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To my family

ABSTRACT

Jutta Huvila

Prognostic biomarkers in endometrioid endometrial carcinoma

University of Turku, Faculty of Medicine, Department of Obstetrics and Gynecology, Department of Pathology, University of Turku Doctoral Program of Clinical Investigation, Turku, Finland (2017)

Endometrial cancer is a heterogenic group of malignancies with differences in pathogenesis and clinical behavior. Correct risk stratification of these patients is essential for successful allocation of treatment modalities. Currently, classification of endometrial cancer is based solely on clinicopathological parameters. The aim of this thesis was to study the genomic heterogeneity of endometrioid endometrial carcinoma (EEC) and to identify prognostic immunohistochemical biomarkers for disease stratification.

This study is based on patient material derived from 640 patients with up to 30 years of follow-up. Gene expression profiling using two different microarray platforms revealed Apolipoprotein E (APOE) to be the most overexpressed gene in poorly differentiated EEC when compared to well-differentiated carcinoma. Immunohistochemical analysis of early stage EEC specimens suggested that progesterone receptor (PR) has an independent role in prognostication. Further studies suggested that l-asparaginase (ASRLG1) could serve as a novel prognostic biomarker in EEC. In an attempt to produce a clinically useful prognostic panel of biomarkers, immunohistochemical stainings of tissue microarrays combined with a machine learning-based method were employed. The results demonstrate that EEC patients can be stratified into three groups with significantly different clinical behavior using p53 and ASRGL1 stainings.

In summary, the study highlights the importance of PR, p53 and the novel biomarker ASRGL1 in EEC prognostication. The present findings suggest that the panel of tissue biomarkers developed can be used for identification of patients who are at risk of aggressive disease course and an unfavorable outcome of EEC.

Keywords: endometrial cancer, endometrial endometrioid cancer, tissue biomarker, tumor heterogeneity, ASRGL1, PR, p53

TIIVISTELMÄ

Jutta Huvila

Ennusteelliset merkkiaineet endometrioidissa endometriumin karsinoomassa

Turun yliopisto, Lääketieteellinen tiedekunta, Synnytys- ja naistentautioppi sekä Patologia, Turun yliopiston kliininen tohtoriohjelma, Turku (2017)

Kohdun limakalvon syöpä eli kohdunrunkosyöpä on heterogeeninen tautiryhmä, jonka patogeneesissä ja kliinisessä käyttäytymisessä on eroja. Potilaan syövän ennusteen arvio on tärkeä, jotta oikeat hoitomuodot kohdentuvat niitä tarvitseville potilaille. Nykyisellään, ennusteen arvioinnissa käytettävät riskiluokitukset perustuvat sekä kliinisiin että patologis–anatomisiin muuttujiin eikä niissä käytetä merkkiaineita (”biomarkkereita”). Tämän tutkimuksen tavoitteena oli selvittää tavallisimman kohdunrunkosyövän, endometrioidin endometriumin syövän (EEC) heterogeenisyyttä sekä määrittää ennusteellinen, immunohistokemiallisiin värjäyksiin pohjautuva merkkiainepaneeli taudin luokittelemiseksi.

Tutkimusaineisto muodostuu 640 potilaasta, joista on enimmillään 30 vuoden seuranta-aika. Kahdella eri mikrosirualustalla toteutettu geeniekspressioanalyysi osoitti Apolipoproteiini E:n (APOE) olevan korkeimmin ylieskpressoitunut geeni verrattaessa huonosti ja hyvin erilaistunutta tautia. Kohtuun rajoittuneen EEC:n immunohistokemiallinen analyysi viittaa progesteronireseptorin (PR) itsenäiseen ennusteelliseen rooliin. Myöhemmät tutkimukset viittasivat l-asparaginaasin (ASRGL1) mahdolliseen rooliin taudin käyttäytymistä ennustavana merkkiaineena EEC:ssä. Kliinisesti käyttökelpoisen, immunohistokemiallisiin värjäyksiin pohjautuvan ennusteellisen merkkiainepaneelin muodostamiseksi analysoitiin monikudosblokkeilla tehtyjä immunohistokemiallisia värjäyksiä kone-oppimiseen pohjautuvien analysointimenetelmien avulla. Analyysin tulokset osoittivat että p53- ja ASRGL1- värjäysten avulla voitiin EEC -potilaat luokitella kolmeen kliiniseltä taudinkuvaltaan ja ennusteeltaan eroavaan ryhmään.

Tämä tutkimus korostaa PR, p53 ja ASRGL1 –merkkiaineiden merkitystä EEC:n riskinarvioinnissa. Kehitetyn merkkiainepaneelin avulla oli tässä tutkimuksessa mahdollista tunnistaa ne potilaat, joiden taudinkulku oli aggressiivinen ja ennuste epäsuotuisa.

Avainsanat: kohdunrunkosyöpä, endometriumin karsinooma, tuumori heterogeenisyys, ennusteelliset tekijät, ASRGL, PR, p53

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ABBREVIATIONS

AJCC American Joint Committee on Cancer
BMI Body-mass index
BT Brachytherapy
BSO Bilateral salpingo-oophorectomy
CTL Cytotoxic $CD8^+$ T-lymphocyte
DFS Disease free survival
DSS Disease specific survival
EC Endometrioid carcinoma
EEC Endometrioid endometrial (adeno)carcinoma
EIN Endometrial intraepithelial neoplasia
ER Estrogen receptor
ESGO European Society of Gynecological Oncology
ESMO European Society of Medical Oncology
ESTRO European Society of Radiotherapy & Oncology
FIGO Fédération Internationale de Gynécologie et d'Obstétrique – The International Federation of Gynecology and Obstetrics
FFPE Formaline-fixed, paraffin-embedded
GOG Gynecological Oncology Group
H Hysterectomy
LND Lymph node dissection
LNM Lymph node metastasis
LVI Lymphovascular invasion
MI Myometrial invasion
MMR Mismatch repair
MRI Magnetic resonance imaging
MSI Microsatellite instability
NEEC Non-endometrioid endometrial carcinoma
OS Overall survival
PR Progesterone receptor
qRT-PCR Quantitative real-time polymerase chain reaction
TAM Tumor associated macrophages
TCGA The Cancer Genome Atlas
TMA Tissue microarray
WHO World Health Organization
WPRT Whole pelvic radiation therapy

Several genes and their encoded proteins are mentioned this thesis. They are referred to by their official abbreviations ("gene IDs") as listed in international databases. In several cases, different names have been suggested for one and the same gene, as explained in the text.

LIST OF ORIGINAL PUBLICATIONS

I Huvila J, Brandt A, Rojas CR, Pasanen S, Talve L, Hirsimäki P, Fey V, Kytömäki L, Saukko P, Carpén O, Soini JT, Grénman S and Auranen A. Gene expression profiling of endometrial adenocarcinomas reveals increased apolipoprotein E expression in poorly differentiated tumors. *Int J Gynecol Cancer* 2009; 19(7), 1226-1231.

II Huvila J, Talve L, Carpén O, Edqvist PH, Pontén F, Grénman S and Auranen A. Progesterone receptor negativity is an independent risk factor for relapse in patients with early stage endometrioid endometrial adenocarcinoma. *Gynecol Oncol* 2013; 130(3), 463-469.

III Edqvist PH*, Huvila J*, Forsström B, Talve L, Carpén O, Salvesen HB, Krakstad C, Grénman S, Johannesson H, Ljungqvist O, Uhlén M, Pontén F and Auranen A. Loss of ASRGL1 expression is an independent biomarker for disease-specific survival in endometrioid endometrial carcinoma. *Gynecol Oncol* 2015; 137(3), 529-537.

IV Huvila J*, Laajala TD*, Edqvist PH, Talve L, Pontén F, Grénman S, Carpén O, Aittokallio T and Auranen A. Combined ASRGL1 and p53 immunohistochemistry as an independent predictor of survival in endometrioid endometrial adenocarcinoma. *Submitted.*

* Equal contributors

1 INTRODUCTION

Endometrial cancer (EC) is the most common gynecological malignancy in the developed world and the second most common gynecological malignancy worldwide after cervical cancer. The survival rates vary between 80% in early disease and 50% in advanced disease (Kitchener and Trimble, 2009). A majority, approximately 75%, of endometrial cancer cases are found in an early stage. However, currently more than 10% of stage I cancers, generally thought to have a good prognosis, relapse (Creutzberg et al., 2000; Zeimet et al., 2013; Green et al., 2015).

Endometrial cancer is divided into low-, intermediate- and high-risk disease: first, according to histological subtype; and second, according to the staging and grading of the International Federation of Gynecology and Obstetrics (FIGO) (Zaino et al., 1995; Pecorelli, 2009). Endometrioid-type endometrial cancer (EEC) is the most prevalent histological subtype, and this group is further divided into risk groups primarily according to stage and grade, whereas non-endometrioid type EC is always considered a high-risk disease.

Treatment of EC has remained relatively static over the past 40 years (Kitchener, 2006), and surgery remains the most important mode of treatment (Amant et al., 2005). The mortality rates in Finland and elsewhere in Europe have declined slightly over the past 30-40 years (Bray et al., 2005; Finnish Cancer Registry, 2016), which may be the result of improved early detection of the disease. However, similar improvements have not been seen in the USA (Siegel, Miller and Jemal, 2016). The extent of surgery is determined by risk stratification and patient-related comorbidities, and this includes hysterectomy, salpingo-oophorectomy and optional pelvic and para-aortic lymphadenectomy. Adjuvant therapy, such as whole pelvic radiation therapy (WPRT), brachytherapy or chemotherapy, is generally provided when intermediate or high-risk disease in suspected (Colombo et al., 2016; NCCN, 2016).

While non-endometrioid EC (NEEC) is always considered a high-risk disease and is intensively treated, successful allocation of accurate treatment in the wide range of EEC cases remains a challenge. The currently used risk stratification method fails to identify a subgroup of EEC patients who, despite the supposed low-risk nature of the disease, still suffer from a recurrence or die of EEC. Furthermore, it is possible that a considerable number of intensively treated EEC patients do not experience a relapse but are subsequently predisposed to the side effects of adjuvant treatment modalities, such as lower extremity lymphoedema or neuropathy. Quality of life following treatment is an important issue for endometrial cancer survivors, of whom there are currently more than 12,000 in Finland (Finnish Cancer Registry, 2016) and more than 750,000 in the USA (Miller et al., 2016).

During the past two decades, medicine has undergone a revolution that is expected to fundamentally transform future medical research and practice. Technological developments (such as genetic, high-throughput genomics and other "omics" analyses and tissue microarrays) have rendered molecular analysis of human samples cheaper and more efficient. Molecular characterization of many disease phenotypes has demonstrated unexpectedly high heterogeneity of molecular mechanisms leading to common diseases, including cancers (Cancer Genome Atlas Research Network et al., 2013b; Burrell et al., 2013). Accumulation of huge amounts of molecular "omics" data and other digitized data from registries and electronic health records is leading to an exponentially evolving phenomenon called big data.

Undoubtedly, the big data revolution and personalized medicine now influence EC research and treatment as well. Currently, however, only a few clinical and histopathological biomarkers are integrated into clinical guidelines (Tangjitgamol et al., 2009; Colombo et al., 2016; NCCN, 2016) despite continuous efforts to study clinical, histopathological, immunohistochemical, serum and molecular biomarkers and distinguish between low- and high-grade disease. Yet only a small proportion of studies have focused exclusively on EEC, which is the most prevalent type of endometrial cancer. Subsequently, there is a substantial need for prognostic biomarkers. These biomarkers are especially needed to guide treatment decisions, both surgical and adjuvant, in order to truly begin treating endometrial cancer patients in accordance with personalized treatment strategies.

The current thesis attempts to incorporate the approaches of personalized medicine (and big data) in research on EC. Currently available literature in the field provides plenty of data on individual biomarkers and risk factors. In light of our present knowledge, it seem likely that individualized treatment of EEC patients can be achieved only by employing a carefully selected and validated panel of biomarkers and other disease-relevant data.

2 REVIEW OF THE LITERATURE

2.1 Epidemiology in endometrial cancer

2.1.1 Incidence and mortality worldwide

Endometrial cancer is the most common gynecological cancer of the Western world, with nearly 170,000 new cases annually and nearly 320,000 worldwide (Torre et al., 2015). The highest incidence is in North America (22.0/100,000) and Europe (11.8 to 12.5/100,000) (Parkin et al., 2005). Survival is generally good: in developed countries, the age-adjusted survival rate is 82%, whereas in developing countries, it is 67% (Parkin et al., 2005).

The incidence of endometrial cancer is increasing. This increase is thought to be associated with the current epidemic of obesity, increased life expectancy, a decline in fertility, and previous use of estrogen-based hormone replacement therapy in particular without progestins (Bray et al., 2005; Kitchener and Trimble, 2009). Contrary to post-menopausal women, the incidence of endometrial cancer is declining in pre-menopausal women in a majority of Northern European countries. In Finland, however, the incidence of endometrial cancer continued to increase in pre-menopausal women born after the Second World War (Bray et al., 2005), although according to more recent register data, the incidence of EC in pre-menopausal women no longer demonstrates a rising trend (Engholm et al., 2015).

2.1.2 Incidence of EC and prognosis in Finland

In 2014, uterine cancer (ICD-10 code C54) was diagnosed in 836 Finnish patients, making it the fourth most common cancer in Finnish women after breast, colon and lung/bronchial cancer. The age-adjusted incidence of and cancer-specific deaths from uterine cancer in 2014 were 13.2/100,000 and 2.2/100,000, respectively. Endometrial cancer was typically found at an early stage, and in more than half of the cases, the disease was restricted to the uterus. The average age of patients with newly diagnosed EC (in 2010-2014) was 69 years, ranging from two cases in the 30–34 age group to 65 cases in patients 85 years and older. Approximately 90% of EC patients were aged 55 and older. During follow-up (2012–2014), the disease-specific survival rate was 93% one year after diagnosis and 84% five years after diagnosis. (Finnish Cancer Registry, 2016).

2.2 Etiology of endometrial cancer

2.2.1 Dualistic classification and risk factors

Division of endometrial carcinogenesis into two principal types (1 and 2) was presented more than 30 years ago (Kurman, Kaminski and Norris, 1985). Type 1 comprises 70–80% of endometrial cancer cases and is associated with estrogen-related endometrioid histology,

whereas type 2 is associated with non-endometrioid histology. Patients with type 2 cancer are more often of advanced age compared to those with type 1. Type 1 patients are more frequently obese and nulliparous (Amant et al., 2005), whereas type 2 patients are associated with advanced age, multiparity (Brinton et al., 2013) and antiestrogen (tamoxifen) use (primarily for serous adenocarcinoma). The aforementioned differences between the two principal types of EC have triggered a number of studies to identify molecular markers associated with these subtypes (summarized in **Table 2.1)**.

Table 2.1 Characteristic features and biomarkers in type 1 and 2 EC

	Type 1	**Type 2**
Proportion	60–80%	20–40%
Histology	EEC	High-grade EEC and NEEC
Aneuploidy	Rare	Frequent
Estrogen stimulation	Related	Unrelated
Clinical behavior	Indolent	Aggressive
5-year survival	86%	59%
ER, PR loss of expression	27–30%	76–81%
PTEN inactivation	37–78%	1–11%
Microsatellite instability	20–45%	0–5%
p53 mutations	10–20%	90%
HER2 overexpression	Rare	45–80%
E-cadherin alterations	10–20%	80–90%
ARID1A mutation	25–48%	6–11%
PIK3CA mutation	36–52%	24–42%

Modified from Liu et al. (2007), Engelsen et al. (2009), Murali et al. (2014) and Werner et al. (2014)

2.2.2 Precursor lesions

Types 1 and 2 cancers are associated with distinct precursor lesions. Endometrial hyperplasia is a precursor lesion for type 1 cancer. It is associated with exposure to unopposed estrogen and it is histologically categorized as non-atypical or atypical endometrial hyperplasia according to WHO guidelines (Kurman, 2014). In 1% to 3% of cases, endometrial hyperplasia without atypia leads to well-differentiated EC, whereas in up to 29% of cases, atypical hyperplasia is the antecedent lesion of cancer (Kurman et al., 1985; Lacey et al., 2010). Moreover, around 40% of patients with atypical hyperplasia in fact have an underlying carcinoma (Trimble et al., 2006; Reed et al., 2010). The differential diagnosis between a precursor lesion and well-differentiated EC is difficult (Silverberg, 2000).

Type 2 cancers arise from atrophic endometrium and are associated with an atypical endometrial lesion limited to the epithelium (intraepithelial lesions) (Kurman, 2014). Also in EEC, a co-existing atrophic endometrium has been associated with an adverse behavior

(Sivridis, Fox and Buckley, 1998; Geels et al., 2012), and it has been postulated that an endometrioid adenocarcinoma arising from an atrophic background would represent a third, separate type (Sivridis et al., 1998) associated with more frequent loss of E-cadherin (Geels et al., 2015).

2.2.3 Hereditary syndromes

Various hereditary syndromes are associated with an increased risk of endometrial carcinoma, and it has been estimated that 5% of EC cases result from a hereditary cause (Lu, 2008). Lynch syndrome (hereditary nonpolyposis colorectal cancer, or HNPCC) is the most common cause of familial EC and is responsible for 2% of endometrial cancers (Ollikainen et al., 2005; Hampel et al., 2006); in young patients, the incidence is even higher (Lu et al., 2007). Lynch syndrome has an autosomal-dominant inheritance pattern resulting from germline transmission of a defective DNA mismatch repair gene (*MSH2, MLH1, MSH6* or *PMS2*). The fact that defects of the mismatch repair genes as well as hypermethylation of the *MLH1* gene have also been detected in sporadic EC speaks to the importance of the DNA repair mechanism in the maintenance of endometrial integrity and in the development of EC, as will be reviewed below. The lifetime risk of endometrial cancer for Lynch syndrome patients is 20% to 71% as reviewed by Vasen et al. (2007) and Garg and Soslow (2011). In addition to the elevated risk of EC, 30% of Lynch syndrome patients develop a second primary tumor within 10 years of the first cancer (Kitchener and Trimble, 2009).

