prospectively collected and well-annotated endometrial cancer dataset.

2.3b. Study 2

Specific background: There is an urgent need in endometrial cancer to better stratify patients for their risk of recurrence, as up to 20% of patients with presumed low risk, localised disease will face recurrences and subsequently poor prognosis. In endometrial cancer, contrasting many other cancers, preoperative histology is easily obtained and usually of sufficient quality to study. However, routinely, only the final operative histological specimen is used for estimation of prognosis and guidance towards adjuvant treatment. Discrepancy between preoperative and operative histological results are frequent and estimated to reach 15-32% in various studies[79,151-153], including both discrepancies in grade and histological subtype.

The specific aim of this study was to, rather than exploring how well preoperative histology predicts final operative histology, specifically question the information content of the concordance and discordance between preoperative and operative histology reports, focusing on metastatic spread and prognostic significance.

2.3c. Study 3

Specific background: Genome analysis techniques such as whole exome sequencing, genome wide expression or methylation analyses are able to give a wealth of information, as shown in multiple studies[57,154-157]. For most studies snap-frozen tumour tissue with high tumour-cell content is required (often arbitrarily >80%) to obtain samples with little stromal contamination[147,158]. However, so far, it has not been investigated whether this requirement may introduce a systematic selection bias for specific tumour subtypes or histological grading.

The specific aim of this paper was to compare tumours above and below the routine 80% tumour-cell content cutoff (on haematoxylin-stained frozen sections) for associations with clinicopathological characteristics and survival.

2.3d. Study 4

Specific background: In endometrioid and clear cell ovarian cancer, *ARID1A* was recently identified as a potential tumour suppressor gene with frequent somatic truncating or missense mutations and concomitant loss of protein[159,160]. *ARID1A* is a key member of the switch/sucrose non-fermenting (SWI/SNF) chromatin remodeling complex, which mobilises nucleosomes, altering accessibility of promoters and ultimately resulting in facilitated or repressed transcription. *ARID1A*, supposedly, confers specificity to the regulation of gene expression in this process[161-163].

The specific aim of this paper was to study the relationship between loss of *ARID1A* and standard clinicopathological variables in endometrial carcinoma, and specifically in the endometrioid and clear cell subtypes as they resemble the ovarian cancer subtypes in which the tumour suppressor gene was discovered. Additionally, we analysed the distribution of *ARID1A* loss in consecutive stages of

tumour progression; atypical hyperplasias, primary tumours and metastases to get insight in the timing of the inactivation of the gene.

2.3e. Study 5

Specific background: STMN1 is, amongst others, a regulator of microtubule dynamics and essential for all cells to proceed through mitosis. In many cancers, stathmin1 levels are elevated[164-168] and elevated stathmin1 levels have been associated with poor prognosis and increased risk of lymph node metastasis; identifying stathmin1 as a prognostic biomarker[55,155,166,169-172]. There is preclinical evidence, mainly in breast cancer, suggesting stathmin1 could additionally be a predictive biomarker for response to taxanes treatment[173-176]. REMARK reporting recommendations are recommended and were followed in our study to ensure rigourous reporting and more generalisability of results[83].

The specific aim of this paper was, studying both endometrial cancer cell lines and our prospective clinical database, to question whether stathmin1 has potential as a predictive biomarker for response to paclitaxel containing chemotherapeutic regimes in endometrial cancer. We also compared stathmin1 levels in metastatic and primary tumours to explore potential changes in biomarker status on tumour progression.

3. GENERAL DISCUSSION

3.1. Materials and methodological considerations: Patients

3.1a. Patient series

For a schematic overview of the various patient series used in the different studies, see Figure 6.

Study	Patient series: inclusion and follow-up time	Material investigated	Biomarker	Biomarker type	Platform used
1	1268	FFPE	stage	prognostic	NA
2	1374	FFPE	pathology	prognostic	
3	553	snap-frozen tissue	purity	prognostic	
4	641	FFPE	ARID1A	prognostic	IHC
		snap-frozen tissue	ARID1A		RNA array
	77	FFPE			IHC
	31	FFPE			IHC
5	603	FFPE	STMN1	predictive	IHC
	77	FFPE			IHC
		cell lines			in vitro studies

2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014

- Primary tumour Haukeland University Hospital series
- Primary tumour MoMaTEC series
- Metastasis
- Hyperplasia
- Follow-up

Figure 6. Overview of used patient series, materials investigated, (types of) biomarkers and platforms in this thesis.

Haukeland University Hospital series

A population based series of prospectively collected endometrial cancer cases of patients diagnosed with and treated for the disease has been constructed at Haukeland University Hospital, Bergen, Norway as previously reported[25]. Haukeland University Hospital is a tertiary referral hospital for gynaecological oncology, covering Hordaland county, with a population just over 505 000, approximately 10% of the Norwegian population[177], but also serving as referral centre for two adjacent counties (Rogaland and Sogn og Fjordane).

All patients consented to participation prior to inclusion. Through the prospective character of the series, optimal data collection is guaranteed and selection or collection bias prevented. Patients are all treated following routine clinical guidelines and studies thus performed on (prospectively collected) archival tissue.

Samples are extensively clinically annotated, including, but not limited to, FIGO stage (1988 and 2009), histological subtype, grade, BMI, primary and adjuvant treatment, follow-up status and localisation of and treatment for any recurrent disease. The follow-up period extends for at least 5 years in surviving patients. Due to ongoing inclusion of patients and prolonged follow-up, in the various studies where this database was used (studies 3, 4 and 5), the total patient number and follow-up length varies. Figure 6 gives an overview of the various types of material and platforms applied for these studies.

Molecular Markers in Treatment in Endometrial Cancer (MoMaTEC)[178] series)

This is a multi-centre (n=10), multi-national (n=3) observational cohort study, investigating molecular markers in preoperative biopsies, in well-annotated patients with endometrial cancer and up to 5 years follow-up. Haukeland University Hospital is the coordinating centre and all patients included in the Haukeland series are automatically included in the MoMaTEC series too. As this study continues to recruit new patients, numbers of patients vary amongst the different studies (studies 1 and 2) where these data were used (Fig. 6).

Hyperplasia cohort

This is a series of prospectively collected cases of consented patients, diagnosed with endometrial hyperplasia at Haukeland University Hospital. All underwent hysterectomy and bilateral salpingoooophorectomy for that reason between 2001 and 2009. Similar clinicopathological information is collected as indicated for the endometrial carcinoma patients. Patients with hyperplasia were not subjected to follow-up visits and follow-up data is therefore lacking. A total of 38 patients were included in this series, of which 31 were diagnosed with complex hyperplasia with atypia.

The ability to study and compare the molecular characteristics of endometrial cancer in different stages of development (hyperplasia with atypia, primary tumour and (paired) metastasis) with the clinical annotations as indicated, makes this biobank an immensely rich source for the study of endometrial cancer.

3.1b. Biobank material

For the endometrial cancer biobank in Haukeland University Hospital, snap-frozen tumour tissue is collected alongside tumour tissue for the pathology department (FFPE tissue, subsequently collected in TMA for the biobank), urine and blood samples and recently also MRI imaging in patients who all have well-annotated clinical information. Laboratory information and (routinely) imaging results are routinely and consistently available. For the MoMaTEC series, curettage material is collected together with blood samples with similar rigorous clinical annotation. This allows for the performance of a wide variety of studies. Also in recurrent disease, FFPE tissue and/or snap-frozen tissue are collected whenever possible and can be paired with the related primary tumour samples for analysis (study 4 and 5).