Tumor suppressor phosphatase and tensin homolog (PTEN) is an inhibitor of the PI3K/AKT pathway and also controls proliferation. Conversely, the inactivation of PTEN results in increased proliferation and reduced apoptosis. The role of PTEN in EC is further emphasized by observations of somatic *PTEN* mutations in EC, as will be reviewed below. Cowden syndrome is a rare disorder associated with a germline mutation of the *PTEN* gene that carries an increased genetic susceptibility to EC, especially in younger patients (Eng, 2003; Blumenthal and Dennis, 2008).

BRCA1 (breast cancer 1) mutations, which are strongly associated with hereditary breast and ovarian cancer, do not increase overall risk for EEC (Shu et al., 2016). Even though the role of BRCA1 mutations remain inconclusive in serous carcinoma as reviewed by Garg and Soslow (2011) recent evidence indicates that the risk of serous or serous-like EC is increased in women carrying BRCA1 mutations (Shu et al., 2016).

2.3 Histology and grading

2.3.1 Histology

The heterogeneity of endometrial cancer is also illustrated by its histological classification into several subtypes according to WHO (Kurman, 2014). This classification is summarized in **Table 2.2**.

Table 2.2 Histological classification and incidence of endometrial carcinoma according to WHO (Kurman, 2014).

Endometrial carcinomas	Incidence
Endometrioid carcinoma	70–80%
Mucinous carcinoma	1–9%
Serous intraepithelial carcinoma	rare*
Serous carcinoma	5–10%*
Clear cell carcinoma	2 %
Neuroendocrine tumours	<1%
Mixed cell adenocarcinoma	
Undifferentiated carcinoma	uncommon
Dedifferentiated carcinoma	uncommon

* (Wheeler et al., 2000; Kitchener and Trimble, 2009; Murali et al., 2014; Matias-Guiu and Davidson, 2014)

2.3.2 Grading of endometrioid endometrial adenocarcinoma

The endometrioid subtype of endometrial cancer is divided into three grades according to differentiation of the tumor, as compared to normal proliferative endometrium. In 1995, an international Gynecologic Oncology Group (GOG) (Zaino et al., 1995) suggested a grading system in which both architectural grade and nuclear grade were evaluated, and both had an independent role in the determining the grade.

EEC is divided into well (grade 1), moderately (grade 2) and poorly differentiated (grade 3) carcinomas according to the amount of solid, non-gland forming nests of neoplastic cells. By definition, grade 1 cancers consists of <5%, grade 2 of 5% to 50%, and grade 3 cancers of more than 50% of solid growth of the neoplastic component, as exemplified in **Figure 2.1**.

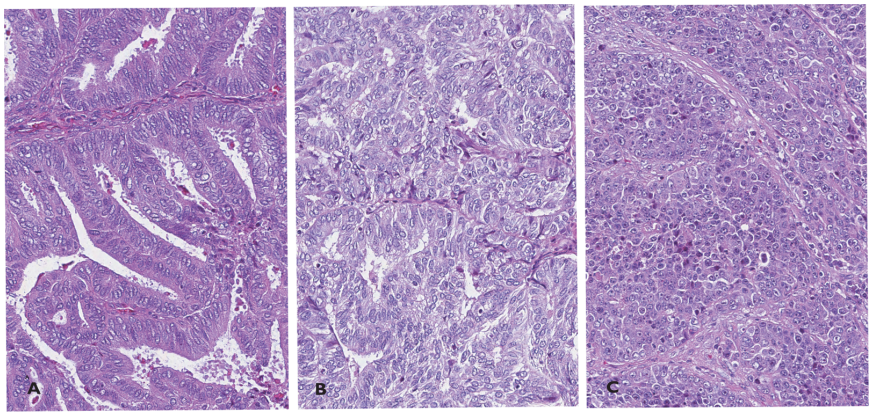


Figure 2.1 Photomicrograph of **a)** well, **b)** moderately, and **c)** poorly differentiated EEC

Squamous differentiation should not be considered solid neoplastic growth. Notable nuclear atypia, defined as large pleomorphic nuclei with coarse chromatin, and irregular nucleoli raise the grade of a grade 1 or 2 tumor by one.

2.4 Diagnosis, imaging and staging

2.4.1 Symptoms and diagnostic procedures

Endometrial cancer often presents as abnormal or unexpected uterine bleeding (Amant et al., 2005). The presence of abnormal bleeding is more common in poorly differentiated EC, however, without prognostic significance (Seebacher et al., 2009). In advanced cases, symptoms such as pelvic pain or abdominal distension may be present (Morice et al., 2016).

The cornerstones of endometrial cancer diagnostics are histopathological analysis of endometrial biopsy material and imaging. A sample for histological analysis is obtained either by miniature endometrial biopsy (e.g., Pipelle ®) or endometrial curettage, which can be performed concurrently with hysteroscopy (Colombo et al., 2016). For diagnostic evaluation, an endometrial biopsy assessed under hysteroscopic guidance remains the gold standard, especially when carcinoma is clinically suspected despite negative results from initial investigation (SGO et al., 2014).

2.4.2 Imaging

Radiological imaging is widely used in EC for exclusion of metastatic disease and detection of enlarged or suspicious lymph nodes. During a clinical examination, transvaginal ultrasound is routinely used to evaluate the depth of myometrial invasion (MI). Additionally, computerized tomography (CT) or, alternatively, magnetic resonance imaging (MRI) is performed. Fluorodeoxyglucose positron emission tomography/CT (FDG PET/CT) imaging can also be performed on a case-specific basis to evaluate the possible spread of disease outside the uterus. (Haldorsen and Salvesen, 2016; Morice et al., 2016). However, the benefit of routine imaging in low-grade EC has not been demonstrated, and routine preoperative imaging is not considered necessary (SGO et al., 2014; Baker et al., 2015).

2.4.3 Staging

In 1988, the FIGO Committee on Gynecological Oncology introduced a surgical staging system for cancer of the uterine corpus. The FIGO staging system was revised in 2009 (Pecorelli, 2009), and it is presented in **Table 2.3**.

2.5 Treatment

Surgery is the primary curative treatment of EC. Hysterectomy (H) and bilateral salpingo-oophorectomy (BSO) are the standard procedures, which are combined with lymph node dissection (LND) on a case-specific basis. In developed countries, endometrial carcinoma

is usually surgically addressed using a laparoscopic or robot-assisted laparoscopic approach. After surgery and histopathological evaluation, adjuvant therapy is provided according to Asian, European and American guidelines (Tangjitgamol et al., 2009; Colombo et al., 2016; NCCN, 2016).

Table 2.3 The FIGO staging system of 2009, TNM classification of EC and five-year survival

FIGO stage		TNM	% five-year survival
I	Tumor confined to the uterine corpus		
IA	Tumor limited to endometrium or invading less than half of myometrium	T1aN0M0	96
IB	Tumor invades one half or more of myometrium	T1bN0M0	87
II	Tumor invades stromal connective tissue of the cervix but does not extend beyond uterus	T2N0M0	80
III	Local and/or regional spread as specified below:		
IIIA	Tumor invades the serosa of the corpus uteri or adnexae (direct extension or metastasis)	T3aN0M0	48
IIIB	Vaginal or parametrial involvement (direct extension or metastasis)	T3bN0M0	53
IIIC1	Metastasis to pelvic lymph nodes	T1-3N1M0	60
IIIC2	Metastasis to para-aortic lymph nodes with or without metastasis to pelvic lymph nodes	T1-3N2M0	53
IV	Tumor invades bladder and/or bowel mucosa, and/or distant metastases		
IVA	Tumor invades bladder and/or bowel mucosa	T4 Any N M0	57
IVB	Distant metastasis (excluding metastasis to vagina, pelvic serosa or adnexae, including intra-abdominal metastases and/or inguinal lymph nodes)	Any T Any N, M1	16

Modified from FIGO, American Joint Committee of Cancer (AJCC) and WHO (Pecorelli, 2009; Edge, 2010; Werner et al., 2012; Kurman, 2014).

2.5.1 Surgical staging

To determine the extent of surgery needed, patients with EEC are classified as low-, intermediate- or high-risk according to preoperative investigations, as presented in **Table 2.4**. All patients with non-endometrioid histology are considered to have a high-risk disease.

The necessity and benefits of lymphadenectomy in EC have been widely discussed (Colombo et al., 2016). In the current Finnish guidelines, pelvic and para-aortic lymph node dissection (LND) is performed depending on the histology, grade and preoperatively estimated depth of MI, as presented in **Table 2.4** (FIN-GOG, 2016).

Table 2.4 Preoperative risk stratification

Risk group	Preoperative stage and grade	Primary treatment
Low	MI <50%, grade 1–2	H+BSO
Intermediate	MI <50%, grade 3; MI ≥50 % grade 1–2	H+BSO+LND
High	MI ≥50%, grade 3; cervical stromal invasion or advanced disease	H+BSO+LND

Adopted from ESMO-ESGO-ESTRO guidelines (Colombo et al., 2016) and FIN-GOG guidelines (FIN-GOG, 2016). MI: myometrial invasion, H: hysterectomy, BSO: bilateral salpingo-oophorectomy, LND: lymph node dissection (pelvic and para-aortic).

The role of LND, both as a staging procedure and as a potentially curative treatment, has been widely discussed. The presence of lymph node metastasis (LNM) is associated with an adverse prognosis (**Table 2.3**), which has been shown to be independent of other uterine-related risk factors, such as MI and grade (Barrena Medel et al., 2011). However, the therapeutic value of LND continues to be debated (Koskas, Rouzier and Amant, 2015).

Several studies have demonstrated that intermediate and high-risk, as well as advanced EC patients, benefit by means of survival from LND (Chan et al., 2006; Todo et al., 2010), but the findings concerning supposed low-grade EEC remain controversial. In several studies, low-risk EEC patients demonstrated no survival benefit from LND (Trimble, Kosary and Park, 1998; Chan et al., 2006; Todo et al., 2010; Wright et al., 2016). Similarly, two randomized clinical trials have been performed (Benedetti Panici et al., 2008; Kitchener et al., 2009), and neither demonstrated a survival benefit from LND. However, these studies have been criticized over patient selection and quality issues, such as the (in)sufficient number of lymph nodes removed (Guntupalli et al., 2012). LND is currently not considered to be a curative treatment for EC. However, LND plays an important role in surgical staging (SGO et al., 2014).

The feasibility of LND, including sensitivity and specificity of sentinel lymph node biopsy, has been evaluated in the treatment of EC (Kang et al., 2011; Cormier et al., 2015). As a middle ground solution between performing LND and not performing LND, sentinel lymph node biopsy, performed, for example, in breast and vulvar cancer surgery, has been proposed to be useful in EC treatment (Cormier et al., 2015). Sentinel lymph node mapping, particularly in conjunction with a surgical algorithm to guide the process (Barlin et al., 2012), and pathologic ultrastaging (Delpech et al., 2007; Cormier et al., 2015) have been found to be a potential staging strategy in low or intermediate grade disease (Delpech et al., 2007; Ballester et al., 2011; Daraï et al., 2015). This strategy provides information on nodal metastasis and in the future may possibly replace traditional LND as a staging procedure, which may aid in reducing morbidity and postoperative complications. Ongoing prospective trials to evaluate the optimal technical performance as well as the clinical utility of sentinel lymph node mapping (ClinicalTrials.gov, 2016b; ClinicalTrials.gov, 2015) will

hopefully provide insight into how sentinel lymph node mapping guides adjuvant treatment.

A frozen section from EC tissue can be prepared perioperatively to assess risk factors, such as deep MI, and to guide the subsequent need for LND. When assessing deep MI, discordance rates of approximately 15% have been reported between the frozen section and the formalin-fixed paraffin embedded (FFPE) material measurements (Sinno et al., 2016; Soslow, 2016) However, in a prospective setting, clinically significant discordance was found in only 1.3% of cases (Kumar et al., 2012). The frozen section is most often prepared to guide LND in low- and intermediate-risk cases. However, currently the benefit of LND in these cancers has been questioned, and it has been suggested that sentinel node mapping could obviate frozen sections in EEC diagnostics (Soslow, 2016).

2.5.1.1 Sampling of surgical specimens

Very little research data is available on proper sampling of hysterectomy and lymphadenectomy specimens. Most of the available research data focus on the consistency between preoperative samples and hysterectomy specimens, which demonstrate that in 2% to 4% of cases, a clinically relevant upgrading of grade 1 EEC to grade 3 EEC or NEEC occurred (Leitao et al., 2009; Neubauer and Lurain, 2011; Helpman et al., 2014).

The Manual of Surgical Pathology presents the requirements for adequate tissue sampling (Lester, 2010). Altogether, a minimum of 20 samples should be taken from the ovaries, fallopian tubes, parametrium, cervix, lower uterine segment and tumor. However, the sampling procedure supposedly varies considerably between treatment centers, and as a practical matter, it is never reported as a qualitative factor in scientific papers despite its consequential role in staging. Non-adequate tissue sampling for biochemical analyses and for extraction of DNA, RNA, proteins and other molecules for research purposes is obviously a major factor affecting the reliability of subsequent analyses, but it is seldom addressed in research reports.

Cervical involvement is important in the evaluation of the spread of the disease. The extent of cervical sampling, i.e., whether two cervical samples are enough or the entire uterine cervix should be processed, has been systematically assessed in a scientific context (Nayar et al., 2008; Syed, Reed and Millan, 2015). The results indicate that two cervical samples, an anterior and a posterior sample, are adequate to identify stromal invasion to the cervix.

2.5.2 Adjuvant therapy

The need for adjuvant therapy is based on postoperative risk assessment, as presented in **Table 2.5**. In 2016, the Finnish guidelines for adjuvant therapy, as determined by the Finnish Gynecological Oncology Group (FIN-GOG), were updated to comply with the

renewed ESMO-ESGO-ESTRO consensus statement, which included a new high-intermediate risk group with lymphovascular invasion (LVI).

In addition to the recommendations, WPRT can be considered in cases when patient-related issues, such as obesity or poor general health, lead to exclusion of lymphadenectomy.

The effect and the allocation of postoperative radiation therapy in stage I have been evaluated in several large studies (Creutzberg et al., 2000; Keys et al., 2004; Nout et al., 2010a) and in a meta-analysis (Kong et al., 2012), which have shown that radiation therapy, – both WPRT and brachytherapy – reduce locoregional recurrences but do not affect overall survival (OS) or disease-specific survival (DSS). The effect of adjuvant radiation therapy was evaluated in a recent meta-analysis (Gupta et al., 2016). The study suggested that patients with high-intermediate risk, as determined the American Society for Radiation Oncology (ASTRO) criteria, would benefit from adjuvant radiation therapy in terms of OS.

The use of chemotherapy as well as hormonal treatment, such as progestin and antiestrogen therapy, is restricted to management of advanced, inoperable or relapsed disease (FIN-GOG, 2016).

Careful consideration of treatment allocation is important, not only to improve survival, but also to avoid overtreatment and to ensure adequate quality of life. Radiotherapy and chemotherapy are associated with side effects including hair loss, emesis, gastrointestinal and genitourinary problems, lymphedema, as well as increased mortality (Paulsson et al., 2009; Nout et al., 2010b; Joly et al., 2014).

Table 2.5 Post-operative risk stratification for EEC

Risk stratification	Stage and grade	Adjuvant therapy	
		LND not performed	LND performed
Low-risk	Stage IA, grade 1–2, LVI -	None	
Intermediate risk	Stage IA, grade 3; Stage IB grade 1–2, LVI -	± Brachytherapy *	
High-intermediate risk	Stage I grade 1–2, LVI +; Stage IA, grade 3	WPRT G3 and LVI – → BT	± Brachytherapy *
High-risk	Stage IB, grade 3; Stage II–III (no residual)	WPRT	Brachytherapy
Advanced	Stage III residual disease; Stage IVA	Case-specific	Not applicable
Metastatic	Stage IVB	Case-specific	Not applicable

* + Especially if ≥ 60 years old, BT: brachytherapy, WPRT: whole pelvic radiation therapy
Adopted from ESMO-ESGO-ESTRO guidelines (Colombo et al., 2016) and FIN-GOG guidelines (FIN-GOG, 2016).

2.6 Prognostic factors in endometrioid endometrial cancer

2.6.1 Systematic approach to literature review

Endometrial cancer has been intensively studied during the past three decades, with approximately 30,000 articles on EC listed in the PubMed database. There is great variation in the extent and quality of these publications. Additionally, our knowledge of the pathogenesis and classification of EC has increased during these years, bolstering the requirements of study design to ensure that the results are interpretable and comparable. To best approach the current state of prognostic markers in EEC, a systematic literature review was performed as part of this thesis work. The literature review was complemented with studies outside the scope of the inclusion criteria for the systematic literature review when they were considered relevant. Additionally, some relevant studies published outside the time frame of the systematic review were included.

2.6.1.1 Criteria for systematic literature review

Studies were considered eligible if they met the following criteria: (1) a human study consisting of ≥150 EEC patients; (2) the study assessed the value of demographic or clinicopathological factors or serum, protein or gene biomarkers; (3) the study had disease relapse or death of disease as a reported outcome; and (4) the study included a separate multivariate analysis of EEC cases.

Studies with a mere focus on treatment or imaging, predictive studies or staging procedures were excluded. Only studies published in peer-reviewed scientific journals and written in English were included, and review papers and letters were excluded.

2.6.1.2 Search strategy

Two approaches were used to retrieve literature relevant for this study. First, the Medline (PubMed) database was searched over a time frame spanning more than 30 years (from May 5, 1985, to September 5, 2015). The search was performed to find all papers published in English that had endometrial cancer/carcinoma or endometrial adenocarcinoma in the title and prognosis, prognostic factor(s) or biomarker(s) in the title or abstract field.[1] The algorithm in **Figure 2.2** illustrates the search process. As described in the algorithm, from a total of 1,597 articles identified 38 were targeted for further analysis.

Those 38 articles were subjected to a reference search. This complementary search identified an additional three publications that met the inclusion criteria. These articles were not found in the original search because studying the outcome of the disease was not the primary aim of the study.

[1] ((endometrial ca*[Title] OR endometrioid[Title] OR endometrial adenocarcinoma[Title]) AND (prognosis[Title/Abstract] OR prognostic factor*[Title/Abstract] OR biomarker*[Title/Abstract]) AND "English"[Language])

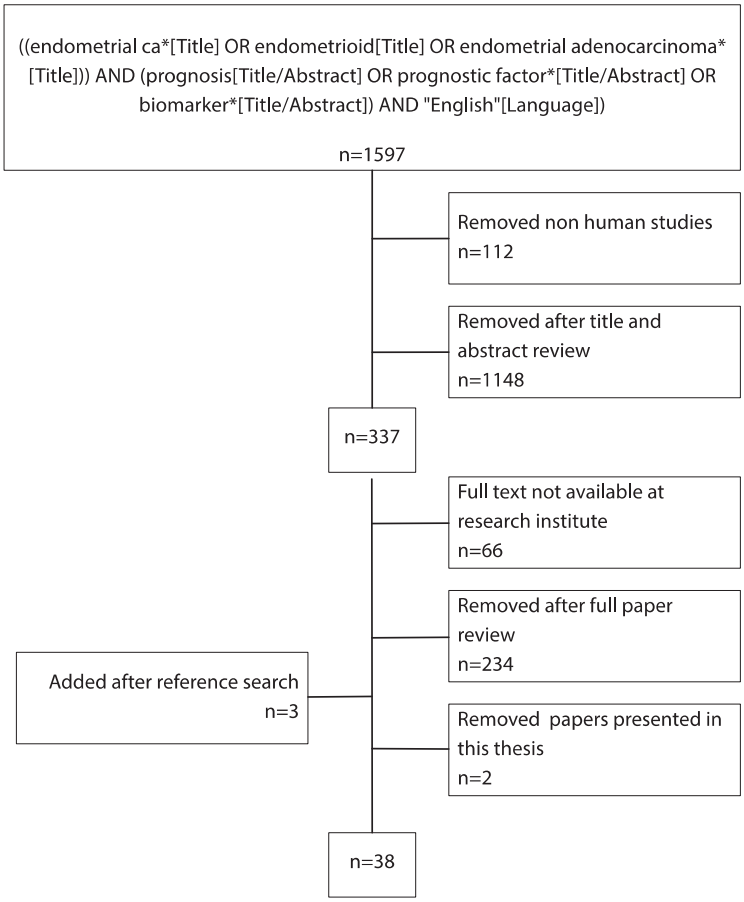
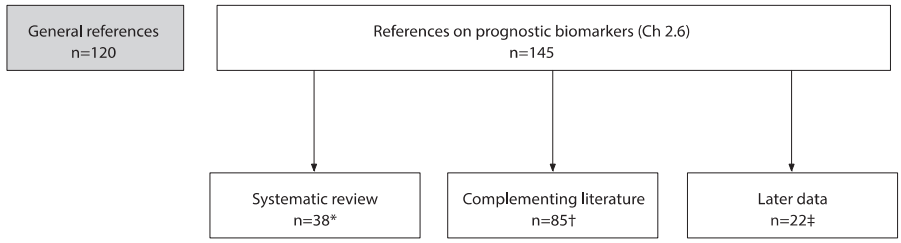


Figure 2.2 Algorithm used for systematic literature review

The articles identified cover a wide spectrum of prognostic factors, from the clinical, such as age, to novel tissue and gene biomarkers. Two studies that are included in this thesis were excluded and will be reviewed later in the results section (Huvila et al., 2013; Edqvist et al., 2015). The 38 studies deemed eligible for inclusion are summarized in **Table 2.6**. The distribution of the referred literature according to the inclusion justification is presented in Figure **2.3**.



**Systematic review: papers fulfilling the inclusion criteria for the systematic review of the literature.*
†Complementing literature: literature published in the same time frame as the papers forming the systematic review but not fulfilling the inclusion criteria.
‡Later data: paper published after the determined timeframe included in the systematic literature review

Figure 2.3 The distribution of the referred literature according to the inclusion justification.

2.6.2 Clinicopathological prognostic factors

2.6.2.1 Age, menopausal status and obesity

The mean ages from the reviewed studies are presented in **Table 2.6**. The mean age for all patients was 62 years. However, there was a clear difference between the mean ages of Asian and European populations, at 55 and 65 years, respectively. In 12 studies, age – managed either as a continuous variable or determined using a threshold age – was found to be an independent prognostic factor in EEC and was found to be non-significant in eight studies. In two studies, the results diverged in terms of the prognostic effects of age and DFS and OS or DSS, as presented in **Table 2.7.**

Table 2.6 List of the 38 studies used in the systematic review of literature

Author	Country	Treatment time	Cases EEC(/EC)	Stage I
Alektiar et al. (2002)	USA	1987–1998	251	251(100%)*
Benedetti Panici et al.(2014)	Italy	NR	514	386(75%)
Bosse et al.(2015)	The Netherlands	1990–2006	926	100%*
Brennan et al.(2014)	Australia	2005–2007	316/372	311(85%)
Chattopadhyay et al.(2012)	United Kingdom	2000–2007	288	200(70%)
Chattopadhyay et al.(2013)	United Kingdom	2000–2007	216	216(100%)*
de Jong et al.(2009)	The Netherlands	1984–2004	316/368	201(55%)
de Jong et al.(2012)	The Netherlands	1984–2004	306/355	196(55%)
Engelsen et al.(2008)	Norway	1981–1990	209/230	192(83%)*
Geels et al.(2012)	The Netherlands	1999–2009	527	513(97%)
Geels et al.(2013)	The Netherlands	1999–2010	335	287(86%)
Green et al.(2015)	Sweden	2001–2007	1140	100%*
Guntupalli et al.(2012)	USA	1991–2007	757	60%
Honkavuori-Toivola et al.(2013)	Finland	1992–2000	225/238	149(66%)
Huang et al.(2015)	Taiwan	2007–2010	169	118(70%)
Jongen et al.(2009a, 2009b)	The Netherlands	1984–2004	315	186(59%)
Kasamatsu et al.(2003)	Japan	1990–1998	280	199(72%)
Kim et al. (2010)	Korea	1996–2008	413	304(74)
Krakstad et al.(2015)	Norway	2001–2012	463/564	474(84%)*
Kübler et al.(2014)	Germany	1995–2008	163	128(79%)*
Liu et al.(2015)	China	2008–2009	206	166(81%)
Nakanishi et al.(2001)	Japan	1978–1997	255	NR
Nofech-Mozes et al.(2008)	Canada	1999–2004	513	100%
Pfisterer et al.(1995)	Germany	1982–1990	162	162(100%)
Saga et al.(2006)	Japan	1988–2001	307	269(88%)
Santala et al.(2014, 2015a, 2015b)	Finland	1992–2000	211	140(66%)
Schmid et al.(2007)	Austria	1995–2005	403	315(78%)
Stefansson et al.(2004)	Norway	1981–1990	237	187(79%)
Stefansson et al.(2006)	Norway	1981–1990	246/274	220(81%)*
Steinbakk et al.(2009)	Norway	1989–2004	258	242(94%)*
Steinbakk et al.(2011)	Norway	1989–2004	224	224(100%)
Weinberg et al.(2013)	USA	1996–2010	336	313(93)*
Westin et al.(2015)	USA	2000–2009	187	115(61%)
Wik et al.(2013)	Norway	1981– 1990	239/286	200(84%)*
Zeimet et al.(2013)	Germany	NR	1021	1021(100%)

NR: not reported, IQR: intraquartile range, SD: standard deviation

Stage II–IV	Operated/LA	Mean age (range)	Follow(months)	*
-	100%	60(29–86)	58	* stage IB
120(24%)	100%/50%	NR	49(27–29)	
-	100%/0%	68(41–90)	160/89**	* high-intermediate risk ** 2 patient sets
58(15%)	100%/55%	61	37	
88(30%)	100%/86%	66(SD 10)	66.5	
-	100%/24%	66(SD10)	80(34–131)	* stage I
166(45%)	95%/42%	65(31–89)	53(0–258)	
159(45%)	100%/44%	64(IQR 56–73)	until 2010	
38(17%)*	NR	NR	108(60–180)	*stage I–II; stage III–IV
14(3%)	100%/43%	62(25–92)	50(0–128)	
48(14%)	100%/17%	64(24–93)	47(1–128)	
-	100%/NR	68(32–92)	107	* stage I
40%	100%/83%	NR	49.5(1–195)	
76(34%)	NR*	65	77(0–136)	
51(30%)	NR	55 (± 12)	NR	
128(41%)	98%/42%	65(32–89)	60(0–258)	
76(28%)*	100%	56(27–81)	62(12–135)	*stage II and IIIA with positive cytology
109(26%)	413(100%)	52(25–83)	26.5(1–168)	
90(16%)*	NR	63	NR	*stage I–II; stage III–IV
35(21%)	100%/42%	68 (±10)	95	* cases with no MI excluded
40(19%)	100%/100%	53 (± 8)	69(9–78)	
NR	100%	57(28–80)	87.0	
-	100%/23%	63(28–94)	28(2–144)	
-	100%	67(45–86)	53	
38(12%)*	100%	57(27–81)	61(45–92**)	* stage II
71(34%)	100%/NR	64(37–98)	77(0–136)	
88(22%)	100%, LA 36%	NR	NR	
50(21%)	94%	64(33–87)	108(60–180)	
53(19%)*	NR	NR	108(60–180)	*stage I–II; stage III–IV
16(6%)*	100%/0%	67(37–94)	63(1.209)	*stage I–IIA; stage IIB
-	100%/0%	66(37–94)	66(1–209)	
23(7%)	100%/73%	66(26–93)	59(6–183)	* stage I–II with risk factors
72(39%)	100%/NR	NR	28.3	
39(16%)*	NR	64 (median)	108(60–180)	*stage I–II; stage III–IV
-	100%/48%	64(34–96)	64	

In two of the reviewed studies, patient age was the primary focus (Nakanishi et al., 2001; Benedetti Panici et al., 2014). In one study, advanced age was an independent prognostic factor for overall as well as disease-free survival (Benedetti Panici et al., 2014) together with cervical invasion, tumor grade and pelvic lymph node invasion. In Nakanishi's work (2001), advanced age, determined by the postmenopausal phase, was related to higher frequency of MI with less frequent obesity, and it was speculated that age would be of greater significance in patients with early invasion.

Age has been known to be associated with non-endometrioid histology and thus poor prognosis. Tumor grade did not demonstrate an association with advanced age in either of the reviewed studies (Nakanishi et al., 2001; Benedetti Panici et al., 2014).

One study focused on the prognostic effect of the state of non-malignant endometrium adjacent to cancerous tissue (Geels et al., 2012) and stated that atrophic endometrium was an independent prognostic factor in well-differentiated EEC. Additionally, atrophic endometrium was associated with older age and lower BMI, and these patients were more likely to have an advanced-stage disease. In multivariate analysis, older age, advanced FIGO stage and the presence of atrophic endometrium were independent negative predictors of DFS.

Obesity is a well-described risk factor for type I EEC, and the epidemic of obesity has been blamed for an increase in the rising incidence of EEC (Bray et al., 2005). Of those papers reviewed, only Nakanishi et al. (2001) included BMI or obesity in the multivariate analysis. Thereafter, obesity has been associated with poor prognosis in older women (Benedetti Panici et al., 2014) as well as with an increased risk of death in women with low-grade EEC (Felix et al., 2015). In a meta-analysis, obesity was associated with an adverse overall survival rate (Secord et al., 2016). However, the effect of obesity on progression-free survival or disease-specific survival has not been demonstrated (Arem and Irwin, 2013).

It has been proposed that older patients (≥70 years) would have an intrinsically more aggressive EC (Alektiar et al., 2003), although contradictory data also exist (Fleming et al., 2011).

2.6.2.2 Staging and peritoneal cytology

In 23 of the 38 studies reviewed, stage was entered in the final multivariate model, and in 17 papers, it was found to be an independent prognostic factor for DFS and/or DSS/(OS) in EEC. In three studies, stage was not found to be an independent prognostic factor. However, in two of these papers, only stage I or stage I–II were studied. Additionally, in two papers stage was found to be an independent prognostic factor in DSS but not in DFS (**Table 2.7**). Although there were variations in the categorization of study material, stage was the most unanimous prognostic factor in the literature.

Until the new WHO 2009 staging was published, WHO 1988 was the guideline for standard care. New scientific evidence led to some changes in the staging (Creasman, 2009). Endocervical glandular invasion was no longer considered cervical invasion. Absence of myometrial invasion and invasion into less than half of the myometrium were joined into stage IA, and pelvic and para-aortic metastasis divided into different groups IIIC1 and IIIC2, respectively. Additionally, positive peritoneal cytology was no longer considered stage IIIA. The prognostic power of WHO 2009 staging was found to be improved when compared to the WHO 1988 staging (Lewin et al., 2010; Cooke, Pappas and Gaffney, 2011; Werner et al., 2012; Kim et al., 2012). However, in another study, the prognosis of the previous stage IA (without MI) cancer was diminished when restaged to the FIGO 2009 stage IA, thus highlighting the persistent need for individualized risk prediction models and nomograms in EC (Abu-Rustum et al., 2011).

Peritoneal cytology is a cytological sample taken from the peritoneal cavity in the first phase of the surgery. In the systematic literature review, it was found that peritoneal cytology was investigated in two studies, both of which were performed on EEC confined to the uterus. The results were inconsistent. In one of the studies, peritoneal cytology was found to be an independent prognostic factor [CI 4.66(1.13–12.47); p=0.031] (Saga et al., 2006). In the other study, this was not the case (Kasamatsu et al., 2003). In both studies, unlike MI, FIGO grade was found to be an independent prognostic factor. In the study by Saga et al. (2006), LVI was also assessed but was not found to be independently prognostic.

The prognostic power of peritoneal cytology was assessed in a study by Werner et al. (2012) which demonstrated that patients who had positive peritoneal cytology (FIGO 1988 stage IIIa) without serosal or adnexal involvement had clearly better five-year survival rates when compared to cases that were IIIa due to serosal invasion or adnexal metastasis (FIGO 2009 stage IIIa), 90% and 59%, respectively. Recently, a multidisciplinary panel stated that peritoneal cytology is no longer mandatory for staging endometrial carcinoma (Colombo et al., 2016). Still, according to Garg et al. (2013), to ensure accurate disease stratification, peritoneal cytology status should be determined in early stage EEC.