3.1c.Treatment

Routinely, patients were subjected to hysterectomy and bilateral salpingo-oophorectomy upon a preoperative histological confirmation of endometrial cancer, unless surgery was contraindicated due to severe comorbidity. Routine lymph node sampling was limited to pelvic lymphadenectomy for the larger part of the study period, with para-aortic sampling only when suspicious nodes were encountered during the operation. Adjuvant treatment was offered according to clinical guidelines, which means that in Norway, adjuvant treatment was not routinely offered in stage 1 or 2A (FIGO 1988) disease, but either chemotherapy or radiation was administered for high risk stage 1 and 2A patients (including non-endometrioid, grade 3 and deeply infiltrating tumours) as well as stage 2B (FIGO 1988) in the first half of the study period. Starting 2009, these patients were

offered chemotherapy only after surgery. Participating Swedish and Belgian centres offered radiation and chemotherapy to a higher percentage of patients, also in earlier years of study participation.

3.1d. Survival

Survival for the Haukeland series for the various FIGO stages (including both 1988 and 2009) is indicated in Table 5 and compared to various national recordings.

Table 5. Five-year disease specific survival data in the Haukeland
University Hospital series and various national registries

FIGO 2009	HUS series	National registries		
		SEER[2]	Norway[3]	
	% dss	% os	% dss	
Stage I	92.2	95.3	93.4	localised
Stage II	84.7	67.5	73.3	regional
Stage III	51.4			
Stage IV	21.4	16.9	42.3	distant

of note: The Haukeland University Hospital series forms roughly 10% of the total Norwegian data
HUS series: Haukeland University Hospital series

3.1e. Absence of healthy tissue comparator

In some cancer types, such as kidney or breast, healthy tissue is often removed together with the tumour and can serve as optimal comparator to investigate changes caused by the cancerous process, even when considering that normal tissue is not entirely comparable to tumour tissue, being usually less cell dense and more stroma rich f.e.. However, in endometrial cancer, there is usually no normal (non-cancerous) tissue available for comparison, in patients, nor in healthy women, since the endometrium is atrophic after menopause (when the majority of cancers arise; median age at diagnosis is 65 years in the Haukeland University Hospital as well as other series). This implies that in this cancer, comparisons are often made between different patient categories or subtypes. This has important implications,

as the reference population will in part determine which differences become visible. An interesting alternative and relatively easily feasible in endometrial carcinoma, is studying drivers of carcinogenesis during disease progression from the precancerous through to the metastatic stage, using hyperplasias with atypia (the precursor lesions of endometrioid endometrial carcinoma), primary tumours and metastatic lesions, or primary and metastatic tumours only, something that was explored in this thesis (study 4 and 5).

3.1f. Absence of central review

Central review has not been applied for those studies included in the thesis that used material of the multi-centre MoMaTEC study (study 1 and 2). Although there are many valid arguments in favour of doing so, we aimed to mirror the daily routine in a multicentre setting, with its associated diagnostic accuracy. The high quality of routine reporting in academic tumour boards of the academic hospitals including the far majority of the MoMaTEC samples, speaks in favour of this 'real world' approach.

3.2. Methods and methodological considerations: techniques used

3.2a. IHC

TMA

To generate TMA, first, the area of highest tumour aggressiveness of each tumour is identified on hematoxylin/eosin slides to ensure tumour representativity. Then, 0.6mm diameter tissue cylinders (three for primary tumour; one for metastasis) are punched out of the corresponding original FFPE tissue block and mounted in a recipient FFPE block using a custom-made precision instrument (Beecher instruments, silver spring, MD, USA). The validity of this method has been demonstrated in several studies[179,180].

Immunohistochemistry technique

Five μm thick TMA sections were first dewaxed in xylene and dehydrated in graded ethanol series, followed by antigen retrieval (routinely performed by microwave cooking in TRS pH6 (S1699 Dako Target Retrieval Solution, 20 minutes). Then slides were blocked for peroxidase and non–specific binding proteins (Dako S2023, 8 minutes). Primary antibody incubation time and dilution for IHC varied per antibody (stathmin1: 1h, dilution 1:50 (Cell signaling #3352), study 5 and ARID1A: 2h, dilution 1:100 (Biosite: AT1188a), study 4). EnVision+ system, HRP secondary antibody was used in both cases, followed by DAB+ chromogen (DAKO K4011) for detection. Finally, slides were counterstained with hematoxylin (Dako, S2020). In IHC, antibody specificity for the protein of interest is crucial, therefore positive (samples with known high protein level)[181,182] and negative (omitting primary antibody) controls are stained in each immunohistochemical experiment.

Staining evaluation

Both the intensity of staining and the proportion of tumour cells with this intensity are considered using standard light microscopy and assigned a classification from 0-3 (intensity: 0 (no staining) to 3 (intense staining) and area of tumour with this staining from 0-3 (0; no area stained positive, 1; <10%, 2; 10-50% and 3; >50% area positive). The final score is then the product of intensity and area of tumour staining with this intensity, and varies from 0-9. Staining can be (mostly) cytoplasmic, nuclear, membranous and even shift with tumour progression. As IHC staining evaluation is a semi-quantitative method, slides are, as a rule, scored by two persons, blinded for patient characteristics and outcome[90,183]. To quantify the reproducibility, kappa values are calculated for all IHC series, having the same two persons score a random set of samples of the series independently. Determination of cutoff values for loss or overexpression for the individual proteins is data-driven (with the data usually grouped in tertiles or quartiles), focusing on survival differences and individual subgroup size amongst others. Evaluating cases with multiple metastatic lesions, in case of different scores in the

various samples, the assumed most pathological score was, after testing multiple options, chosen to be representative and relevant.

ARID1A staining is predominantly nuclear. We decided on, in accordance with the first published article on the marker in New England Journal of Medicine[160], a nuclear staining score '0' as the optimal cutoff for loss of protein (assuming non-tumour cells and stroma scored positive in the sample). If both tumour and normal tissue were negative, the sample was excluded from analysis, and technical failure assumed. All other scores were called 'positive'. This cutoff was used in hyperplasias, primary tumours and metastases.

Staining for stathmin1 is cytoplasmic. A cutoff value for high expression had previously been determined in both tumour and curettage material[55]. After evaluating our metastatic stathmin1 samples, the tumour cutoff value (cytoplasmic staining score 9) appeared optimal to ensure consistency in scoring, and the most reasonable to use in view of the earlier studies.

A weakness in any study using immunohistochemistry (study 4 and 5) is that comparisons with existing literature can be complicated by diversity in methodology (including antibodies, antigen retrieval and formalin fixation time), obscuring cancer specific hypotheses[184,185] as well possibly different application of cutoffs. Table 6 illustrates this diversity for studies on ARID1A. As such we consider it important to try and adhere to standards used by authors of what can be called 'marker papers' in that specific field and increase the likelihood that observed results are related to signal (cancer specific differences or similarities) rather than noise (methology dependent).