Table 2.7 A summary of statistically significant differences in age, FIGO stage and grade in studies where such information was presented

Author	Recurred	DOD	Survival analysis		Variables			Used cut-off and addition details
					Age	FIGO Stage	FIGO Grade	
Alektiar et al. (2002)	16(6%)	21(8%)	DFS	RR	4.0(1–17); p=0.02*		4.0(1–13); p=0.03 **	*60, ** G1–2/G3
			OS	RR	5(2–16); p=0.001*		4(1–11); p=0.01 **	
Benedetti Panici et al. (2014)	67(13.8%)	67(13%)	DSS	HR	2.57(1.30–5.06), p=0.006*	3.02(1.49–6.11), p=0.002	2.11(1.08–4.13), p=0.01	* age 65
Bosse et al. (2015)	NR	NR	DFS*	HR	NS		3.72(2.12–6.53), p=0.045**	*distant relapse, **grade 3
			OS	HR	3.19(2.15–4.74), p<0.001		1.79(1.30–2.48), p<0.001	
Brennan et al. (2014)	43(12%)	16(6%)	DFS	HR		3.87(1.50–9.96), p=0.005	2.45(1.01–5.97), p=0.048	
Chattopadhyay et al. (2013)	18(8%)	11(5%)	DFS	HR			NS	
			DSS	HR			NS	
de Jong et al. (2012)	86(24.2%)	61(17.2%)	DFS	HR		1.84(1.06–3.21), p=0.032*	NS	* advanced stage III/IV
			DSS	HR		4.29(1.96–9.38), <0.001		
Geels et al. (2012)	21(3.8%)	7(1.2%)	DFS	HR	1.06(1.01–1.12)	8.47(1.73–41.57), p NR		
Geels et al. (2013)	44(13%)	23(6.9%)	DFS	HR		5.12(2.65–9.89), p NR	4.39(1.74–11.08), p NR*	* grade 3 significant
			DSS	HR		2.93(1.16–7.36), pNR	5.74(1.52–21.72), p NR*	
Green et al. (2015)	NR	105(9.2%)	DSS	HR	4.3(2.1–8.5), p<0.001*	2.5(1.6–4.0), p<0.001**	2.3(1.3–4.0), p=0.004***	*≥76 (vs <65, 65–75), ** Ib, *** grade 2–3
Guntupalli et al. (2012)	NR	NR	DFS	HR	1.04(1.02–1.05), p<0.001		1.56(1.30–1.88), p<0.001	
			OS	HR	1.04(1.02–1.05), p<0.001		1.59(1.31–1.91), p<0.001	
Honkavuori-Toivola et al. (2013)		35(16%)	DSS	HR		p<0.001*	p<0.003*	HR reported separately for every stage and grade
Huang et al. (2015)	NR	NR	DFS	HR		NS		
Jongen et al. (2009a)	65(20.6%)	38(12.1%)	DSS	OR		2.69(1.13–5.88); p=0.014	NS	
			RFS	OR		NS	2.38(1.19–4.72); p=0.014	
Jongen et al. (2009b)	65(20.6%)	38(12.1%)	DSS	OR		NS	NS	
			RFS	OR		NS	3.57(1.53–8.30); p=0.003	
Kasamatsu et al. (2003)	14(5%)	NR	DFS	HR	NS		3.46(1.21–9.92); p=0.02 *	* grade 3
			OS	HR	NS		11.02(3.02–40.27); p<0.001*	
Kim et al. (2010)	NR	NR	OS	HR	NS	11.89(2.11–66.99); p<0.01*	2.45(0.97–6.35); p=0.02*	* Highest presented
			DFS	HR	NS	60.55(4.8–76.7); p>0.01*	1.08(1.17–6.82); p=0.04*	

Author	Recurred	DOD	Survival analysis		Variables			Used cut-off and addition details
					Age	FIGO Stage	FIGO Grade	
Krakstad et al. (2015)		38(8.2%)	DSS	HR	1.03(1.02–1.09), p<0.001	17.1(8.4–34.8), p<0.001	NS	
Kübler et al. (2014)	20(12%)	NR	DFS	HR		NS	NS	
			OS	HR		19.6(2.03–189.4), p<0.02	NS	
Liu et al. (2015)	76(37%)	NR	DFS	HR	NS	15.66(4.74–51.66), p<0.001*	2.83(1.56–5.13), p=0.001**	* stage IV, **G3
			OS	HR	NS	14.03(4.08–48.27), p<0.001*	3.34(1.66–6.72), p=0.001**	
Nakanishi et al. (2001)	44(17.3%)	29(11.4%)	OS	OR			1.85(1.18–2.88); p=0.007	
			DFS	OR	3.04(1.56–5.93); p<0.001		1.59(1.09–2.32); p=0.017	
Nofech–Mozes et al. (2008)	67(13%)	NR	DFS	RR	NS		NS	
Pfisterer et al. (1995)		26(16%)	RFS	RR	1.04(1.00–1.08); p=0.031*		NS	* age/year
Saga et al. (2006)		13(4.2%)	DSS	HR	NS		4.63(1.12–12.10); p=0.031 *	* G1/G2–3
Santala et al. (2014)	NR	33(15.6%)	DSS	HR		14(3.7–51), p <0.001*	3.4(1.3–9.2), p=0.047**	*stage IV, reported for every stage, **grade 3
Schmid et al. (2007)	46(11%)	12(3%)	OS	HR	1.1(1.0–1.1); p=0.001*	2.1(1.5–2.9); p<0.001	1.8(1.2–2.8); p=0.009	*continuous variable
			DFS	HR	1.1(1.0–1.1); p=0.002*	2.0(1.5–2.8); p<0.001	1.7(1.1–2.6); p=0.02	
Stefansson et al. (2004)	NR	41(17%)	DSS	HR	NS	7.9; p<0.001*	2.7 and 7.7; p=0.003**	* stage I–II/III–IV, ** G2 and G3
			DSS	HR	NS	6.2; p<0.001*	2.0; p=0.037**	
Stefansson et al. (2006)		68(25%)*	DSS	HR		SNR	SNR	
Weinberg et al. (2013)	58(17%)	25(7.4%)	DFS	HR	1.29(1.00–1.65), p=0.048		NS	
			DSS	HR	NS		NS	
Westin et al. (2015)	NR	NR	RFS	HR		11.74(3.7–37.31), p<0.001*	NS	*stage IV, reported for all stages
Wik et al. (2013)			DSS	HR	1.05(1.02–1.07), p=0.001	12.6(7–22.6), p<0.001	NS	
Zeimet et al. (2013)	117(11.5%)	NR*	DFS	HR	2.03(1.35–3.05), p<0.001	NS	NS	*deaths 99(9.7%)
			OS	HR	1.79(1.15–2.81), p=0.01	NS	NS	

Detailed data not available: Chattopadhyay (2012), de Jong (2009), Engelsen et al. (2008), Santala et al. (2015a,b), Steinbakk et al. (2009, 2011),

DOD=died of disease, NR=not reported, NS=not significant, SNR=significance not reported

2.6.2.3 Grade

In all of the 38 papers reviewed, distribution of FIGO grade was reported. FIGO grade was found to be an independent prognostic factor in 16 studies and was determined to be non-significant in 10 studies. Additionally, in two studies grade was associated with DSS but not with DFS (**Table 2.7**).

One of the reviewed papers focused on tumor grading. The prognostic significance of grading was assessed using both the FIGO grading system and the binary grading system (Stefansson et al., 2004). Both grading systems were found to be independent prognostic factors without any dramatic difference in prognostic power.

From those papers that presented a non-selected material, which means that all tumor grades and stages were entered in the study, the grade distributions are shown in **Table 2.8**. There was significant variation, especially in the grades 1 and 2 distributions, across the reviewed papers. This is striking, considering that the histopathological evaluation of patient cohorts in scientific research reports is often performed by dedicated pathologists with an orientation or specialization in gynecological pathology. Consequently, the grading of EEC in a routine clinical setting may be even more variable. A similar observation was made in the PORTEC 1 study, where 79% of the cases were centrally reviewed. Initially, 32% of tumors were considered grade 1, 68% grade 2 and 11% grade 3, whereas after the review, the distribution was as follows: 60%, 32% and 8%, respectively (Creutzberg et al., 2000). However, the new grading results did not have an effect on prognosis. Furthermore, Kwon et al. (2007) reported significant discrepancies between original pathology and formal review, with a discrepancy rate of 42.7%.

The aforementioned observations reflect the previous findings in which the reproducibility of FIGO grading, both architectural and nuclear, has proven challenging (Zaino et al., 1995; Lax et al., 2000; Sagae et al., 2004; Stefansson et al., 2004). In a review article by Clarke et al. (2010), the inter-observer variability of the FIGO grading system was 0.41–0.65, with an intra-observer variability of 0.66–0.73. Additionally, the reproducibility of the overlapping of grade 3 EEC and NEEC has been shown to be challenging (Gilks, Oliva and Soslow, 2013; Hussein et al., 2016).

A new binary grading system was presented by Lax et al. (2004) to facilitate a more reproducible grading system, and both systems were found to perform as independent prognostic factors. According to the new grading system, if the tumor presented two of the three high-grade patterns – namely diffusely invasive growth pattern, tumor cell necrosis or solid growth in >50% of tumor area – it justified the tumor's consideration as high-grade. The binary grading system has been shown to be slightly more reproducible than FIGO grading (Lax et al., 2000; Sagae et al., 2004; Stefansson et al., 2004). Additionally, it has succeeded in identifying a subset of FIGO 1–2 cancers that, according to the binary system, are considered high-grade and have a reduced survival rate.

New techniques and increased knowledge regarding EC pathogenesis have facilitated the discussion pertaining to categorization of EC. New findings argue that a proportion of grade 3 EEC would in fact have such clinicopathological and behavioral characteristics as well as an immunohistochemical staining profile, advocating that they could and should be considered as type 2 cancer (Zannoni et al., 2010; Voss et al., 2012; Geels et al., 2012; Brinton et al., 2013).

Currently, the grading of EC is performed according to FIGO grading protocol. However, in risk assessment and treatment guidelines, FIGO grades 1 and 2 are managed similarly (Colombo et al., 2013; NCCN, 2016).

Table 2.8 Grade distribution from studies with non-selected patients. Patient cohorts that were used in several studies are shown together.

	Grade		
Author(s)	1	2	3
Benedetti Panici et al.	8%	59%	33%
Brennan et al.	49 %	31 %	20 %
Chattopadhyay et al.	33 %	40 %	27 %
de Jong et al.	45–51%	26–30%	19–28%
Engelsen et al., Stefansson et al., Wik et al.	19-25%	43-64%	11-38%
Geels et al.	42 %	41 %	17 %
Guntupalli et al.	54 %	31 %	15 %
Honkavuori-Toivola et al., Santala et al.	52–53%	31–33%	15-16%
Huang et al.	66 %	14 %	20 %
Jongen et al.	51 %	31 %	18 %
Kim et al.	59 %	26 %	15 %
Krakstad et al.	44 %	38 %	18 %
Liu et al.	39 %	34 %	27 %
Nakanishi et al.	87 %		13 %
Schmid et al.	47 %	38 %	15 %
Westin et al.	13 %	65 %	21 %
median	47 %	34%	20 %
range	8–66%	14–65%	15–38%

2.6.2.4 Tumor size

Tumor size has been considered as a prognostic factor for more than 50 years. Commonly, tumor size has been determined as the greatest diameter or by calculating the average of the two largest diameters from the tissue specimen (Schink et al., 1987; Chattopadhyay et al., 2013).

In two of the studies reviewed, tumor size was evaluated in a multivariate analysis. In one study, tumor size was found to be a better predictor of distant failure and DSS than MI (both assessed as a continuous value). However, there was a statistically significant correlation between size and MI (Chattopadhyay et al., 2013). In the study by Nakanishi et al. (2001), tumor size did not demonstrate any independent prognostic value.

Tumor size has been found to correlate with the presence of extrauterine disease (Shah et al., 2005) and LNM (Schink et al., 1991; Doll et al., 2014). However, none of the studies found tumor size to be an independent prognostic factor for recurrence. In early stage EEC, tumor size was found to be an independent predictor of LNM (tumor ≥2cm) and DSS (tumor ≥5cm) (Mahdi et al., 2014).

2.6.2.5 Myometrial invasion

Myometrial invasion (MI) refers to endometrial cancer invading into the underlying myometrium and further on to the serosal surface. MI can be assessed as absolute depth or a percentual depth compared to the thickness of the myometrium. Under the current WHO staging guidelines, an invasion of half or more into the myometrium is considered a cut-off point for deep invasion.

In 18 studies, MI was entered in a multivariate analysis; 15 of these demonstrated that MI (assessed as percentual invasion of the myometrium) did not have an independent role in EC prognostication, whereas two studies (Guntupalli et al., 2012; Chattopadhyay et al., 2013) (MI assessed as outer third and 50%, respectively) suggested an independent prognostic role for MI. In one study, the significance of MI was not reported. Additionally, two papers included tumor-free distance and absolute depth of invasion in the myometrium in the multivariate analysis. Tumor-free distance was found to be significant in one paper (Chattopadhyay et al., 2012), and absolute depth of invasion was significant in the other (Geels et al., 2013). The values defining MI cut-off have varied over the years according to FIGO guidelines, and similarly there were variations in the MI cut-off values used in the reviewed articles, which may contribute to this confusing finding. In 17 of the 18 papers, MI was adjusted to FIGO stage and/or grade, both of which are likely to correlate with deep MI, which probably explains the unobtrusive effect of MI on prognosis in the multivariate analysis.

Three of the reviewed papers focused primarily on the assessment of MI. One assessed the differences between MI in the first third (as determined in the FIGO staging prior to 1988) or the first half. No significance in prognostic capability was found, nor was MI associated with DFS or OS (Alektiar et al., 2002). One study indicated that tumor-free distance from the serosa would be a better prognostic indicator than MI (Chattopadhyay et al., 2012), whereas the third stated that depth of invasion is a superior prognostic factor over tumor-free distance (Geels et al., 2013).

Deep MI has long been recognized as a prognostic factor for lymph node metastasis and OS (Goff and Rice, 1990), especially in cases where disease is limited to the uterus (Prat, 2004). In stage I disease, deep MI has been associated with a risk for distant failure (Mariani et al., 2002). Additionally, it is a crucial risk factor in staging patients and guiding treatment choices (Kwon et al., 2009; Barrena Medel et al., 2011). In most cases, assessing MI is uncomplicated, however, it is sometimes overstated (Ali, Black and Soslow, 2007).

2.6.2.6 Lymphovascular invasion

Lymphovascular (space) invasion (LVI) is defined as the spreading of cancer cells to blood vessels and/or lymphovascular spaces. However, another interpretation of LVI, which means sole invasion to the lymphatic vessels, is sometimes used (Weber et al., 2012). Considerable variation (between 4% and 37%) in LVI has been observed in EC, as reviewed by Gemer et al. (2007). This wide range has been speculated to relate to difficulties in recognizing LVI (Koskas et al., 2015), although contrary opinions, i.e. straightforward recognition of LVI, have been expressed (Soslow, 2016). Inadequate sampling can reduce the detection level of LVI, whereas artifacts, such as vascular pseudoinvasion, can result in a false interpretation of LVI (Folkins et al., 2010). In one of the reviewed studies, the results of the LVI reevaluation were reported, and the frequency of LVI increased from 6.9% to 13.9%.

The correlation between LVI and lymph node metastasis has been frequently debated. Several studies advocate the independent role of LVI in predicting lymph node metastasis (Guntupalli et al., 2012; Koskas et al., 2013; Jorge et al., 2016), although contradictory results have also been reported (Neal et al., 2016). In a comparison between different prognostic models for LNM, the model that included LVI identified the largest group of patients who did not have LNM (Kang et al., 2012). Additionally, LVI has been associated with adverse survival in FIGO stage I disease (Aristizabal et al., 2014).

The prognostic effect of LVI was assessed in 17 studies in which LVI was entered in the final multivariate analysis. These papers are summarized in **Table 2.9**. The proportion of positive LVI cases varied between 3.2 and 32.8% across the studies. The great variation reflects the difficulties inherent in the evaluation of LVI. In most studies, the definition of LVI was not described, or LVI was defined as any tumor cells in endothelial-lined space. In a study by Bosse et al. (2015), different LVI scoring systems were evaluated, and substantial LVI (using a three-tire scoring system) had the strongest impact on prognosis. Similarly, in a work by Stefansson et al. (2004), LVI was assessed as tumor invasion to none, 1 vessel or ≥2 vessels. Invasion in ≥2 vessels was considered positive LVI, as it was a better predictor of survival. In the two studies referenced above, the frequency of LVI was 4.8 and 4.6%, respectively, which is significantly less than the 21.8% median LVI in the reviewed papers. The lowest frequency of LVI, 3.2%, was identified in a study by Geels et al. (2012) in which only WHO grade I tumors were studied.

In nine studies, LVI was not found to have an independent prognostic role, whereas in seven papers, LVI was significantly associated with adverse survival (**Table 2.9**). Additionally, in one paper, LVI was associated with OS but not with DFS (Kim et al., 2010). LVI did not have a prognostic effect in study populations limited to stage I or II, with the exception of two studies that only included patients with other high-risk factors (Weinberg et al., 2013; Bosse et al., 2015).

2.6.2.7 Conclusion on clinicopathological prognostic factors for EEC

According to this review, the FIGO stage retains its status as a cornerstone in EEC risk stratification. The greatest challenge regarding staging is the paradox of correct staging prior to and during surgery in order to determine the extensiveness of surgery (including lymphadenectomy). It appears obvious that more tools are needed to guide surgical staging.

Age, menopausal status and BMI are all easily assessable values exhibiting varying prognostic effects. It can be speculated that advanced age, in conjunction with lower BMI and thus lower estrogen production, is associated with a disease more likely arising from an atrophic background and of a more advanced stage, therefore having an adverse prognosis.

The value of WHO grading remains an unsolved question. One of the challenges is the poor reproducibility of the grading, which reduces the reliability of the results. The binary grading proposed by Lax et al. (2000) might be a solution to poor reproducibility, especially as the treatment guidelines already manage grades 1 and 2 cases similarly. In the future, new genomic and other biomarkers are expected to bring new insights into the differences between these grade groups and determine their role in risk assessment. Similar reproducibility issues were unearthed when evaluating LVI. The range of LVI is rather wide, and the definition of LVI was heterogeneous. What seems obvious is that substantial LVI is more reliable in prognostication, and LVI is a reliable prognostic factor in more advanced, stage III and IV disease processes.

In a majority of studies in which MI was assessed, it was not an independent prognostic factor in EEC. However, in a majority of these studies, it was adjusted for stage and grade, both correlating with MI. The invasion depth remains one of the primary factors guiding the choice of surgical treatment, especially LND.

Table 2.9 A summary of the prognostic role of LVI on survival in studies where such information was presented

			LVI		
Author	**Patients**		**Significance**	**pos/all**	**%**
Alektiar et al (2002)	stage I B	DFS	NS	19/251	8
		OS			
Bosse et al (2015)	*stage IB grade 2–3	DFS	3.61(1.90–6.84), p<0.001	44/926	**4.8**
	stage IC grade 1–2, IIA	OS	2.02(1.30–3.12), p=0.001		
Brennan et al (2014)		DFS	NS	57/333	17.1
Chattopadhyay et al (2013)	stage I	DFS, DSS	NS	47/216	21.8
de Jong et al (2012)		DFS	2.74(1.58–4.76), p<0.001	43/156	27.6
		DSS	2.94(1.40–6.21), p=0.005		
Geels et al (2012)	grade 1	DFS	NS	17/527	3.2
Geels et al (2013)		DFS DSS	NS	68/335	20.3
Guntupalli et al (2012)		DFS	2.19(1.62–2.96), p<0.001	239/757	31.6
		OS	2.04(1.49–2.79), p<0.001		
Jongen et al (2009a,b)		DFS, DSS	NS	73/295	32.8
Kim et al (2010)	no MI cases excluded	DFS	NS	75/413	18.2
		OS	2.75(1.12–6.77); p=0.03		
Liu et al (2015)		DFS	2.64(1.58–4.41), p<0.001	62/206	30.1
		OS	1.83(1.03–3.26), p=0.039		
Nakanishi et al (2001)	stage I	DFS, OS	NS	44/255	17.2
Nofech-Mozes et al (2008)		DFS	2.81(1.28–6.30), p=0.01	116/513	22.6
Saga et al (2006)	stage I, II	DSS	NS	90/308	29
Stefansson et al (2004)		DSS**	4.3; p<0.001	11/237	**4.6**
		DSS**	6.1; p<0.001		
Weinberg et al (2013)	stage I/II patients with	DFS	2.78(1.51–4.55), p<0.001	99/330	30
	LVI/G2–3/≥50%MI	DSS	6.98(2.71–17.96), p<0.001		

NS: not significant; * FIGO 1988, **two separate DSS models

The bolded LVI percentual numbers represent LVI defined as ≥2 vessels

2.6.3 Prognostic biomarkers

2.6.3.1 Hormonal receptors

Already in 1985, Creasman et al. (1985) reported that estrogen receptor (ER) and progesterone receptor (PR) expressions correlate with disease-free survival in stages I and II endometrial carcinoma. Thereafter, the prognostic value of hormone receptor status has been studied frequently. However, often EEC and NEEC cases are analyzed together, preventing the interpretation of results for EEC patients.