Table 6. Methodological differences in immunohistochemistry technique for ARID1A in literature

| Study | antigen retrieval | antibody supplier | Antibody | | |
			Clonality	Dilution	Incubation time
Wiegand et al.[160]	pH8,CC1/ standard	Abgent, AT1188a	monoclonal	1:50	2h room temperature
Maeda et al.[186]	pH6, autoclave 120 degrees, 5 minutes	Sigma Aldrich, SAB1404575*	monoclonal	1:25	N/A
Mao et al.[187]	pH6, autoclave 120 degrees, 10 minutes	Sigma Aldrich, HPA005456	polyclonal	1:250	4 degrees overnight
Wiegand et al.[188]	pH8,CC1/ standard	Abgent, AT1188a	monoclonal	1:50	2h room temperature
Guan et al.[189]	pH6, autoclave 120 degrees, 10 minutes	Sigma Aldrich, HPA005456	polyclonal	1:250	4 degrees overnight
Fadare et al.[190]	pH?, Bond Max solution, 30 minutes	Santa Cruz sc-32761	monoclonal	1:50	1h room temperature
Samartzis el al.[191]	pH8, CC1	Abgent, AT1188a	monoclonal	1:200	1h room temperature
Yamamoto et al.[192]	pH6, autoclave 121 degrees, 15 minutes	Sigma Aldrich HP005456	polyclonal	1:50	4 degrees overnight
Werner et al.[184]	pH6, microwave, 20 minutes	Abgent (Biosite) AT1188a	monoclonal	1:100	2h room temperature
Allo et al.[193]	N/A	Sigma, clone: BAF250a	N/A	1:75	N/A
Bosse et al.[194]	pH9, microwave treatment	Santa Cruz sc-32761	monoclonal	1:800	4 degrees overnight

*discontinued

N/A: not availabe

2h: 2hours

<u>3.2b. Cell lines studies</u>

Endometrial cancer cell lines were obtained in 2009 from various suppliers and STR profiling, to confirm authenticity, was performed in 2012[195,196]. All cell lines were maintained under the conditions recommended by the suppliers.

Transfection

Cell transfections were performed using lentivirus (Open biosystems, GIPZ lentiviral shRNAmir; stathmin1 knock-down cells) and transfection with non-silencing shRNAmir in a control cell population (control knock-down cells). Making use of the puromycin resistance gene incorporated in the shRNAmir, transfected cell populations were selected treating with puromycin (1ug/ml).

Dose response experiments

Drug treatment assays, performed in triplicate, included 24 hour paclitaxel treatment (range 1-500 nM) and 24 hour paclitaxel (range 1-500 nM) /carboplatin (fixed 200 mM) combination treatment, after which cells were either fixed in formaldehyde (final concentration 2%) for microscopic evaluation of apoptosis; used in a proliferation assay (MTS) or processed for immunoblotting. To evaluate apoptosis, ≥150 cells were counted in three different areas in 96-well plates. Proliferation assays were performed with CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega), adhering to the instructions from the manufacturer. Plates were read in an ELISA plate reader (TECAN Magellan Sunrise) at 490nm.

Immunoblotting

Immunoblots were performed adhering to a standard protocol. In short, cells were cultured and treated in 6-well plates and harvested in lysis buffer after 24 hours treatment. Proteins (25ug) were separated by SDS/PAGE and transferred to a nitrocellulose membrane (Biorad, Norway). Stathmin1 and/or cleaved PARP were detected using cleaved PARP (Asp214; D64E10; #5625, Cell Signaling) and stathmin1 (#3352, Cell Signaling), both diluted 1:1000; β-actin served as a loading control (anti β-actin antibody (AC-15; ab6276, AbCam), diluted 1:10.000; overnight incubation was used for all primary antibodies. Alkaline phosphatase

conjugated secondary antibodies were used (Anti-rabbit IgG (Sigma Aldrich A3687): Anti-mouse IgG (Sigma Aldrich A3562), both diluted 1:10.000) and chemoluminiscence substrate (lumiphos 34150 WB, Thermo scientific) for detection.

3.2c. RNA microarray analysis

Tissue was collected during surgery and snap-frozen in liquid nitrogen. To ensure data quality, all samples were evaluated for tumour purity (haematoxylin stained frozen sections), and included only if purity was at least ≥50% (majority >80%). Following the manufacturer's instructions, RNA was first extracted from snap-frozen primary tumours (RNeasy Mini Kit, Qiagen, Hilden, Germany) and hybridised to Agilent Whole Human Genome Microarrays 44k (Cat. no. G4112F). Then, arrays were scanned and features extracted using the Agilent Microarray Scanner Bundle. Mean spot signals were used as intensity measure and expression data were normalised using median over entire array (quantile normalization). Raw data were imported in J-Express software (Molmine, Norway) for analysis. Genes differentially expressed between groups were identified by SAM analysis and considered significant if FDR <0.01.

3.2d. Statistical techniques

Statistical analyses were all performed using the PASW18 and PASW20 Statistics software package (SPSS inc. Chicago, USA). Categorical variables were evaluated using the Pearson chi-squared or Fisher's exact test, where applicable. Univariate analyses of time from primary treatment to death due to endometrial carcinoma (disease specific survival) were performed by the Kaplan-Meier (product-limit) method, with the log-rank test to compare survival between groups. Patients who deceased due to other causes, or surviving at the last day of follow-up, were censored. The Cox proportional hazards model was used for multivariate survival analyses (proportionality assumption confirmed), evaluating our variables of

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interest corrected for known important clinicopathological variables. The Mann–Whitney U test was used to correlate protein and RNA expression data. Two-sided P-values of ≤0.05 were considered significant.

4. SUMMARY OF RESULTS

4.1. Study 1

Using clinical information from the patients included in the MoMaTEC study, we staged a patient series including 1268 patients twice, according to FIGO 1988 and FIGO 2009 criteria. The correct assignment of patients to FIGO stages benefitted from the high percentage of lymphadenectomy (performed in 72% of stage 1 tumours), reducing the chance of occult stage 3 disease. We show in this large and well-annotated dataset that the removal of 1988 stages 1A and 2A as well as a part of the inclusion criteria for stage 3A, major changes effectuated in the FIGO 2009 staging system, improved the prognostic predictions, as judged by stage-dependent 5-year disease specific survival. Surprisingly we noted that the myometrial infiltration depth showed a stronger prognostic impact in those patients who did not undergo lymphadenectomy. In this subgroup (n=243), deep myometrial infiltration reduced 5-year disease specific survival 28% (p=0.001); contrasting the group who had had lymphadenectomy performed, where this survival difference was minimal and not statistically significant (mainly the cases with deep myometrial infiltration here showed improved survival).

FIGO 2009 staging was demonstrated to be a stronger, independent prognostic variable for stage 1 tumours in a Cox proportional hazards model together with histological subtype and grade, with and without deep myometrial infiltration (FIGO 2009 HR 3.91; CI 1.35-11.36, p=0.001 vs. FIGO 1988 HR 2.3; CI 0.88-5.99), and for the subgroup with non-endometrioid histology (FIGO 2009 8.96; CI 2.25-35.62 vs. FIGO 1988 5.86; CI 1.87-18.33).

4.2. Study 2

Histology results from preoperative and operative specimens were investigated in 1288 patients who had complete information for both specimens; follow-up data was available for 1165 out of 1288. Lymphadenectomy was performed in 74% of patients; in 72% of cases pelvic lymphadenectomy only. Histology results for both

preoperative and operative specimen were categorised as low risk (grade 1 or 2 endometrioid histology or specific for the preoperative specimen presumed hyperplasia only) or high risk (grade 3 endometrioid histology or non-endometrioid histology) and concordant or discordant between preoperative and operative results; ultimately leading to four risk categories: low risk/low risk (concordant low, n=761), low risk/high risk (discordant, n=148), high risk/low risk (discordant, n=50) and high risk/high risk (concordant high, n=206). In total in 16% (n=207/1288) of cases there was discordance (grade or histological subtype) between preoperative and operative histology.