Two of the reviewed papers assessed the effect of hormone receptor status on survival. Jongen et al. (2009a) reported that the absence of PR-A in tumor tissue is associated with adverse DSS, whereas ER-alpha, ER-beta or PR-B did not have an independent prognostic value. In a study by Wik et al. (2013), ER-alpha was an independent prognostic factor [HR 3.5(CI 1.2–3.7), p<0.001] when adjusted for age, grade and stage. Additionally, in one paper, it was argued that the GATA binding protein 3 (GATA3), a transcription factor, together with ER-alpha, has an independent prognostic impact (Engelsen et al., 2008). Jongen et al. (2009a) determined ER and PR positivity as >10% positive tumor cells with moderate to strong intensity. Wik et al. (2013) determined ER positivity as the lower quartile of the dataset, which represented a staining index over 4, corresponding with >10% positive tumor cells with moderate to strong intensity.

In addition to the articles reviewed, the prognostic value of hormone receptors has been clarified in several other studies (Fukuda et al., 1998; Saito et al., 2006; Trovik et al., 2013; Tangen et al., 2014; Backes et al., 2016) in which ER and/or PR have been shown to have independent prognostic significance. These studies, however, did not fulfill the inclusion criteria for this systematic review and were excluded. In a recent study, PR positivity was associated with improved survival in grade 3 EEC as well as in serous carcinoma (Köbel et al., 2016).

The determination of hormonal receptors in EC tissue may be helpful in guiding the use of hormonal therapy. Current guidelines recommend using progestin therapy in fertility-preserving treatment. Progestin or anti-estrogen therapies are used in the treatment of advanced or recurrent disease (Tangjitgamol et al., 2009; Colombo et al., 2016; NCCN, 2016).

2.6.3.2 DNA ploidy

Altered DNA ploidy, typically aneuploidy, has long been known to be associated with cancer (Storchova and Pellman, 2004). In their review paper, *Hallmarks of cancer: the next generation*, Hanahan and Weinberg (2011) considered genome alterations as characteristics that enable cancer. However, whether aneuploidy is the cause or the consequence remains ambiguous (Storchova and Pellman, 2004).

The role of DNA ploidy and S-phase fraction in EEC was studied in two of the reviewed papers, both focusing on stage I disease. The percentage of aneuploidy in tumors was 14% and 17%, respectively (Pfisterer et al., 1995; Green et al., 2015). Aneuploidy was associated with histopathological risk factors such as hormone receptor negativity and higher grade. Neither of the studies demonstrated that aneuploidy had an independent prognostic role. However, flow cytometric S-phase fraction (>5.5%) had an independent prognostic role [2.3 (CI1.4–3.7), p=0.001] (Green et al., 2015). Additionally, in one study performed on curettage samples from stage I patients, ploidy did not have a significant prognostic role in univariate analysis (Steinbakk et al., 2011).

The role of DNA ploidy in EC has been assessed in several settings, as reviewed by Mauland et al. (2014), and has been found to be prognostically significant in a proportion of the studies. DNA ploidy has also been assessed in a prospective setting, where a non-diploid curettage sample was associated with aggressive clinicopathological phenotype as well as poor survival (Njølstad et al., 2015). However, no subanalysis on EEC cases was performed.

2.6.3.3 Proliferation markers

Sustaining chronic cell proliferation is one of the most fundamental traits of cancer (Hanahan and Weinberg, 2011). Ki-67 protein is a cellular marker for proliferation that is widely used in diagnostics. In only one of the reviewed papers, Ki-67 expression was entered in a multivariate model. Stefansson et al. (2004) demonstrated that Ki-67 expression was an independent prognostic factor, whereas mitotic count was not. Both variables were adjusted for stage, grade and vascular invasion.

Cyclin A, B and E function as activators of the cell cycle and are thus associated with cellular proliferation. In a series of studies, a high expression of cyclin A (Santala et al., 2014) was found to be associated with DSS in EEC [HR 2.3(CI 1.0–5.3, p=0.043)], whereas cyclin B or E (Santala et al., 2015a; Santala et al., 2015b) did not have an independent prognostic effect. Cyclin E expression was also assessed in a study by Steinbakk et al. (2009), where it was not found to have an effect on survival in a univariate analysis.

2.6.3.4 Tumor protein p53

Tumor protein p53 regulates the cell cycle and functions as a tumor suppressor. Mutations in and deletions of *TP53* are known to be involved in a wide range of human cancers. In EC, aberrant p53 expression is known to be associated with type II disease, as reviewed above, and to also be a late event in EEC (Hoang et al., 2013).

In the systematic literature review, two studies reported on p53 expression in EEC (Steinbakk et al., 2009; Jongen et al., 2009b). In both studies, aberrant p53 expression was shown to have an independent effect on DSS. In the study by Steinbakk et al., p53 expression was evaluated as part of a panel, together with p21 and survivin (see Section 2.6.3.5). In the study by Jongen et al. (2009b), in addition to p53, three other biomarkers (aromatase, HER2 and COX-2) were entered in the multivariate analysis but did not demonstrate an independent effect on DSS. In both papers, aberrant p53 expression was defined as overexpression using different cut-off values.

Several studies have shown the prognostic value of p53 expression. In most cases, the studies have included non-endometrioid cancer cases, where p53 alteration is known to be present in most of the cases. Additionally, multiple different threshold values have been used to assess p53 mutations by immunohistochemistry. In ovarian cancer, where

alterations in immunophenotype of p53 are pathognomonic, completely negative p53 expression is also associated with *TP53* mutations, and these are usually nonsense, frameshift or splice-site mutations (Lassus and Butzow, 2007; Yemelyanova et al., 2011). This interpretation of aberrant expression rather that overexpression of p53 has been extended to EEC. However, most of the previous publications did not consider completely negative p53 staining as aberrant, which might affect the prognostic value of p53 in previous studies.

2.6.3.5 Microsatellite instability (MSI)

MSI is a result of impaired DNA mismatch repair (MMR), which can be due to germline mutations or somatic inactivation of the MMR genes *MSH2, MSH6, MLH1* and *PMS2*. Inactivation of MMR genes occurs in 20% to 45% of sporadic endometrial tumors (Peltomäki et al., 1993; MacDonald et al., 2000), which is in most cases caused by methylation of *MLH1* (Salvesen et al., 2000).

One of the reviewed papers demonstrated that MSI high genotype, in conjunction with low p21, a cell cycle suppressor, and high survivin, an apoptosis inhibitor, was associated with an adverse prognosis in stage I EEC (Steinbakk et al., 2011). All 34 MSI cases had a diploid genome, and aneuploidy was not associated with survival, as previously stated (2.6.3.2)

The results in Steinbakk et al. (2011) are similar to those in Salvesen et al. (2000) and show the connection between MSI and diploid genome as well as p53 overexpression. However, the negative prognostic effect was not verified. Results showing improved survival associated with MSI have been published (Cohn et al., 2006; Resnick et al., 2010). In a recent study, McMeekin et al. (2016) demonstrated that MMR defects do not affect the overall outcome of EEC patients, despite the fact that MMR defects were associated with higher grade and more frequent LVI.

2.6.3.6 PTEN

Mutations of the *PTEN* gene are among the most frequent genetic lesions in EEC. Loss of PTEN expression has been connected to endometrial hyperplasia, early tumorigenesis (Mutter et al., 2000; Kimura et al., 2004) and improved survival (Risinger et al., 1998; Mackay et al., 2010) in EEC.

Of the papers reviewed, two assessed the effect of PTEN loss in EEC. Westin et al. (2015) reported that in obese patients, loss of PTEN was associated with favorable prognosis when adjusted for stage, grade and obesity. In a study by Steinbakk et al. (2009), PTEN loss did not have an effect on survival in univariate analysis and was not entered in a multivariate analysis.

The reliability of PTEN antibodies has been questioned, and the interpretation is considered complicated (Garg et al., 2012). Although these problems now seem to have

been overcome (Garg et al., 2012), they may cast doubt on the earlier results. Perhaps more than a prognostic marker, PTEN has been proposed to be useful as a diagnostic marker, distinguishing EEC from NEEC (Hussein et al., 2016), and additionally, to contribute to the integrated classifications system, as will be reviewed later.

2.6.3.7 Vascular proliferation

Despite the essential role of angiogenesis in cancer and its potential as a therapeutic target (Hanahan and Weinberg, 2011), only one of the reviewed papers focused on angiogenesis. Stefansson et al. (2006) found that the vascular proliferation index, as measured by Ki-67/factor VIII co-expression, was an independent prognostic factor for DSS (OR 2.2, p=0.008).

2.6.3.8 Tumor-infiltrating lymphocytes and macrophages

Immune inflammatory cells that infiltrate cancer tissue play an established role in a tumor microenvironment and have been suggested to have an effect on cancer progression (Hanahan and Weinberg, 2011). Additionally, inflammatory mechanisms offer an intriguing target for immunotherapeutic strategies in cancer (de Jong et al., 2009; Hanahan and Weinberg, 2011; de Jong et al., 2012; Kubler et al., 2014).

Cytotoxic $CD8^+$ T-lymphocytes (CTL) have the ability to recognize and kill cells presenting foreign proteins on their surfaces, such as tumor cells. As for regulatory T-cells (T_{reg}, $Foxp3^+$), they inhibit the actions of CTLs. High CTL count as well as low T_{reg} count have been associated with improved survival, and the CTL/T_{reg} ratio has been shown to be an independent prognostic factor in EEC (de Jong et al., 2009).

Tumor-associated macrophages (TAMs) have been suggested to be attracted to and sustained by the neoplasm and beyond to support malignant growth by enhancing immune suppression and facilitating invasion. Kübler et al. (2014) showed that $CD163^+$ TAMs were associated with a higher stage and grade of EEC and also had an independent effect on DFS and OS. A similar association was not found with T_{reg} cells, despite the correlation between these variables. T_{reg} cells were, however, associated with the presence of LVI and LNM.

De Jong et al. (2012) also evaluated the expression of the indeleamine-2,3.dioxygenase (IDO) gene in EEC prognostication. The increased expression of IDO by tumor cells is reported to be an important immune escape mechanism for cancer. In EEC subtype analysis, IDO was not found to be an independent prognostic factor.

2.6.3.9 HER2

The human epidermal growth factor receptor 2 (HER2) is a member of the epidermal growth factor receptor (EGFR) family. It is perhaps best known as a biomarker for breast

cancer and is used in guiding adjuvant therapy. In EC, HER2 overexpression is primarily associated with type II EC and is considered a rare event in type I EC, as reviewed above (**Table 2.1**). Of the papers reviewed, two assessed the prognostic value of HER2 (Steinbakk et al., 2009; Jongen et al., 2009b); in neither of the studies was HER2 overexpression an independent prognostic factor of EEC. Subsequently, unlike in breast cancer, HER2 overexpression has not been successful as a predictive biomarker for targeted therapy in EC (Fleming et al., 2010).

2.6.3.10 L1CAM

The L1 cell adhesion molecule (L1CAM; CD171) was introduced as a biomarker for stage I EEC in a large retrospective study in 2013 by Zeimet et al. (2013). The results indicated that L1CAM positivity was associated with adverse survival when evaluating DSS and OS (Zeimet et al., 2013). L1CAM has been suggested to provide guidance in choosing patients for adjuvant therapy as well as a potential therapeutic target in the future. However, the exact molecular mechanisms leading to the aggressive phenotype remain, unclear (Zeimet et al., 2013), although the mechanism is speculated to be related to induction of epithelial-mesenchymal transition and cell migration (Kiefel et al., 2012).

Similar prognostic effects of L1CAM have been seen in previous studies (Fogel et al., 2003; Huszar et al., 2010). Following encouraging results by Zeimet et al., several papers evaluating the usability of L1CAM in EEC have been published. L1CAM has been found to be an independent prognostic factor for distant recurrence in EEC that is limited to the uterus (Bosse et al., 2014). Additionally, L1CAM positivity has been shown to be related to advanced disease (Pasanen et al., 2016) and to correlate with aberrant p53 expression (Van Gool et al., 2016) and with ER and PR negativity (Huszar et al., 2010). L1CAM has also been shown to be frequently expressed in type II EC and non-endometrioid histology (Geels et al., 2015; Geels et al., 2016).

Additionally, at the gene expression level (RNA-Seq), L1CAM expression has been associated with higher grade, non-endometrioid histology, advanced stage and poor survival (Dellinger et al., 2016).

2.6.3.11 Novel tissue biomarkers

A number of other tissue biomarkers have been evaluated as prognostic markers in EC. In this literature review, four of the included papers introduced novel findings that are neither previously described nor thus far validated in EEC.

The overexpression of lysine-specific demethylase 1 (LSD1), previously connected with decreased differentiation and aggressive tumor biology, was associated with an unfavorable prognosis in EEC patients (Liu et al., 2015). Previously, LSD1 inhibition has been studied as a therapeutic strategy in poorly differentiated EC cell lines and found to be promising (Theisen et al., 2014).

The overexpression of the ATPase family, AAA domain containing 2 (ATAD2), was found to be related to aggressive EC (Krakstad et al., 2015). The authors speculated that this finding was linked to regulators of cell cycle progression. ATAD2 protein acts as a transcriptional co-regulator through interactions with androgen and estrogen receptors (Hsia et al., 2010).

The urokinase-type plasminogen activator (uPA) functions as a modulator of extracellular matrix and contributes to angiogenesis and metastasis. Additionally, it has been recognized as a potential therapeutic target (Jiang et al., 2015). In EEC, a high expression of uPA was shown to have an independent, adverse prognostic effect on disease-free survival, a finding that was speculated to be related to degradation of the extracellular matrix, tumor invasion and metastatic potential (Huang et al., 2015).

Honkavuori-Toivola et al. assessed the significance of MMP-2 and TIMP-2, a matrix metalloproteinase and a tissue inhibitor of matrix metalloproteinase associated with tumor microenvironment, and found that strong MMP-2 and weak TIMP-2 were not significantly associated with DSS (Honkavuori-Toivola et al., 2013).

2.6.3.12 Current state of tissue biomarkers in EEC

A broad spectrum of different tissue biomarkers has been studied in EEC. Hormonal receptors ER and PR have shown a reasonably reproducible association with prognostic significance and are worth studying in a prospective setting.

According to this literature review, the prognostic value of ploidy and HER2 expression in EEC are limited and do not add sufficiently to prognostication. The prognostic role of Ki-67, vascular proliferation and inflammatory mechanism requires further confirmation. Most of the novel biomarkers lack any validation. Only L1CAM, a fairly new biomarker in EEC, has been repeatedly shown to have an independent prognostic role in EEC, and like ER and PR, it deserves to be further validated.

The increasing knowledge regarding the genomic and genetic features in EC has highlighted the role of the p53, PTEN and the MMR genes as surrogate markers for genetic classification. In this literature review, p53 was observed to have independent prognostic value, whereas the latter two did not. These markers will be further discussed in Section 2.6.5 of this literature review.

2.6.4 Serum biomarkers

Serum biomarkers are indicators that are measurable from blood. Currently, serum biomarkers do not have an established role in the diagnosis of EEC. Three of the studies reviewed focused on serum biomarkers and their usability in pre-operative risk assessment.

Serum carbohydrate antigen 125 (CA-125), a biomarker in clinical use for epithelial ovarian cancer diagnosis and follow-up, was found to be an independent prognostic marker for

DSS and DFS when the value was high (>70 U/mL). However, there were variations in both the specificity and the negative predictive value of CA-125. Its usefulness was relatively low when predicting poor prognosis and was highest when predicting adnexal involvement (Kim et al., 2010).

Human epididymis protein 4 (HE4) is a serum marker that, much like CA-125, is used in differential diagnosis and also in monitoring recurrence or progressive disease in patients with epithelial ovarian cancer. HE4 was evaluated in conjunction with CA-125 as a prognostic factor in one of the reviewed studies, and the results indicate that elevated HE4 (>70 pmol/L) is associated with MI and advanced disease and has an independent prognostic value superior to that of CA-125 (Brennan et al., 2014).

C-reactive protein (CRP) is traditionally associated with rapid response to proinflammatory cytokines. Additionally, it has been shown to be involved in cell death and in the development of malignant disease. The relationships between CRP, CA-125 and EEC were studied in one of the reviewed papers. Elevated CRP levels, assessed as a continuous variable, were related to reduced DFS and OS in multivariate analysis, whereas CA-125 did not have prognostic significance (Schmid et al., 2007).

A panel consisting of both CA-125 and HE4 has been found to be useful in predicting the presence of metastatic disease (Saarelainen et al., 2013). Similarly, the panel of both CA-125 and HE4 was useful in pre-operative risk assessment and also in a prospective setting (Antonsen et al., 2013). Mutz et al. (2012) showed that in EEC, HE4 was of better prognostic value than CA-125 when assessing OS. However, in a recent meta-analysis, HE4 and CA-125 were compared, and HE4 was found to be superior when assessing screening accuracy (Hu et al., 2016).

In conclusion, none of the studied serum markers demonstrate an indisputable role in EEC prognostication. In a recent review article by Bendifallah et al. (2016), various predictive models were evaluated, and none of the models predicting survival included a serum biomarker.

2.6.5 Reclassification of EC using integrated approach

In 2013, the Cancer Genome Atlas Research (TCGA) Network published a paper that introduced a novel classification system for endometrial cancers (Cancer Genome Atlas Research Network et al., 2013a). Based on integrated genomic characterization, in specific genomic, transcriptomic, and proteomic analysis, endometrial cancer was classified into four different subgroups: ultramutated, hypermutated and copy-number low and high subgroups. These subgroups are presented in **Table 2.10**.

Table 2.10 Summary of EC classification based on The Cancer Genome Atlas data

TCGA classifications	Description	Histology	Genetic alterations
POLE "ultramutated" (≈7% of cases)	High incidence on hotspot mutations in exonuclease domain of POLE	EEC, G3-2-1*	POLE, P1K3CA and PTEN mutations
Hypermutated (≈28% of cases)	MSI	EEC, G2/3-1*	ARID1A mutations MLH1 promoter hypermethylation
Copy-number low (≈39% of cases)	MSS	EEC, G1-2-3*	PR receptor overexpression, PTEN mutations, ß-catenin mutations
Copy-number high (≈26% of cases)	Extensive somatic copy number alterations	Serous carcinoma and serous-like EEC (25% of G3 EEC)	High incidence of p53 and PPP2R1A mutations. Lack of ARID1A mutations

Adopted from Cancer Genome Atlas Research Network et al. (2013a),
* in order of frequency
POLE: DNA polymerase epsilon, catalytic subunit, MSS: microsatellite stable

The four TCGA subgroups were shown to have significant (p=0.02) prognostic differences. The ultramutated subgroup was associated with strong progression-free survival, whereas the copy-number high group was associated with the worst progression-free survival. The remaining two groups presented intermediated prognoses.

One of the interesting findings of the TCGA work was that up to 25% of the tumors that were histologically categorized as high-grade endometrioid cancers in fact had a molecular phenotype of serous carcinomas, including frequent *TP53* mutations and somatic copy number alterations, and were grouped into the copy-number high group (Cancer Genome Atlas Research Network et al., 2013a). The original histology was revisited, and it revealed that the copy-number high group is a heterogenic, diagnostically challenging group of high-grade EC, of which only a part are true endometrioid-type cancers (Hussein et al., 2016). To date, the differences in prognosis between copy-number high EEC and NEEC remain unclear.

One of the novel findings in this new classification was the *POLE* mutations group. The effect of genomic variations of *POLE*, a catalytic subunit of DNA polymerase epsilon involved in nuclear DNA replication and repair, has been previously described in colorectal cancer (Palles et al., 2013), where it was associated with a hypermutant and microsatellite stable colorectal cancer. In endometrial cancer, *POLE* mutations are associated with high-grade endometrioid tumors (Church et al., 2013; Meng et al., 2014). EEC that presents with a *POLE* mutation is associated with a good prognosis (McConechy et al., 2016), even though they often seem to harbor p53 mutations (Cancer Genome Atlas Research Network et al., 2013a; Hussein et al., 2015). Currently, some results indicate that the prognostic effect would be limited to the poorly differentiated subgroup (Meng et al., 2014; Church et al., 2015). When assessing the morphological differences in *POLE* vs. non-

POLE mutated, the *POLE* group more often had heterogeneous or ambiguous tumor morphology and obvious lymphocytic infiltrates (Hussein et al., 2015). The increase of intratumoral T-cells has been speculated to be one of the mechanisms leading to favorable prognosis (van Gool et al., 2015).

Despite the varied grade distribution over the four recognized subgroups, there were significant differences in the survival rate. This finding highlights the necessity of recognizing these subgroups and the need for surrogate markers for the subgroups. Stelloo et al. (2015) presented a classification system where the classes were defined as follows: 1) p53 mutated, 2) microsatellite instable, 3) *POLE* mutated, and 4) no specific molecular profile. This was accomplished by using immunohistochemistry, mutational analysis and methylation-specific PCR. These subgroups were further tested in a larger series of early stage EEC, together with clinicopathological prognostic factors and L1CAM expression. The study revealed that when *POLE*, L1CAM, MSI and *CTNNB1* were integrated with histopathological factors, risk assessment was improved (Stelloo et al., 2016). Talhouk et al. (2015) suggested a classification system in which patients would be categorized in four groups as follows: 1) MMR abnormal, 2) *POLE* mutated, 3) p53 wild type, and 4) p53 aberrant. The preoperative applicability of this classification system was further tested in respective preoperative samples, and it demonstrated a strong concordance with the original classification system (Talhouk et al., 2016).

In conclusion, the genomic characterization of EC has given rise to a completely novel method of categorizing EC. Future studies and prospective trials are needed to evaluate to prognostic role of these genetic classes, as well as the clinical usability of the surrogate markers.

3 AIMS OF THE PRESENT STUDY

The primary aim of this study was to find prognostic biomarkers in a retrospective cohort of endometrioid endometrial cancer patients with complete clinical and long-term survival data. In addition to promising existing biomarkers, the aim was to find novel biomarkers that might be useful in prognostication and the classification of EEC. Finally, the aim was also to create a clinically useful panel of immunohistochemical markers for the classification of endometrioid endometrial cancer patients.

The specific aims of this study were:

1) To evaluate the differences between well- and poorly differentiated EEC at the level of gene expression in order to identify potential new biomarker candidates.

2) To investigate the role of tissue biomarkers (ER, PR, p53, Ki-67, PTEN, MHL1, HER2) that have been shown to have a prognostic effect in EEC in a subgroup of patients where the disease is limited to the uterus (stage I–II).

3) To confirm the observation of a l-asparaginase (ASRLG1) protein expression in endometrium and to evaluate its potential as a novel prognostic biomarker in EC.

4) To create a clinically useful immunopanel for prognostication of EEC patients using tissue biomarkers studied in this thesis as well as a novel prognostic biomarker L1CAM.

4 MATERIALS AND METHODS

The materials and methods are described in detail in the original publications.

4.1 Patients

4.1.1 Patients (I)

For gene expression profiling and immunohistochemical stainings in study I, fresh frozen and FFPE tissue samples from 34 patients were used: 10 FIGO grade 1, 9 FIGO grade 2 and 15 FIGO grade 3 EEC. Samples from well (FIGO grade 1, n=6) and poorly differentiated (FIGO grade 3, n=7) cases were subjected to RNA extraction microarray hybridization and immunohistochemistry. All 34 samples were subjected to RNA extraction and qRT-PCR (quantitative reverse transcriptase-polymerase chain reaction). Additionally, normal controls of benign endometrium were used for qRT-PCR. The fresh frozen tissue material was obtained from the tissue bank of the Department of Obstetrics and Gynecology, and the FFPE samples from the tissue bank of the Department of Pathology, Turku University Hospital. Permission to use the samples was granted by the Finnish National Authority for Welfare and Health, and the study was approved by the ethical committee of the Southwestern Finland Hospital District.

4.1.2 Patient cohorts (II–IV)

In studies II–IV, the patient material consisted of three patient cohorts, including a total of 674 patients, as presented in **Table 4.1**. Clinicopathological data and surgical treatment are presented in **Table 4.2**. All patients entered in this study were subjected to hysterectomy, and 96.2% were also subjected to salpingo-oophorectomy.

Table 4.1 Use of samples from patient cohorts 1–3 for studies II–IV

	Cohort(s)
Study II	Cohort 1, only EEC stage I–II (n=182)
Study III	Cohort 1 (n=229) and a separate validation from Bergen, Norway (n=286)
Study IV	Cohort 1 and cohort 2, only EEC (n=306)

Table 4.2 Characterization of patients in cohorts 1–2 and validation cohort

	Cohort 1	Cohort 2	Validation cohort
Cohort size	229	91	286
Treatment years	2004–2007	2001–2004	1981–1990
Mean age (years)	67	65	65
Grade			
Grade 1	117(51.1%)	49(53.8%)	64(22.4%)
Grade 2	62(27.1%)	25(27.5)	163(57%)
Grade 3	37(16.2%)	16(17.6%)	30(10.5%)
NEEC or mixed type	13(5.6)%	1(1.1%)	29(10.1%)
Stage *			
Stage I	186(81.2%)	71(78.0%)	211(74.0%)
Stage II	5(2.2%)	5(5.1%)	19(6.7%)
Stage III	29(12.7%)	13(14.3%)	42(14.7%)
Stage IV	9(3.9%)	2(2.2%)	13(4.6%)
Lymphadenectomy			
Performed	182(79.5%)	74(81.3%)	NA
Not performed	47(20.5%)	17(18.7%)	NA
Relapsed	31(13.5%)	13(14.3%)	46(16.1%)
Died of disease	25(10.9%)	12(13.2%)	74(25.9%)

* Norwegian cohort missing 1 FIGO stage

NA: not assessable

All patients were restaged to comply with the FIGO 2009 staging presented in the literature review. The patients underwent surgery, and adjuvant treatment was allocated according to prevailing hospital guidelines. From cohorts 1 and 2, 34 (10.7%) of patients did not receive any adjuvant treatment, whereas 116 patients (36.5%) received WPRT, 147 (46.2%) received brachytherapy, and 21 (6.6%) received both WPRT and brachytherapy. Eighty-one (25.5%) patients received postoperative chemotherapy.

The FFPE samples for cohorts 1 and 2 were obtained from the tissue bank of the Department of Pathology, Turku University Hospital. Permission to use the samples was granted by the Finnish National Authority for Welfare and Health (permissions # 6550/05.01.00.06/2010 and 3616/05.01.00.06/2011). The study was approved by the ethical committee of the Southwestern Finland Hospital District.

To validate the original finding in study III, a prospective cohort was obtained from Haukeland University Hospital, Bergen, Norway. The cohort is described in **Table 4.2** and in the original paper (III). The study was approved by the Regional Committee of Medical and Health Research Ethics (REK), Western Norway.

4.2 Methods

4.2.1 RNA extraction (I)

RNA was extracted from tumor and control samples using a commercial reagent kit (Qiagen RNeasy Mini Kit; Qiagen, Hilden, Germany). All samples were treated with RNase-free DNase, and the concentration of RNA was measured spectrophotometrically using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, LLC, Wilmington, DE, USA). The quality of the RNA was further determined by capillary electrophoresis using an Experion apparatus (Bio-Rad, Hercules, CA, USA).

4.2.2 Microarray hybridization (I)

Microarray analyses were carried out at the Turku Center for Biotechnology using Affymetrix U133plus2.0 GeneChips and Sentrix Human WGA-6 Expression BeadChips (V1, Illumina). For Affymetrix microarray analysis, biotinylated complementary RNA (cRNA) was synthesized for 5 μg of total RNA using the standard One-Cycle Kit (Affymetrix). Quantitation of the biotinylated cRNA was performed by hybridization to Affymetrix U133plus2.0 GeneChips. For Illumina hybridization, 500 ng of total RNA was used for preparation of biotinylated cRNA using the Illumina RNA amplification kit (Ambion). Hybridizations were performed according to the instructions of the manufacturer. Washing and scanning were performed according to the Illumina BeadStation 500x manual (revision C). Illumina expression data were extracted using BeadStudio version 1.5.0.34 applying default settings.

4.2.3 First-strand synthesis of complementary DNA (I)

First-strand synthesis of complementary DNA (cDNA) was performed for all of the samples and controls. Quantitative RT-PCR was used to validate and confirm the gene expression of apolipoprotein E (APOE) in the endometrial cancer samples. The specific primers for APOE were designed with Primer Express Software (Applied Biosystems) in accordance with probes from the Universal Probe Library (Roche Applied Science). All PCRs were performed using ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems).

4.2.4 Tissue microarrays (TMAs) (II–IV)

Generation of TMAs, immunohistochemistry, and slide scanning were performed on TMAs at the Swedish Science for Life Laboratory (SciLifeLab) facilities in the Department of Immunology, Genetics, and Pathology at the Rudbeck Laboratory of Uppsala University (Sweden), in accordance with protocols used in The Human Protein Atlas project (www.proteinatlas.org). In brief, formalin-fixed, paraffin-embedded tumor samples were selected, and corresponding hematoxylin-eosin stained histologic slides were reviewed to select areas for production of TMAs representing the 306 EEC specimens. To construct the TMAs, two 1.0-mm diameter cores from each donor block (duplicate samples) were

taken and assembled in an array format in a recipient TMA block using TMArrayer™ (Pathology Devices, Westminster, MD, USA) or the Beecher Instruments Manual Tissue Arrayer MTA-1 (Estigen OÜ, Tartu, Estonia). Seven TMA blocks were prepared, each typically containing 120 cores assembled from donor blocks corresponding to 60 individuals.

4.2.5 Immunohistochemical stainings

In study I, all immunohistochemistry was preformed on FFPE tissue samples. Sections were cut at 4 μm, deparaffinized in xylene, rehydrated through a graded series of ethanol and briefly rinsed in Tris-buffered saline (TBS). Immunohistochemical stainings for ER, PR, p53, and PTEN were performed using a TechMate 500+ immunostaining instrument and Labeled Streptavidin Biotin (LSAB) peroxidase/ diaminobenzidine multilink detection kit (Dako, Glostrup, Denmark), and for MLH1, MSH2, and MSH6 and β-catenin using the Ventana BenchMark XT immunostaining instrument and Ventana ultraView Universal DAB Detection Kit. Antibodies and dilutions used are presented in **Table 4.3**.

Table 4.3 Antibodies and dilutions used in study I

Antibody	Antibody and source	Dilution
p53	M7001, Dako, Glostrup, Denmark	1:300
PTEN	M3627, Dako, Glostrup, Denmark	1:100
ER	M7047, Dako, Glostrup, Denmark	1:40
PR	NCL-PGR, Novocastra Laboratories, Newcastle, UK	1:20
MLH1	G168-15, BD Pharmingen, San Jose, CA, USA	1:5
MSH2	G219-1129, BD Pharmingen, San Jose, CA, USA	1:30
MLH6	44, BD Pharmingen, San Jose, CA, USA	1:200
β-catenin	CAT-5H10, Zymed Laboratories, Waltham, MA, USA	1:200

Evaluation on immunohistochemical stainings for hormonal receptors was done as in clinical practice, and positive nuclear staining in ≥20% of tumor cells was considered positive. Immunostaining for p53 was scored according to the proportion of positive tumor cell nuclei as follows: – (<5%), + (5–50%) and ++ (>50%). For PTEN immunohistochemistry, the proportion of positive tumor cells were scored using the following criteria: less than 5% = –, 6% to 25% = ±, 26% to 75% = +, and greater than 76% = ++. For MLH1, MSH2, MSH6, and β-catenin, any nuclear staining in tumor cells was considered positive.

In studies II–IV, TMAs were sectioned at 4-μm intervals using a Rotary Microm HM355S equipped with a Section Transfer System (Microm International GmbH, Walldorf,

Germany), collected on SuperFrost Plus slides (Menzel-Gläser GmbH, Braunschweig, Germany), and baked at 60°C for 45 min. Sections were stored at -20°C until use. Antibodies and dilutions used in studies II–IV are presented in **Table 4.4**.

Table 4.4 Antibodies and dilutions used in studies II-IV

Antibody	Provider	Product name	Purity	Dilution	Study
ER	Atlas Antibodies [1]	HPA001070	pAb	1:50	II, IV
PR	Atlas Antibodies [1]	HPA008428	pAb	1:15	II, IV
p53	DakoCytomation [2]	M7001	mAb	1:1000	II, IV
Ki-67	DakoCytomation [2]	M7240	mAb	1:200	II, IV
PTEN	Cell Signaling [3]	9559	mAb	1:75	II
HER2	Atlas Antibodies [1]	HPA001383	pAb	1:100	II, IV
MLH1	Zymed [4]	39-3200	mAb	1:150	II, IV
L1CAM	Sigma-Aldrich [5]	L4543	mAb	1:500	IV
ASRGL1	Atlas Antibodies [1]	HPA029725	pAb	1:375	III, IV

[1] Stockholm, Sweden, [2] Glostrup, Denmark, [3] Danvers, MA, USA, [4] San Francisco, CA, USA, [5] Saint Louis, MO, USA

After staining, the TMA slides were scanned and blinded to clinical data. Immunohistochemical stainings were assessed using ImageScope (Aperio, Vista, CA, USA) using cut-offs presented in **Table 4.5**. Non-malignant areas and stromal tissue in tumor specimens served as controls.

4.2.6 Silver-enhanced in situ hybridization (SISH) (II)

Silver-enhanced in situ hybridization was performed with VENTANA HER2 DNA and INFORM®Chromosome 17 (Chr17) probes (Ventana Medical Systems, Tucson, AZ, USA) on TMA sections using the Benchmark® XT automatic immunostaining device according to the manufacturer's protocol. The signals for HER2 and Chr17 were counted in more than 20 non-overlapping nuclei per sample. HER2 was considered amplified if the HER2 gene copy number was over 6 or an HER2/Chr 17 ratio was over 2.2.