Discordance between preoperative and operative histology translated in an intermediate 5-year survival. Both discordant categories showed similar survival percentages, positioned between those for the concordant low and concordant high risk categories, in the overall study population (75 and 80% 5-year survival vs. 94 and 58%, p<0.001) as well as specifically in stage 1 disease (87 and 93% 5-year survival vs. 98 and 66%, p<0.001). The combined risk classification confirmed to have independent prognostic value, in a Cox proportional hazards model adjusted for FIGO (FIGO 1-2 vs. 3-4) and age, showing a hazard rate 2.7 and 2.9 for the discordant groups and 5.1 for the concordant high risk group; a better independent prognostic factor therefore than either preoperative (HR 2.1; CI 1.3-3.2) or operative histology (HR 2.4; CI 1,3-3,9) on their own.

This pattern of improved stratification with the combined preoperative/operative histology classification repeated itself estimating risk of positive lymph nodes that were present in 7% for the concordant low risk group, 23% for the concordant high risk group, whilst the discordant groups had positive nodes in 14 and 20% (p<0.001).

4.3. Study 3

We demonstrate in this correspondence, evaluating endometrial cancer patients with snap-frozen tissue availability (n=273/533 total dataset) that (upfront) purity

criteria for high throughput studies, which are arbitrarily set to include samples with little stromal contamination, introduce a selection bias, favouring samples from patients with aggressive disease. The dataset included samples with a tumour cell content ranging from 5-100% (mean 75% and median 80%). High tumour cell content was associated with high age (p<0.01), post-menopausal state (p=0.03), high-grade (p=0.03), non-endometrioid histology (p=0.03) and deep myometrial infiltration (p=0.04) but not with FIGO (2009) stage or presence of metastatic lymph nodes.

For patients with tumour cell content (>80%), 5-year survival dropped to 76%, from 86% in patients with tumour cell content <80% (p=0.02). Five-year disease specific survival for all endometrial cancer patients in the series (n=553 patients, including patients with no snap-frozen tissue) was 82%.

4.4. Study 4

For this study we examined all 535 patients with IHC results for ARID1A, from a total series of 641 patients. Concerning demographical variables, patients with and without ARID1A staining showed similar results, and systematic selection bias of our cohort was therefore unlikely. ARID1A staining is nuclear and moderate to intense in normal tissue[182,197]. Loss of ARID1A staining, highly correlated with mutational status as published previously[160,186], was noted in none of the hyperplasias without atypia, 16% of the hyperplasias with atypia (endometrioid subtype precursor lesions), 19% of the primary tumours and 28% of the metastases. Testing of paired primary tumours and metastases showed a borderline significant increase in loss of protein (p=0.054).

Endometrioid (p=0.031) or clear cell (p=0.049) histology, (younger) age (p=0.005), high differentiation grade (grade 1 and 2) (p=0.007), diploid tumour status (p=0.002), but also deep myometrial invasion (p<0.001) were significantly associated with loss of ARID1A in our dataset. No relation was found with patient outcome (disease specific survival or recurrence free survival), nor with (lymph

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node) metastasis. Correlation of mRNA expression data and protein levels by immunohistochemistry results was statistically significant (p=0.01), and mRNA expression was related to endometrioid histology (p=0.02) and myometrial infiltration depth (p=0.06). Exploring the intriguing association with deep myometrial infiltration for a link to EMT, we examined two gene signatures related to EMT[102,198] for their relation with ARID1A protein levels or mRNA expression, without finding any support for such association.

4.5. Study 5

Using two endometrial cancer cell lines, we demonstrated increased sensitivity to paclitaxel treatment after stathmin1 knock-down and observed PARP cleavage at a lower treatment dose after knock-down (compared to the stathmin1 wild-type cell line).

In our patient series, identifying 18 patients only in a series of 607 clinically well-annotated patients, who fulfilled all criteria including recurrent or residual disease response data according to RECIST criteria, treatment with medication of interest, and stathmin1 immunohistochemistry data available, we related treatment response to paclitaxel with stathmin1 protein level in the tumour. Response to paclitaxel, but not to other treatments, was markedly better in patients with normal stathmin1 level compared to high stathmin1 level and statistically significant (p=0.027) in spite of the low patient numbers. Further, in the poor prognosis subgroup of patients with metastatic disease, those who were treated with paclitaxel and had high stathmin1 level, had a significantly reduced survival compared to patients treated similarly but with normal stathmin1 level (p=0.03). Survival was unrelated to stathmin1 protein level when alternative treatments had been administered (p=0.76). Also, in a multivariate model, corrected for FIGO stage and histological subtype, stathmin1 remained an independent predictor of survival, in the subgroup of patients treated with paclitaxel only (HR 2.3, CI 1.1-5.2).

Finally we observed frequent discordance (26%) in stathmin1 level between paired primary and metastatic samples, but overall a significant increase in stathmin1 during tumour progression (p<0.001).

5. STUDY APPROVALS AND GRANT SUPPORT

NORWEGIAN **CANCER** SOCIETY

6. DISCUSSION OF THE RESULTS

This thesis is based on 5 publications evolving around the identification and validation of prognostic and predictive biomarkers in endometrial cancer using a variety of research approaches. Aware of the genetic similarities across diseases, such as between endometrial cancer and ovarian cancer[57] or the origin of low grade endometrioid and clear cell ovarian tumours from endometriosis[199], this included exploration of the potential of a novel ovarian cancer biomarker (*ARID1A*) in endometrial cancer. On the other end we have emphasised the wealth of not always optimally exploited information available in clinical data and have focused on the development of predictive and prognostic biomarkers using translational research.

6.1. Clinical risk stratification in the molecular era

The prognostically most important variables in endometrial cancer are FIGO stage, histological subtype and differentiation grade; no single other (clinical or molecular) biomarker has shown to have more independent prognostic value to date[16]. For the clinician they provide an important tool to stratify patients and to advise on various types of surgical and adjuvant treatment. In both **study 1** and **2** we benefit from the large size of the patient series and the quality of the clinical and follow-up data. The rationale for FIGO to revise the endometrial cancer staging system followed directly from analysis of a large database of endometrial cancer patients (n=42.000) reported to the organisation[71]. Using patient data from over 1200 patients, we show that FIGO 2009 improves as well as simplifies the prognostic stratification compared to the previous version that dates back to 1988. Contrasting some large US register studies[72,73], we were able to restage all patients, increasing the internal validity of our results; as although these studies only struggled with a small percentage of the datasets (≤6%); it concerned those patients from stage 2A and 3A that actually had shifted stage. Downstaging these tumours should not affect the prognosis of the stages negatively, which we were

able to show. Additionally we included all subtypes, which may explain some of the differences with other studies, limited to endometrioid tumours only[200]. Our population-based approach is less biased by the limitations in diagnostic accuracy for histological subtyping and grading and the moderate reproducibility between pathologists, as these may lead to exclusion of difficult cases in studies requiring histopathologic revision and inclusion of only certain subtypes.

In our study, the n=34, mostly endometrioid, patients that were downstaged from 1988 stage 2A to 2009 stage 1A and 1B, showed a prognosis equivalent to the other cases in their new stages, understriking the correctness of downstaging this group. However, although many studies agree[72,73], some do not[201,202], but Cohn then showed a better overall prognosis for all stage 2 patients. Attempting to understand this discordance a few studies are of particular interest. McCluggage stressed the low inter-observer agreement amongst gynaecological pathologists in cases with deemed difficult cervical assessment ($\kappa=0.49$)[76]. Another, very recent, study noted poor agreement on routine cases with cervical involvement between routine pathologists focusing on different patterns of cervical involvement such as stromal or glandular, although there was a high level of agreement (83%) on overall cervical involvement[203]. In the latter series, a comparison with closely matched stage 1 and 2 patients, except for cervical involvement, did not show any survival differences, although cases likely have been handled differently clinically. Both studies discussed issues including the absence of an anatomical barrier between the lower uterus and the cervix, the lack of clear definitions by FIGO and difficulties in separating stromal and glandular involvement[76,203]. This raises concerns regarding the comparability of different studies in literature.