Table 4.5 Summary of interpretation of immunohistochemical stainings

Antibody	Analyzed staining	Evaluated intensity (int)	Evaluated frequency (fre)	Cut-off (II) and values used for prognostic modeling (IV)
ER	nuclear	0–3*	continuous	fre > 10% (II) int and continuous fre (IV)
PR	nuclear	0–3	continuous	fre > 10% (II) int and continuous fre (IV)
p53	nuclear	0–3	semi-quantitative (<10%; 10–50%; >50%)	int 2 > 50% or int 3 in 10% tumor cells (II) as above or tumor cells completely negative (IV)
Ki-67	nuclear	-	continuous	continuous variable (II, IV)
PTEN	nuclear	0–3	-	>0 int (II, IV)
HER2	membranous	0–3	negative/incomplete/ complete	complete membranous staining in > 10%, int 2–3 (II) complete membranous staining in > 10%, int 3 (IV)
MLH1	nuclear	0–3	-	>0 int (II, IV)
L1CAM	membranous	-	negative, threshold or positive 0–2 (<10%; ≈10%;>10%)	semi-quantitative (IV)
ASRGL1	cytoplasmic	0–3	semi-quantitative 0–6 (0%; 1–10%; 11–25%; 25–50%; 51–75% and >75%)	fre >75% (II) int and semi-continuous fre (IV)

* negative; week; moderate; high
fre: frequency, int: intensity
For references used for cut-off determination see the original publications.

4.3 Statistical analysis

4.3.1 Microarray data (I)

Statistical analysis was performed using the R/Bioconductor open software package. For Affymetrix, the CEL files were loaded into the software and preprocessed using the simpleaffy package, and a Robust Multi-Array Average expression measure was computed, including quantile normalization. For Illumina, the data were imported, assessed for their quality, and quantile was normalized using the beadarray package (version 1.2.2). Using the limma package, a linear model was fitted to the normalized data. For the estimated coefficients, a moderated t-statistics and log-odds of differential expression were computed. The resulting gene list was filtered according to p=0.001. Genes were annotated, and output files were created using the biomaRt package. Finally, the gene lists generated by the analysis of these two platforms were joined, retaining only genes that were included in both lists.

4.3.2 RT-PCR (I)

Relative quantitation (RQ) analysis was performed in two separate parts with the CT method by using the SDS RQ Manager Software 1.2 (Applied Biosystems) and a 99% confidence level. Statistical significances of differences of the group-specific means were tested using 2-sample t-test for means at a 95% confidence level with the alternative hypothesis as not equal and by using pooled variance (SYSTAT 11, version no. 11.00.01; SYSTAT Software, Inc.). Box plots from the data were prepared by using R/Bioconductor, version 2.5.1.

4.3.3 Descriptive statistics and survival analysis (II and III)

Categorical variables were characterized using frequency and percentages and were analyzed using the chi-square and Fisher's exact test. Continuous variables were characterized using the median and range, and differences between the groups in non-normally distributed continuous variables were tested using the Kruskal-Wallis one-way analysis of variance.

Time-to-event was defined as DFS or DSS from initial hysterectomy to date of EEC relapse or death from disease, respectively. A univariate Cox regression model was used to examine prognostic factors for survival and was quantified using hazard ratios (HRs) with 95% confidence intervals. Factors significantly associated with survival in univariate models were included in a multivariate Cox regression model. The Kaplan-Meier method was used to generate disease-specific survival curves, and differences between groups were analyzed using the log-rank test. All tests were two-tailed, and a $p < 0.05$ was considered statistically significant. In study III, a ROC curve was used to assess the cut-off point for high ASRGL1 levels. A Cox regression model was used to examine prognostic factors for DSS. Statistical analyses were performed with IBM SPSS version 19.0 (II) and version 21.0 (III) (IBM Corp., Armonk, NY, USA).

4.3.4 Clustering and survival analysis (IV)

Penalized LASSO logistic regression and its Cox regression extension were used to create predictive models for the two studied panels, as described in detail in the original paper. Heatmap clustering was conducted using complete linkage coupled with the Euclidean distance to visualize the immunohistochemical stainings and their co-expression, and the cut-offs were derived from the identified pre- and post-operative models. Fisher's Exact Test was used to evaluate p-values for the pre-operative panels, and Cox proportional hazards regression was used to identify statistically significant differences in post-operative risk. The R statistical software (version 3.3.1) was utilized to conduct statistical analyses, in conjunction with the extending R-packages glmnet (version 2.0-5) and hamlet (version 0.9.5).

5 RESULTS

5.1 Gene expression profiling of EEC specimens (I)

When this study was initiated, the different commercial platforms for gene expression profiling (microarrays) were still under development. Therefore, two different platforms (Affymetrix and Illumina) were used in parallel to analyze selected grade 1 and grade 3 tumor samples. Both suppliers advertised their microarrays as "whole genome" platforms, which means that at least some transcripts for a majority of human genes were analyzed. Analysis of the results from the two platforms revealed eight genes that were clearly upregulated in grade 3 EEC (n=7) when compared to grade 1 EEC (n=6) with both technologies (Table 3, study I). These were: *APOE* (apolipoprotein E), *UBXD4* (UBX domain containing 4), *IFI30* (interferon, gamma-inducible protein 30), *DDX59* (DEAD box polypeptide 59), *FLJ13848* (hypothetical protein FLJ13848), *NISCH* (nischarin), *CDC42BPB* (CDC42 binding protein kinase beta [DMPK-like]), and *TORC2* (transducer of regulated cAMP response element-binding protein 2).

Although the results concerning the eight most highly overexpressed genes were generally in strong agreement (Figure 3, study I), a number of differences were also observed between the results obtained with the two microarray platforms (Huvila, 2007).

5.2 Apolipoprotein E in EEC (I)

As *APOE* was the most differentially expressed gene on both Affymetrix and Illumina platforms, the expression of this gene was further studied in an extended study group using qRT-PCR. As shown in Figures 1 and 2 in the original study I, the *APOE* expression was higher in grade 3 compared to grade 1 cases.

Increased *APOE* expression was related to poorly differentiated EEC, whereas there was no significant difference between the well- and moderately differentiated groups.

In the immunohistochemical evaluation, all of the grade 1 EEC samples were ER- and PR-positive, whereas grade 3 specimens were more heterogeneous, including one ER negative and 2 PR negative cases. In the p53 staining, three (43%) of the grade 3 tumors showed staining in >50% of tumor cells, which was interpreted as overexpression, whereas none of the grade 1 tumors overexpressed p53. PTEN-negative cases were distributed nearly equally in both groups. Three tumors were negative for MLH1; none were MSH2 or MSH6 negative. All of the 13 tumors showed positive staining for β-catenin in tumor cells.

5.3 The prognostic value of stage and grade in EC

Follow-up information on patient cohorts 1 and 2 (total 320 patients) made it possible to produce Kaplan-Meier curves for cumulative survival for each stage and grade. Clear

effects of stage and grade on DSS were observed in combined cohorts 1 and 2; both stage and grade were significantly associated with DSS (**Figure 5.1**).

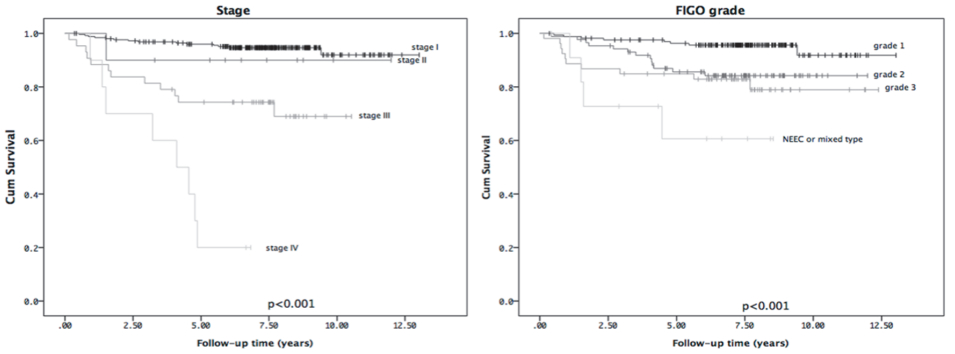


Figure 5.1 FIGO stage- and grade-related survival after EC surgery for cohorts 1 and 2 n=320 (Kaplan-Meier estimation).

In studies II and III, the prognostic value of stage and grade were assessed using COX regression analysis. In both studies, univariate analysis was initially performed, whereafter factors presenting statistically significant prognostic value were entered in a multivariate analysis. In both studies, grade was a significant prognostic factor in univariate analysis but demonstrated no independent prognostic value in multivariate analysis. The independent prognostic value of stage was not assessed in study II, but in study III it also showed an independent prognostic value in multivariate analysis (Table 3, study III).

5.4 Prognostic factors in stage I–II EEC (II)

The value of ER, PR, p53, HER2, PTEN, MLH1 and Ki-67 expression on DFS in early stage (stage I–II) EEC was studied in the second study of this thesis work in 182 EEC patients. Representative photomicrographs illustrating low and high expression of ER, PR, HER2, p53 and Ki-67 are presented in the original article (Figure 1, study II). The results of the immunohistochemical stainings and their effect on DFS are presented in **Table 5.1**. Contrary to FIGO grade (see above), the other evaluated clinicopathological markers (LVI, MI, size, age, BMI, CA12-5) did not exhibit a prognostic role in univariate analysis. Of the immunohistochemical markers, only PR and p53 and had a statistically significant effect on DFS. Therefore, only these markers and FIGO grade were entered in a multivariate analysis in which only PR expression showed an independent prognostic role in multivariate analysis [HR 5.14(CI 1.31–20.12), p=0.019]. The negative prognostic effect of PR negativity was most notable in the intermediate and high-risk subgroups (**Figure 5.2**).

Using HER2 immunohistochemistry, six cases were moderately (2+) HER2 immunopositive and one exhibited strong staining (3+). HER2 amplification was

confirmed with HER2 silver-enhanced in situ hybridization in the only case exhibiting strong staining. Due to a single finding, no statistical analysis of HER2 could be performed.

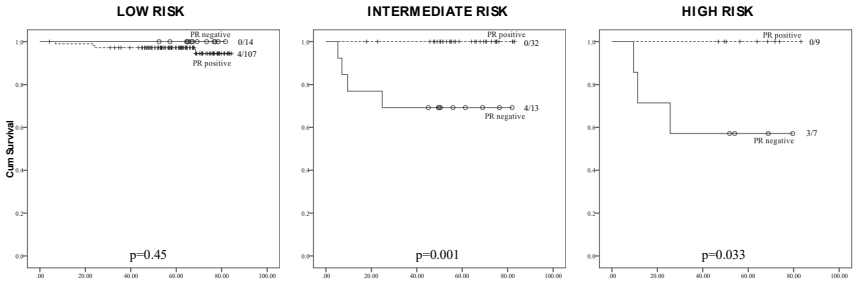


Figure 5.2 Kaplan-Meier estimates on PR-related survival when stratified for clinicopathological risk.

5.5 The role of ASRGL1 expression in EEC (III)

ASRGL1 (also called CRASH, ALP, ALP1) was initially found in a systematic search of The Human Protein Atlas (Uhlen et al., 2005; Uhlen et al., 2010; Ponten et al., 2011) where it demonstrated differential expression across tissues and in malignancies (including EC). Additionally, in unpublished Illumina microarray data, ASRGL1 expression levels were lower in grades 2 and 3 EEC when compared to grade 1 cases (fold change 3.0, $p=0.0014$ and 5.0, $p<0.0001$, respectively). Immunohistochemical expression of ASRGL1 was evaluated in two independent cohorts (a discovery and a validation cohort) using TMAs to validate its prognostic effect in EC.

Representative photomicrographs illustrating ASRGL1 expression are presented in the original article (Figure 3, study III). Decreased ASRGL1 expression was significantly associated with the stage and grade in both cohorts and with age and MI in the validation cohort. In the discovery cohort, low ASRGL1 expression was significantly associated with LVI, a variable that was not available for the validation cohort. Additionally, decreased ASRGL1 levels were associated with poor outcomes in both cohorts. In a subanalysis of non-endometrioid cases, no prognostic effect was detected, and further analysis focused only on EEC cases. In a univariate analysis, age, stage, grade and ASRGL1 had an independent prognostic role on DSS in both cohorts and were entered in a multivariate COX regression analysis. The results are presented in **Table 5.2**. The negative prognostic effect of ASRGL1 negativity in the combined cohort is presented in **Figure 5.3**.

Table 5.1 Prognostic value of immunohistochemical staining results for DFS in 182 EEC patients.

		COX regression		
		Univariate		
(%)	**Event/total**	**HR**	**95% CI**	**p-value**
ER				0.16
Neg (80.2)	13241	2.44	0.71–8.34	
Pos (19.8)	7/146	ref		
PR				**0.001**
Neg (81.3)	12601	8.24	2.41–28.16	
Pos (18.7)	4/148	ref		
p53				**0.01**
Neg (6.6)	8/170	ref		
Pos (93.4)	42707	5.71	1.51–21.52	
PTEN				0.57
Neg (54.4)	36342	ref		
Pos (54.6)	30407	1.44	0.42–4.90	
MLH1[b]				0.93
Neg (27.5)	18323	ref		
Pos (72.5)	8/125	0.95	0.25–3.57	
Ki-67 [a]				
		1.04	0.83–1.30	0.75

[a] Ki-67 divided by 10, median 30 (range 0.5–100)
[b] n=207
HR: hazard ratio, CI: confidence interval
ref: reference

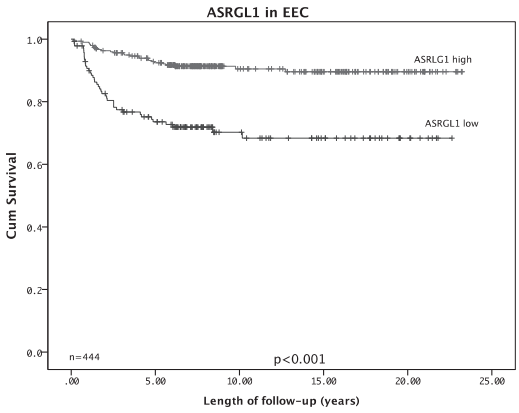


Figure 5.3 ASRGL expression-related survival after EC surgery for the combined discovery and validation cohorts, n=443 (Kaplan-Meier estimation).

Table 5.2 Prognostic value of clinicopathological variables and ASRGL1 expression for DSS in EEC

	Cox multivariate regression analysis							
	Discovery cohort				Validation cohort			
Variable	event/total	HR	95% CI	p-value	event/total	HR	95% CI	p-value
Age		1.05	1.0–1.10	0.65		1.02	0.98–1.06	0.38
Stage				**0.021**				**<0.001**
I–II	8/180	ref			27/213	ref		
III–IV	11/35	3.49	1.21–10.03		28/43	10.06	4.69–21.54	
Grade				0.80				0.59
Grade 1	5/117	ref			6/64	ref		
Grade 2	9/62	1.49	0.44–4.97		36/153	0.47	0.45–5.67	
Grade 3	5/36	1.42	0.39–5.25		14/40	2.06	0.51–8.24	
MI				0.33				**0.006**
≤50%	8/150	ref			10/134	ref		
>50%	11/65	1.67	0.60–4.63		29/81	3.12	1.40–7.26	
ASRGL1				**0.034**				**0.002**
≤75%	14/65	3.55	1.10–11.43			3.23	1.53–6.81	
>75%	5/150	ref				ref		

Statistically significant p-values are bolded

5.6 Immunohistochemical staining panel for risk stratification (IV)

To evaluate the prognostic value of ASRGL1 (study III) in relation to well-described prognostic markers (study II), as well a novel biomarker L1CAM (see 2.6.3.10), a machine learning-based model was used to optimize an immunohistochemical panel for prognostication. This substudy had two different premises: to find an immunohistochemical panel (1) to assess preoperative risk, *e.g.,* to predict clinicopathological findings such as LVI, MI and advanced stage III–IV disease, and (2) to predict the risk of recurrence and the risk of dying of EEC.

Three panels were identified to assess preoperative risk. An ER < 50% was found to predict deep myometrial invasion, and an ER (cut-off 1%) together with a PR (cut-off 25%) were found to predict LVI. A PR with a cut-off of 10% and an ASRGL1 with two cut-offs (25%; 75%) were the best predictors of advanced disease. The results are presented in study IV, Table 3.

The optimal panel of immunohistochemical markers to predict risk of relapse and death from disease consisted of p53 and ASRGL1. Based on the model-identified prognostic panel, the patients were grouped into three risk categories: low-risk (p53 wild type, ASRGL1 >75%), intermediate-risk (p53 wild type, ASRGL1 ≤75% or p53 aberrant, ASRGL1 >75%) and high-risk (p53 aberrant, ASRGL1 ≤75%). From the low-, intermediate- and high-risk cases, 6 (3.0%), 14 (15.6%) and 11 (61.1%) died of disease, respectively. High-risk patients had a 30-fold risk of dying of EEC when compared to low-

risk patients. Kaplan-Meier estimates on ASRGL1 and p53 related survival are presented in study IV, Figure 1A.

The immunohistochemical staining results were clustered using cut-off values derived from the prognostic models, and a heatmap was produced to illustrate the relationship between the different markers. After clustering, data on the clinicopathological variables and survival data were added to the heatmap (study IV, Figure 2).

There was significant correlation between some of the markers, as was visible in a heatmap. ER and PR as well as ASRGL1 were frequently co-expressed. There was also significant co-expression between p53 and L1CAM stainings ($p<0.001$), and of the 27 (8.9%) L1CAM positive results, 14 (48.1%) also had aberrant p53 expression. The effect of L1CAM and p53 expression on disease-specific and recurrence-free survival is presented in study IV, Figure 1B.