Also the subgroup of stage 3A cancers due to positive cytology only is a small group (11 patients from our 1268 patient series), with a clearly improved prognosis compared to other patients in stage 3A (n=26). Literature mostly agrees[204,205] on this and concerns have been raised that the positive cytology could be artificially introduced due to diagnostic hysteroscopy preceding the

hysterectomy[206]. Contrasting this, a recent study[207] found that positive cytology independently predicted poor prognosis and a different relapse pattern, but did not restrict to patients with positive cytology as the only criterion for stage 3A inclusion. As such, although the results are very interesting and understrike the importance of continued reporting of positive cytology results, they do not change our approach to treating these patients, if no other high risk factors are detected. In spite of the large clinical database, both the 1988 stage 2A and stage 3A (based on cytology only) in our study are small however and inherently, one should be cautious for over-interpretation of results. It is difficult though to find even larger well-annotated clinical databases, especially databases that do not span multiple decades, with concomitant other challenges, such as major changes in primary and adjuvant treatment algorithms over time, affecting survival, and possibility of considerable missing data.

Interestingly we noted that myometrial invasion depth independently added prognostic information in the subgroup of patients who had no lymphadenectomy performed (n=243) (HR 5.4, CI 1.55-18.60) but not in the subgroup of patients who had lymphadenectomy performed (n=649). Perhaps part of the explanation can be related to deep myometrial infiltration being a surrogate marker for lymph node metastasis[100], and understrikes the importance of a critical appraisal of myometrial invasion, especially in those patients that did not undergo lymphadenectomy. The alternative interpretation is that the prognostic information derived from careful assessment of myometrial infiltration renders lymphadenectomy unnecessary, unless the staging procedure is found to improve survival in a randomised clinical trial, which it has failed to do to date[126,127].

As we noted relatively frequently, histology results do not match in grade or histological subtype between preoperative biopsy and hysterectomy. A study on the information content of this discordance and possible clinical importance was

therefore undertaken. Although simplifying the comparison by ignoring lymphadenectomy and myometrial infiltration depth in the risk assessment, our results hold in both the complete dataset and in the stage 1 subset where lymphadenectomy results are all negative. We show that discordance in histological subtype and grade identifies patients with an intermediate risk for survival and lymph node metastasis, irrespective of whether the tumour was qualified 'high risk' in the operative or preoperative specimen. This indicates that we may overtreat a subgroup of patients with high risk hysterectomy but not preoperative specimen results and who have a 17% improved disease-specific survival compared to the subgroup with concordant high risk in both specimens. At the same time, and more worrisome is that we identified a subgroup with 15% worse disease-specific survival than predicted by their low risk hysterectomy results, namely those who showed high risk histology in their preoperative specimens. Although these patients may benefit from adjuvant treatment, this is not addressed in current clinical routines that focus on hysterectomy specimens only. Similar observations were made for the risk of lymph node metastasis where the discordant subgroups showed intermediate risk compared to both concordant groups. Importantly, by Cox proportional hazards models and prediction analysis, our risk stratification has independent prognostic value. In our opinion, the extent of surgery alone is not the (sole) explanation for the different prognoses in the discordant groups compared to the concordant groups, underscored for example by the statistically significant different percentage of lymph node metastases in the groups that had equally extensive surgery based on high risk preoperative biopsies (14% vs. 23%, p<0.001). We therefore hypothesise several other explanations, including tumour heterogeneity encountered through repeated biopsy taking, tumours with mixed molecular or morphological characteristics or the less than optimal reproducibility of endometrial cancer pathology reports compared to the other gynaecological malignancies. The latter is supported by a number of articles, varying from central review of pathology reports[74,208], confirmation of

preoperative diagnosis of hyperplastic disease only[209] or interpretation of cervical involvement among gynaecological pathologists[76], and the opportunity to combine two results may increase the accuracy. Heterogeneity has been demonstrated to be one of the bigger challenges in cancer; comparing primary tumours with their metastatic counterparts[210-216] (this thesis), and equally subclonality within the primary tumour itself (which would be the heterogeneity picked up in this study)[217-219], with single biopsies likely underestimating the tumour diversity. Multiple sampling will logically increase the likelihood of including more tumour subclones and thus tumour diversity.

Several articles have also highlighted mixed tumours, where, in one tumour, serous components are admixed with endometrioid components and which show a worse prognosis[35,47,220,221]; as well as tumours with ambiguous (or hybrid) morphological and molecular characteristics)[47,50,57,222,223], defined as tumours that fail to show prototypic features[222].

The design of our study only allowed for hypotheses for discussing our observations and a truly prospective and randomised study, potentially simultaneously recording a panel of markers that are useful in the differentiation of ambiguous tumours (such as PTEN, ER, PR and TP53, others?) would be very interesting to further explore the potential benefit to these patients, with treatment algorithms including adjuvant treatment for the higher risk cases, the discordant subgroup, we identified to have worse prognosis compared to the concordant low risk cases.

Morphology based diagnoses are, as highlighted in different aspects by the above studies, highly important in terms of clinical outcome. Genetic signatures and molecular characteristics are closely linked to the morphological categories and can support them to improve diagnostic accuracy and treatment algorithms, or may even drive changes in current criteria for histological clasification[222]. Still it is

unlikely they will replace the standardly applied morphology, which remains the foundation of endometrial cancer diagnosis and treatment planning.

6.2. Biomarkers in disease progression

To investigate and address the change in biomarker status, or biomarker switch, between a primary tumour and one or all metastases[210-216] or a pre-cancer stage and a cancer[224,225], is one of the current major challenges in cancer research. Together with the challenge of intra-tumour heterogeneity, it is especially important in this era of targeted treatment, righteously starting to receive more attention. A biomarker switch can potentially signify that a targeted treatment is no longer relevant due to lack of target, such as a cancer which was ER+ in the primary tumour but ER- in the metastasis. Repeated assessment of a potential target during disease development has also become relevant through reported examples of presence of HER2 overexpression in metastases, but absent in the primary tumour.

To better understand the drivers of carcinogenesis and disease progression or to develop new targets for treatment or screening, it is also vital to understand which aberrations are early or late or specifically associated with metastasis.

ARID1A was a specifically interesting gene to investigate due to its recent discovery in ovarian cancer, and an absence of larger studies (in endometrial cancer) at the time of study. Mutations in *ARID1A*, likely a *bona fide* tumour suppressor gene[154,159,160,226-228] occur frequently and throughout the gene, are often truncating and are highly associated with loss of protein[160,186,189,227,229]. In our study we were able to confirm a highly significant correlation between *ARID1A* RNA expression and protein levels, building further evidence that loss of protein by IHC is a valid assessment of *ARID1A* status.

For both ARID1A (study 4) and stathmin1 (study 5) we noted that the biomarker frequency of the defined aberrant (and associated with cancer) protein levels,

increased upon disease progression, from primary tumour to metastasis and in the case of ARID1A we also had the opportunity to investigate and confirm this in hyperplasia with atypia, the endometrioid endometrial cancer preceding stage.

The absence of ARID1A protein already in hyperplasias with atypia (16%), but not in hyperplasia without atypia, may indicate that loss of ARID1A is important already in the earlier phases of carcinogenesis. Also Mao *et al.* noticed focal loss of ARID1A in hyperplasias with atypia[187] and a recent study linked loss of ARID1A with incidence of sporadic MSI (in 75%), an important marker of early stage endometrioid endometrial cancer[194], both supporting this hypothesis. In (atypical) endometriosis (precursor for clear cell and endometrioid ovarian carcinoma)[160,188,191,192], and ductal carcinoma *in situ* (precursor of invasive breast cancer)[230], a similar pattern was demonstrated, with some loss of *ARID1A*, but not as much as in primary tumours of the same series. Interestingly, our observation, that, although *ARID1A* loss can be noted in early stages of carcinogenesis, frequency of loss of protein further increase throughout tumour progression, has been confirmed in both endometrial and breast cancer[187,230]. Although not confirmed in our study, *ARID1A* has been suggested to interact with *TP53* as a negative cell cycle regulator[154,194,229] and additionally (not investigated in this thesis) to be associated with *PI3K* aberrations[154,191,194]. One study strikingly noticed increased *PI3K* activity measured by phosphorylation of AKT and p70S6 in samples with loss of *ARID1A* and no *PI3K* pathway member aberrations[154]. Although literature on *ARID1A* is growing rapidly, few articles relate *ARID1A* loss to clinical variables including survival. However in those that do, including, but not limited to endometrial cancer[186,188,227,230,231], all but one[227] agree there is no significant relation with survival. This may be consistent with the importance of *ARID1A* in early stages of carcinogenesis. In endometrial cancer literature, agreement does exist on the association with the endometrioid[187-189] and clear cell[184,231] subtypes, being most associated with *ARID1A* loss, however so far there

is less consistency on the associations with less aggressive disease, such as lower grade and younger age, which are confirmed by some[189], but not all[187,188].

There is a growing body of evidence that not only *ARID1A*, but chromatin remodeling complex members in general, play an important role in the development of many cancers[232-236], although many important aspects, both concerning mechanistic molecular understanding and clinical relevance still need to be further studied.

As mentioned previously, stathmin1 has been shown to be a prognostic marker in endometrial cancer and related to aggressive disease[155,166,169-172].

In study 5, alongside our main focus concerning a potential role for stathmin1 as a predictive marker in endometrial cancer, we investigated the switch, if any, in biomarker status between primary tumour and metastasis. There was a significantly higher percentage of samples with high stathmin1 level in metastases compared to primary tumours (18% in primary tumours vs. 37% in metastases, $p<0.001$).

Importantly, in paired samples, discordance between primary and metastatic sample was 26%; in 16% there was a switch from normal to high, in 10% from high to normal stathmin1 level. An important consequence of this observation is that biomarker status as determined in the primary tumour cannot automatically be assumed to be similar in a metastasis. This has not only implications for novel targeted treatments, but also for (anti)hormonal treatment that entered the clinic a long time ago. Comparing ARID1A loss in primary tumour and metastatic lesions (unpublished data), in 17% (12/71) there was a change in biomarker status, of which 1% (1/71) from loss to presence of ARID1A and in the remainder 15% (n=11) from presence to loss of protein. In literature, similar percentages of discrepancy in biomarker status have been quoted in endometrial cancer[103,210,213] and in other cancer types[214-216]. In breast cancer, often a cancer 'in the lead', the American Society for Clinical Oncology included in 2007 for the breast cancer

treatment algorithms that, if the outcome would influence treatment choices, repeat biopsies should be considered in metastatic disease[237]. Although part of the discrepancy may be explained by sampling in an heterogeneic tumour[218,219] or interobserver variation, the consistency of discrepancy across different biomarkers, studies and platforms[210-217] assumes there can be a real switch in biomarker status between different stages of tumour progression.

For stathmin1, this increase in overexpression in metastases fits well with its role as prognostic biomarker.

As such, both studies contribute to the discussion regarding biomarker status in cancer (progression) in general, and additionally emphasize the clinical relevance for repeated biopsy taking. A pooled analysis in breast cancer reported repeat biopsy taking may directly impact treatment in 1 in every 7 patients[238]. The often heard concerns about invasive procedures forming a burden for an already sick patient seem irrelevant according to a recent paper where nearly 90% of patients would recommend the biopsy to another patient[239]. The same study showed a technical success rate of biopsy taking of 97%[239].

For article 4, we explored associations between loss of a novel biomarker, ARID1A, and important clinicopathological variables in endometrial cancer. And although we have for example looked into EMT as an explanation for the interesting association with deep myometrial invasion, we have not explored this or other associations extensively further for mechanistical explanation or confirmation. Although not the focus of the article, in hindsight, this could have deepened our understanding of the significance of this particular biomarker in endometrial cancer, over the hypotheses currently posited in the discussion section. Similarly, in article 5, further understanding of the mechanistic relationship between stathmin1 and taxanes will be of importance to develop stathmin1 further as a potential predictive biomarker.

6.3. Predictive biomarkers in endometrial cancer

To increase the frequency of patients responding to treatments in the clinic and fulfill the promise personalised medicine has delivered to us, the identification of predictive markers that can tell us which patients likely will (not) respond to treatment (including in the metastatic setting), is an important challenge; others include better recognition of driver and passenger aberrations, or the design of rational, effective drug combinations. Through identification of likely (non)responders, treatment efficacy can be increased and side effects and effective treatment delay prevented in those who are unlikely to respond. To date, few predictive markers have entered the clinic, as commented previously and include *KRAS* in colorectal cancer, *ALK* rearrangement in non-small cell lung cancer, and *HER2/neu* amplification and overexpresson in breast cancer[84-87,240,241] and none in endometrial cancer[16].

The most reliable way to identify a clinically relevant predictive marker and with reasonable certainty distinguish predictive from prognostic aspects, is through controlled clinical trials. However, considering the time and cost involvement for these trials as well as the impact on cancer patients who would be exposed to a drug with its related side effects and who would assume more benefit from trial than from standard therapy, a lot is at stake and ideally highly likely candidates are lined up through alternative methods. The exploration of large prospectively collected patient series with high-quality clinical annotation combined with preclinical data, such as performed in this thesis, is well suited to fill some of this gap. Limitations in this approach are related to the retrospective analysis of prospectively collected data, and, on occasion, more rigorous imaging documentation at the crucial moments (prior to and after finishing metastatic treatments) could have resulted in inclusion of additional patients and thus a larger study population compliant with RECIST criteria.

A relation between stathmin1 level and response to paclitaxel has been investigated preclinically in a few other epithelial cancers, including breast cancer

and retinoblastoma[173-176], however, without confirming these findings clinically in the same cancer type, deemed necessary in our opinion. In our preclinical experiments using endometrial cancer cell lines, we demonstrated increased sensitivity to paclitaxel when, independent of original stathmin1 protein level, stathmin1 was successfully knocked down, in line with the previously mentioned preclinical studies[173,174,176]. We show this effect to be paclitaxel specific, with no change in sensitivity to other frequently used chemotherapeutic drugs in the endometrial cancer treatment, of which we tested carboplatin.

Potentially, we could have used additional assays to quantify apoptosis better, such as using flowcytometry or ELISA assays. Unavailability of and/ or unfamiliarity with certain techniques prohibited their usage at that moment.

We then show in our prospectively collected clinical dataset, correcting for important clinicopathological prognostic variables, that normal tumour stathmin1 levels, measured by IHC, were associated with markedly and statistically significant improved response measured by RECIST criteria, to paclitaxel containing chemotherapeutical regimes compared to tumours with high stathmin1. We further attempted to separate out potential predictive marker characteristics from prognostic marker ones. In that respect, it was important to observe that, in patients with metastatic disease, who in general all show poor survival, those treated with paclitaxel and high stathmin1 tumour levels had significantly reduced disease specific survival compared to the patients with normal stathmin1 levels treated equally. This contrasted the situation in patients treated differently (including different chemotherapeutic regimes), where differences in disease specific survival were not associated with stathmin1 level, such that an association between high stathmin1 level and poor response to paclitaxel, where stathmin1 level can predict the response to paclitaxel containing chemotherapeutic regimes, may be the underlying explanation.

To further substantiate this biologically; platinum based agents or alkylating agents, of which carboplatin and also cisplatin are well known examples, exert

their action through cell-cycle independent DNA binding; forming cross-links and adducts, affecting DNA conformation and DNA repair[242]. Taxanes on the other hand, of which paclitaxel is the prototype, belong to the family of anti-microtubule agents that bind to microtubules thereby reducing their tendency to depolymerise during cell division, and as such inhibit cell cycle progression, promote mitotic arrest and ultimately cell death[243]. As a critical regulator of the dynamics of these same microtubules, a role for stathmin1 in the prediction of response to paclitaxel, but not for carboplatin is biologically conceivable.

We feel that, in spite of the high quality of some of the purely preclinical studies, our study has added credibility to the assumption that stathmin1 may be(come) a valid predictive maker and could envisage a way forward using mouse models as a next and perhaps last intermediate step between our current stand and testing stathmin1's predictive abilities in a clinical trial setting.

Regarding the necessity of biomarker status retesting in metastatic lesions, very important in this context, I refer to the discussion in paragraph 12b.

Considering that the development from bench to bedside (FDA approval for HER2 overexpressed breast cancers[244]) for a pioneer targeted drug, traztuzumab, took approximately 25 years[245], the process has already substantially improved. Recently, development takes approximately a third or less of this time, and sunitinib (SU11248), of which first publications appeared in 2003[246,247], received FDA approval in 2006 already for advanced renal cell carcinoma and in 2011 for pancreatic neuroendocrine tumours[248].

6.4. Cross-disease focus

An exciting and relatively new idea in cancer research to drive cancer knowledge is cross-disease thinking in multiple areas including cancer drivers and biomarkers that appear important across various cancers, or research on targeted treatment where knowledge in one disease can headstart studies in another (Fig. 3). TCGA is

taking a leader role here, through both individual disease and 'pancancer' papers[57,249,250]. Not only are striking similarities found, but also differences between cancers, giving opportunities to further understand the specific roles biomarkers or cancer drivers have in different tumour types. In this context the HER2 biomarker may serve as an example where knowledge and treatment opportunities learnt in breast cancer, have been applied to other cancer types with results at times contrasting those in breast cancer, including sometimes suboptimal correlation of testing across different platforms (e.g. DNA, protein)[251-254]. A potential different regulation of HER2 therefore, and/ or absence of response to HER2 targeted treatment in various cancer types[255,256] allow for a different role or targetability of the protein in those diseases. And even more, juxtaposing and analysing tumour types with unrelated tumour types based on organ of origin, may potentially highlight certain cancer specificities even more.

In the studies included in this thesis, although no cross-cancer analyses have been performed, some cross cancer aspects can be highlighted.

The direct motivation for the investigation of *ARID1A* in endometrial cancer was that the two subtypes affected by loss of ARID1A both originate from endometriosis, and therefore have a direct link with the endometrium and per consequence endometrioid endometrial carcinoma (study 4). And although loss of ARID1A has since been linked to multiple cancer types, a large number of these seem specifically related to gynaecological cancers[187,188,228,229,231].

Although speculative, this suggests molecular similarity between these tumours, with at least one shared aberration, besides their relation through the tissue of origin; not shared by high grade serous ovarian or endometrial cancers.

The selection for more aggressive tumours using a routine 80% tumour cell content stratification was obvious in endometrial cancer (study 3). The existence of a similar prognostic influence in cervical cancer subtypes has subsequently been investigated, and high tumour purity in the adenocarcinomas but not in the squamous cell carcinomas was shown, and associated with increased risk of

recurrent disease[257]. It is interesting to note that the association only holds in the adenocarcinomas, which may be histologically more related to endometrial carcinoma than squamous cell carcinomas.

As a prognostic tumour marker, stathmin1 has been explored in a variety of cancer types, and importantly, its prognostic significance always related to more aggressive cases, assuming a similar effect on carcinogenesis in all. A predictive role of stathmin1 in different cancer types, apart from the study on endometrial cancer included in this thesis (study 5), has only been investigated preclinically. It is tempting to speculate if our clinical findings may be repeated in these cancers too, in line with the similarity in articles on its prognostic marker role.

7. CONCLUSIONS

In a large, prospectively collected endometrial cancer dataset, the FIGO 2009 classification system both simplified and improved prognostic stratification abilities compared to the previous system from 1988. (**study 1**)

We show in a large, prospectively collected dataset that through integration of the preoperative histology with the final or operative histology, prognostic information can be further improved, especially when discordance between both results exists. This results in the identification of subgroups with intermediate risk for metastatic spread and disease specific death that currently go unnoticed. (**study 2**)

The 80% tumour-cell content cutoff, meant to ensure high tumour purity, is, in endometrial cancer, associated with high risk clinicopathological characteristics and reduced disease specific survival and may thus introduce an unintended selection bias. (**study 3**)

In endometrial cancer, loss of ARID1A occurs most in endometrioid and clear cell subtypes and is, besides associated with deep myometrial infiltration, predominantly linked to clinicopathological parameters of less aggressive disease, but lacks correlation with survival. (**study 4**)

Loss of ARID1A appears to start early in endometrioid endometrial cancer carcinogenesis, judged by the already existing loss in atypical hyperplasia, and continues to increase with tumour progression. (**study 4**)

Integration of preclinical and clinical data supports that stathmin1 has potential as a predictive biomarker for response to paclitaxel containing chemotherapeutic regimes in endometrial cancer. (**study 5**)

Biomarker switch is a frequent phenomenon during endometrial cancrinoma disease progression and re-assessment of biomarker status in metastatic disease may be relevant. (**study 4 and 5**)

8. FUTURE PERSPECTIVES

In the discussion section some future perspectives have already been highlighted, such as an emphasis on cross-disease thinking to further cancer knowledge in paragraph 12e. Research so far has only scraped on the surface of the potential of integration of knowledge across cancer types. Similar to the situation in the clinic, where specialists think in conceptual frameworks and diseases specific to their specialty, in research this is often no different and research groups and meetings included, are often focused on one cancer type or in the case of f.e. gynaecological cancers, the cancers that belong to one specialty, impeding or at least not directly enhancing integration of knowledge available in other diseases. As such, TCGA[258]and IGCG[259] have clear potential to foment this, as well as basic and clinically oriented cancer meetings like AACR or ASCO where at least the physical distance between different topics and specialties has been minimized.

Biomarkers are intrinsically related to personalised medicine, or precision medicine, as a newly coined term. We stand on the doorstep of a new era with personalised treatment in cancer, but many areas need to be further developed before all cancer patients will benefit from precise and personalised treatments, including the identification of relevant targets and associated development of predictive biomarkers[260] (Fig. 5). Rather than finding the best treatments for the average patients (one size fits all; using population based study inclusion, routinely without molecularly characterization), as still the routine in a majority of cancer clinical trials[91,261], the focus should be to find the best treatment for the individual patient, which may be quite different[261]. This will require a different approach to clinical trials, based on a firm molecular biological hypothesis, where biomarkers play an important role already in early clinical development. Incorporated already in phase 1 trials, they can assist in the identification of the target population and response prediction[261]. Phase 2 trials could then be enriched for patiens with the biomarker of interest, and through various alternative trial

designs, accrual of patients and treatment allocation could be further modified according to the likely responding population[261,262]. A more flexible design will increase the chance of positive trial results, considering that in unselected patient populations, trials have a higher likelihood to miss positive effects due to patient heterogeneity, including absence of a necessary genetic aberration.

Further, through an ongoing dialogue between the laboratory and the clinic, drug resistance, tumour biology and unexpected responses can be studied more timely and efficiently, combining patient samples and response data with preclinical testing[91,261].

An approach as featured here, is expected to improve, speed up and potentially reduce the costs involved with the bench-to-bedside development time of new anti-cancer drugs, which, as also emphasised in paragraph 12d, is important for our patients to get speedier access to new, well tested, anti-cancer medication.

Also part of this discussion, directly relating back to study 4 and 5, is a further exploration of the need to reassess biomarker status, and thus the need for repeated biopsy taking, in metastatic disease. It is unknown whether all biomarkers will show the same heterogeneity upon cancer progression, or whether for example certain changes are related to the type of spread, the localisation or timing of metastasis and prior treatment.

Systems biology is a relatively new field where iterative computational modeling of multiple types of patient data (omics, imaging, clinical data *e.g.*) is combined with preclinical testing to drive our understanding of carcinogenesis, enhance the identification and development of drugs for emerging targets or to counter emerging drug resistance[263,264]. Applying systems biology approaches more systematically, will require team science in much larger degrees than research groups may be used to, with an important role for bioinformatics, alongside molecular biologists and doctors, and an emphasis on understanding each other's language and the possibilities and limits of the various techniques. With the

availability of affordable large scale techniques to study DNA, RNA and recently also protein, as well as major advances in computational science, such a "marriage" has potential to drive the field further. This will allow us to perform network analysis rather than focusing on individual proteins or pathways, which is important to better understand intracellular feedback, interactions and signaling across different pathways, and particularly important in cancer; detect potential rewiring of pathways[265,266]. Figure 7 illustrates how systems biology approaches can and have linked different data sources and modeling methods to answer a variety of (clinical) questions.
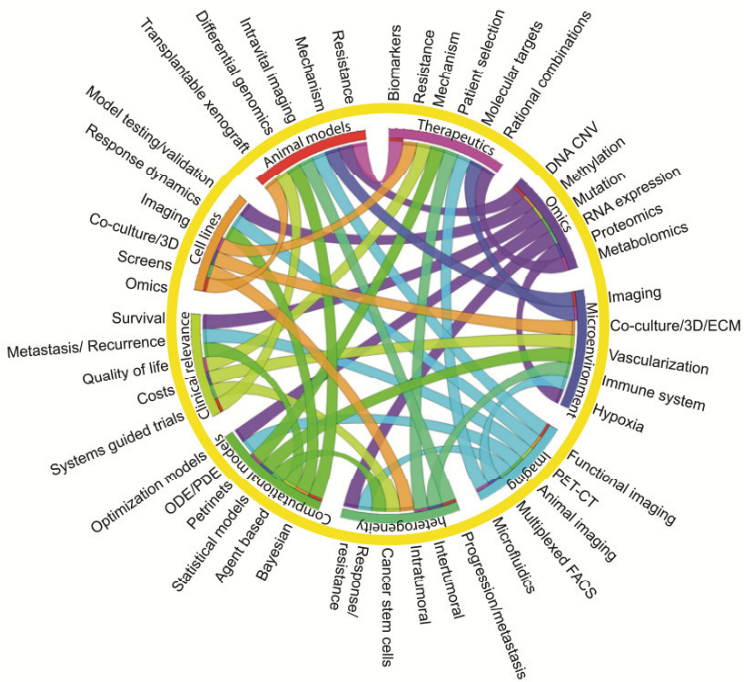

Figure 7. Types of data and systems models in cancer
The circos plot visualises different sources of data, modelling methods and questions that can be addressed using cancer systems approaches. The ribbons represent the multitude of links between data types, models and outcomes.

Practically, and related to this thesis, a large percentage of the samples of our biobank have now been run on a high throughput protein platform, RPPA[267,268], which, through the large number of proteins quantitatively analysed, will hopefully allow us just that. Additionally it will give us the opportunity to integrate these data with RNA expression, sequencing and IHC data, the latter at least to date being more applicable in a routine clinical setting. To study stathmin1 and also *ARID1A* using integrative analysis will further our knowledge and could hopefully further elucidate links between stathmin1 and also *ARID1A* the *PI3K* pathway[154,155,194], and generate novel ones that may have implications for future patient care.

Preclinical models are necessary to study and test hypotheses. Models should be clinically relevant and replicate (part of) the biology in patients. Evidence is accumulating that in order to study some aspects in (endometrial) cancer; current models may be further optimized or combined. Endometrial cancer cell lines, as a model widely used in literature and used in this thesis, are sometimes poorly characterised clinically, and may have been passaged extensively, which can lead to (large) changes in genomic aberrations[269]. Comparing 3D with 2D (monolayer) cell culture, significant differences have been shown in gene expression, predominantly related to the extracellular matrix and related processes such as immune response and cell adhesion, importantly cellular responsiveness to chemotherapy and radiation may be also affected by growth conditions[270,271] and as such, 3D cultures may mimic the *in vivo* situation better. Animal models, often mouse models, with subcutaneous but especially with orthotopic tumours using bioluminescence markers to facilitate imaging, are a further acquisition to the gamut, allowing monitoring tumour progression and response to interventions effectively[272], investigation of the functional interactions between, and specific roles of the tumour and its (natural) microenvironment[273] or for example altered tumourigenesis in the context of obesity.

As also suggested in paragraph 12d, to further the development of stathmin1 as a potential predictive biomarker, mouse models could be very informative, before possibly taking it to clinical trials.

Although not a topic studied in this thesis, the still relatively disappointing results generated by targeted therapies, have not only led to the realisation that we need adequate predictive biomarkers, such as mentioned above, but also to the awareness that carefully studied combination therapies (whether targeted therapies combined with other targeted therapies, hormonal or chemotherapeutic drug) simulataneously blocking various pathways and possible escape (and thus resistance) routes for the tumour, may significantly increase response rates[16,48,91,274].

9. ERRATA

Study 1; page 104 'Eleven cases classified as FIGO 88 stage IIIA based on positive cytology only, were restaged into FIGO 09 IA (n=6), FIGO 09 IB (n=4) and FIGO 09 II (n=1). In this group, tumor subtype' should read: Twelve cases classified as FIGO 88 stage IIIA based on positive cytology only, were restaged into FIGO 09 IA (n=7), FIGO 09 IB (n=4) and FIGO 09 II (n=1). In this group, tumor subtype was endometrioid in 67.7%.

Study 1; page 105 table 2 footnote b 'Five year survival 90% for patients with positive cytology (n=11).' Should read 'Five year survival 90% for patients with positive cytology (n=12).'

Study 4; page 429 'The hyperplasia series consists of a retrospectively collected series' should read 'The hyperplasia series consists of a prospectively collected series'.

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