6 DISCUSSION

Endometrial cancer is the most common gynecological cancer in developed countries, and its the prevalence is on the rise (Kitchener and Trimble, 2009; Torre et al., 2015). As has been presented throughout this thesis, EC is a heterogenic group of tumors. Not only is there heterogeneity in tumor histology, there is also heterogeneity within histologies and, moreover, within the individual tumor. There is phenotypic and genotypic heterogeneity as well as genotype-phenotype inconsistency, both at the intertumoral but possibly also at the intratumoral level.

Accumulated research and clinical data has led to changes in classification and risk stratification of EC, altered the guidelines governing allocation of surgical treatment and adjuvant therapy and, moreover, revealed novel classification modalities based on genomic characterization. Together, these findings have shown that the currently used risk stratification methods are not sufficient, and a biomarker or most likely a panel of biomarkers are needed to correctly classify patients into risk groups for surgery and to allocate further treatment to those patients who require it and will likely benefit from it.

Morphological heterogeneity is a diagnostic challenge. It has been associated with Lynch syndrome, and similarly, MSI-high tumors tend to show considerable intratumoral heterogeneity (Broaddus et al., 2006; Garg et al., 2009). Additionally, morphological ambiguity is associated with the *POLE* mutation (Soslow, 2013). Morphological heterogeneity can increase the risk of discrepancies between preoperative and final diagnosis, which might lead to misguided treatment and be associated with adverse outcomes (Werner et al., 2013).

There is a significant need for improved tools to guide diagnostics. Hoang et al. (2013) demonstrated that particularly in high-grade carcinomas, there is a significant subset of cases in which morphological diagnosis is not in concordance with genotype, arguing further that immunohistochemistry, especially p53 staining, could improve diagnoses. Soslow et al. (2013) stated that we need genetic signatures and expression profiles that are strongly linked not only to morphology-based diagnoses, but also to clinical outcomes. In a recent review, Bendifallah et al. (2016) evaluated the developed predictive tools, such as nomograms, algorithms and risk-scoring systems, and found none of them to be sufficiently accurate. One of the main limitations identified was that all nomograms are based on classical clinicopathological risk factors (Bendifallah et al., 2016). Additionally, none of the models predicting recurrence and survival contained tissue biomarkers.

TCGA data provided a novel insight into the genetic and clinical heterogeneity of EC. The classification presented highlighted the need for "advanced staging" or "integrated or enhanced classification", which by integrating molecular and clinicopathological results

could improve risk assessment (Stelloo et al., 2016) and define biologically and clinically relevant subsets of EC (Murali et al., 2014).

Despite extensive research, however, there is currently no consensus on the optimal panel of prognostic biomarkers. Disappointingly, the results of prognostic studies pertaining to several biomarkers have been inconsistent. Small cohort sizes and heterogenic cohorts might contribute to this deficiency. Several prognostic panels have been suggested, but validation data is scarce. The overarching aim of this thesis was to add knowledge to the still incomplete field of endometrial cancer prognostication.

6.1 Gene expression profiling in identification of new biomarkers

When the first part of this study was initiated, genome-wide microarray analyses were considered to be the most promising new method of finding systematic alterations in the gene expression between different tissues and cell types and in diseases. To improve the reliability of the findings, two independent microarray platforms (Affymetrix and Illumina) were used in the present study, and only those transcripts recognized in both analyses were considered significant. Comparison of gene expression profiles of low-grade and high-grade EEC tumor samples revealed that the most differentially expressed gene on both platforms was *APOE*. This was considered intriguing and also promising, as increased *APOE* expression has also been detected in several other malignancies (Venanzoni et al., 2003; Oue et al., 2004; Chen et al., 2005; Yu, Rustgi and Blair, 2005). The upregulation of *APOE* in poorly differentiated EEC was confirmed by qRT-PCR.

Since the publication of study I, other reports on gene expression profiling of EC and EEC in differential experimental setups have been published (Grønborg et al., 2006; Sakashita et al., 2008; Lindén et al., 2013; Ifere et al., 2013; Rosser et al., 2014; Zhou et al., 2014; Luo et al., 2016). Surprisingly, however, no supporting data on *APOE* upregulation in EC has been published by others. More recently, it has been realized that gene expression profiling alone may no longer be sufficient to study genomic variations in different types of cancer samples. There are several explanations for the reduced applicability of genome-wide microarray profiling. These include the heterogeneity of the starting material, i.e., unknown proportions of normal and tumor tissue in the specimen, and unknown proportions of reactive normal tissue in the sample. Difficulties in grading of tumors, as discussed throughout this thesis, also contribute to this heterogeneity. Additionally, quality issues concerning RNA derived from tissue samples can impair gene expression profiling. Overall, it appears that whole-genome microarrays suffer from high background noise, as illustrated by differences in the results of different groups. Even with these reservations, however, the novel finding in study I of upregulated *APOE* expression in high-grade EEC tumor samples enhances the interesting field of APOE and cancer progression.

The other seven most significant increases observed in the microarray analyses in study 1 involved genes that had not been implicated in other malignancies and were not studied further. After publishing study I in 2009, however, more data has become available for six

of these upregulated genes detected by the microarray platforms. Two of these, *UBDX4* and *FLJ13949*, today known as *UBXN2A* (UBX domain protein 2A) and N(alpha)-acetyltransferase 40 (*NAA40*), respectively, have been linked to apoptosis. The overexpression of *UBXN2A* was shown to trigger p53-dependent apoptosis (Sane et al., 2014) and, in colorectal cancer cells, the depletion of *NAA40* was shown to decrease cell survival by enhancing apoptosis (Pavlou and Kirmizis, 2016). *IFI30*, also known as gamma-interferon-inducible lysosomal thiol reductase (*GILT*), has been found to be associated with antigen presentation, and low *GILT* expression has been associated with adverse survival in breast cancer (Rausch and Hastings, 2015). *NISCH* has been shown to regulate cell migration by inhibiting p21-activated kinase and has been suggested to play an important role in cell invasion (Ding, Milosavljevic and Alahari, 2008). *TORC2*, today known as *CRTC2*, which was previously associated with lipid and glucose metabolism (Screaton et al., 2004), has been recognized as a transcriptional activator of well-established MMR genes (Fang et al., 2015). No complementary data were found for *DDX59*. As new data on these genes continues to accumulate, revisiting the microarray data obtained in study I may become justified.

Because of rapid technological developments, whole genome expression analysis is no longer considered a state-of-the-art method in cancer research. Out of necessity, the field is moving toward very large integrated analyses of big data, as was recently published by the TCGA Research Network (Cancer Genome Atlas Research Network et al., 2013a). In this study, a comprehensive, multiplatform analysis of 373 EC samples was performed, combining analyses of somatic copy number alterations, exome sequence analysis, mRNA and protein expression, microRNA expression and DNA methylation, and somatic chromosomal aberrations determined by whole-genome sequencing of 106 tumor samples. The results confirmed the heterogeneity of genetic and protein biomarkers, but also allowed the authors to classify EC into four groups based on integrated genomic data, including identification of a novel *POLE* mutated subtype in 10% of endometrioid tumors. It appears likely that this type of large integrated analyses of data produced in different projects will contribute to new molecular insights into tumor classification (disease stratification) and lead to treatment recommendations (personalized medicine).

6.2 Apolipoprotein E in EEC

There is a well-established association between APOE (and its isoforms) and cardiovascular disease, metabolic syndrome, Alzheimer's disease and serum lipid levels (Phillips, 2014; Torres-Perez et al., 2016; Shafi, 2016; Sofat et al., 2016). The molecular function of APOE in cancer remains unclear. In one study performed using an ovarian cancer cell line, APOE was shown to be a factor in cancer cell proliferation and survival, and ApoE-specific small interfering RNA led to cell cycle arrest and apoptosis (Chen et al., 2005). However, in one study performed on malignant melanoma, APOE was recognized as a potentially anti-angiogenic and metastasis suppressive factor (Pencheva et al., 2012). APOE has been associated with increased telomerase activity (Shafi, 2016), which is characteristic of cancer cells. Additionally, APOE polymorphism has been associated with

metabolic syndrome and obesity, which are also a known risk factor for EEC (Torres-Perez et al., 2016). Further work is warranted to better understand the role of APOE in cancer and to elucidate the potential shared pathways of carcinogenesis and other APOE-related diseases. The origin of *APOE* also remains unclear, as macrophages in a tumor microenvironment can potentially produce APOE (Driscoll and Getz, 1984).

6.3 Progesterone receptor in early stage EEC

The results of the second study (II) demonstrate that PR status is an independent prognostic factor, and the loss of PR expression is significantly associated with disease relapse in patients with EEC confined to the uterus. PR status was superior as a predictor of relapse over LVI or tumor size. In addition, ER, PTEN, Ki-67, and MLH1 expression were not associated with the risk of relapse. The prognostic role of PR was most evident in the intermediate and high-risk groups, where the identification of patients that require further adjuvant therapy is most warranted.

There is a biological basis for the importance of progesterone signaling in patients with EC (Yang, Thiel and Leslie, 2011), and several possible mechanisms have been suggested to account for the protective role of PR. First, progressive disease was associated with down-regulated PR expression in this and earlier studies. Second, the presence of progesterone has been shown to inhibit migration in cell lines, which is thought to result from inhibition of epithelial-mesenchymal transition (van der Horst et al., 2012). Third, progesterone has been postulated to have a role in attracting tumor-infiltrating lymphocytes in non-progressive endometrial cancer, a finding that may be an important factor in stimulating immunosurveillance (van der Horst et al., 2012).

In a recent meta-analysis, high PR levels were associated with a favorable prognosis in EC (Zhang et al., 2015). However, no cut-off values were reported or evaluated. The question of an optimal cut-off point for PR immunohistochemistry remains unanswered. Cut-offs from 1% (Köbel et al., 2016) to 40% and even 50% (Oreskovic et al., 2004; Yang et al., 2016) have been used to classify tumors as PR negative/positive or low/high-risk. In the present study, a cut-off point of 10% was chosen since it has been used in previous studies focusing on the prognostic value of PR (Fukuda et al., 1998; Saito et al., 2006; Fons et al., 2007). The existence of several cut-off values that all produce statistically significant results could indicate that the loss of PR expression is a rather linear event, and more data is needed to determine the optimal and best reproducible cut-off.

PR immunohistochemistry is a low-cost, routine staining that is widely available in clinical pathology laboratories. Additionally, it is likely to be less susceptible to interpretation errors when compared to, *e.g.,* LVI. Still, biomarkers are not systematically used to guide treatment choices. At present there are at least two ongoing clinical trials, one evaluating the applicability of ER/PR guided lymphadenectomy (MoMaTEC2), and the other

(PIPENDO) aimed at defining an optimal panel of biomarkers (comprising IMP3, p53, ER, PR, MLH1, PTEN, beta-catenin, p16, Ki-67, stathmin, ARID1A and L1CAM) to contribute to risk stratification and clinical decision making in EC (ClinicalTrials.gov, 2016a; Visser et al., 2015). Published information on the planned cut-off values for immunohistochemical staining results was not available. However, in personal communications and conference proceedings, a cut-off of 1 % (PR) has been proposed, at least for the MoMaTEC2 trial. The same cut-off has been used in the evaluation of high-grade EC (Köbel et al., 2016), and it is in wide use in breast cancer research. However, in breast cancer, hormone receptor status is primarily used to guide adjuvant therapy, *e.g.,* the use of tamoxifen treatment for ER positive patients. In such a setting, it is important that all those patients who could potentially benefit from this treatment are found (Hammond et al., 2010).

6.4 ASRGL1 in endometrial cancer

ASRGL1 was initially found in a systematic search of The Human Protein Atlas (Uhlen et al., 2005; Uhlen et al., 2010; Ponten et al., 2011) where it demonstrated differential expression across tissues and in malignancies (including EC). Subsequently, supporting evidence was received from a study in which *ASRGL1* was one of 29 differentially expressed genes in EC in a cluster of down- and upregulated genes that was associated with poor outcome (Salvesen et al., 2009). In addition, data retrieved from the MediSapiens database (Kilpinen et al., 2008) revealed that there is a subgroup of EC cases that show low expression, whereas the majority present high expression. ASRGL1 expression has been reported in EC, but its prognostic value was not addressed (Weidle et al., 2009). In a recent gene regulatory network analysis, ASRGL1 was identified as a hub gene when comparing most differentially expressed genes between EEC and NEEC (O'Mara, Zhao and Spurdle, 2016), emphasizing its potentially important role in EC carcinogenesis.

In the present study, the loss of ASRGL1 expression was significantly associated with an adverse disease-specific survival in EEC, and the finding was confirmed with an independent cohort. Very little data is available on the possible mechanisms by which ASRGL1 could support or promote tumor progression. Evtimova et al. (2004) have demonstrated that ASRGL1 is induced by progesterone, and it could therefore share an expression pattern similar to hormone receptors.

To avoid possible overfitting of the acquired threshold value, the results were validated with an independent patient cohort, and the findings were highly similar. To date, no further verifying data has been published, but work is ongoing (personal communication).

6.5 Prognostic immunohistochemical panel in EEC

The systematic literature review performed for this thesis demonstrates the incompleteness of current risk stratification systems. There are several contradictory findings for most of the prognostic biomarkers and overall poor reproducibility of several clinicopathological

prognostic factors. These imperfections may be considered one of the major incentives for generating a prognostic panel consisting solely of immunohistochemical markers/stainings, as presented in study IV of this thesis. Improved risk stratification is essential for the patient's optimal treatment, survival and quality of life. Another recent study has arrived at the same conclusion: current risk classifications are not good enough (Bendifallah et al., 2015). In today's clinical practice, there is a clear need to distinguish between patients who may benefit from lymphadenectomy and those with low-risk EC where the role of LND has been questioned (Colombo et al., 2016).

Three different models to predict MI, LVI and advanced disease were identified. The models consisted of different markers (ER, PR and ASRGL1) with different cut-off values, which could reflect the differences between the mechanisms leading to the specific pattern of disease spread and invasion. The association between time and increasing MI or disease spread is difficult to evaluate, but it can be speculated that once cancer cells have started invading the myometrium and beyond the process is unlikely to be halted without some extrinsic factor. Although no single preoperative risk panel directly translatable into clinical use could be identified, our results highlight the role of ER, PR and ASRGL1 in preoperative risk assessment.

A panel consisting of p53 and ASRGL1 was shown to be most useful in predicting DFS and DSS. Our data suggest that the currently used definition of aberrant p53 expression in conjunction with decreased ASRGL1 expression (≤ 75%) are strong predictors of adverse survival in EEC. The results confirm the independent prognostic value of ASRGL1 first demonstrated in study III and also reveal that ASRGL1 has an independent prognostic role when compared to well-described prognostic biomarkers. In this study, L1CAM did not demonstrate an independent prognostic role and was not entered in either of the prognostic panels, which was likely due to the strong correlation with p53 expression.

6.6 Methodological considerations and study limitations

The strength of the present investigation is the homogenous, well-described study population(s) whose members have been treated during a limited time period (2001–2007). Complete surgical staging, including lymphadenectomy, has been performed on 80% of the study population. The focus has been on EEC, and all of the statistical analyses have been performed on datasets consisting purely of EEC cases. Follow-up for both relapse and survival was complete, and no patients have been lost during follow-up. Additionally, study III draws strength from two independent population-based cohorts who have been treated during different decades, reducing the impact of altered treatment modalities. The retrospective set-up and the relatively small number of patients, and in particular the small number of end-point events, *i.e.,* disease relapse and death of EC, can be considered a weakness of the study.

The grade distribution of study cohorts II and III, 54% (grade 1), 28% (grade 2) and 18% (grade 3), is in line with the median grade distribution of a wide range of representable and

high-quality EEC studies reviewed in this thesis (**Table 2.8**). Thus, the results can be considered to be comparable to other published investigations.

As discussed in the review of literature (Section 2.5.1.1), adequate tissue sampling is of prime importance for subsequent molecular-level analyses. In most studies, the sampling procedure is inadequately described and may vary considerably. As is the case with the present study, the exact composition of the tissue used for extraction of DNA, RNA, proteins and other molecules remains unknown, even if adjacent tissue has been used for histopathological analysis. This uncertainty may explain some of the discrepancies observed in molecular-level studies of EC. To avoid this problem, studies II–IV of this thesis used immunohistochemistry to analyze tumor samples, which allows the pathologist to determine the proportion of tumor and stromal tissue. For this study, TMAs rather than whole tissue sections were selected due to their efficiency, and also for economical reasons. TM sections have been shown to have a high degree of concordance with whole tissue (Kallioniemi et al., 2001; Bubendorf et al., 2001), and this technique has also been validated for EC (Fons et al., 2007). Rather than preoperative tissue material, tissue derived from the hysterectomy specimen was chosen for TMAs, as the material is more abundant than that found in the preoperative samples. Even though the concordance rate between the preoperative samples and the hysterectomy specimens has generally been found to be good (Barut et al., 2012; Werner et al., 2013; Gungorduk et al., 2014), the applicability of the results to preoperative samples must be assessed with caution. Duplicate cores were derived from every tumor to reduce misinterpretation due to tumor heterogeneity.

6.7 Future perspectives

As discussed throughout this thesis, progression in categorizing EC has been disappointingly slow, as illustrated by the fact that no biomarkers are currently used in international risk stratification guidelines for EEC. Recently, new reports based on The Cancer Genome Atlas have demonstrated the power of big data analyses: integrated analyses (containing genomic, transcriptomic and proteomic data) form the foundation for a new classification system (Cancer Genome Atlas Research Network et al., 2013a). As discussed in the review of literature (Section 2.6.5), molecular biomarkers can be expected to be introduced into the stratification process for EC and EEC patients in the future. Based on the TCGA classification strategy, Talhouk et al. (2016) proposed an immunohistochemical panel of MLH1, MSH2, MSH6, PMS2 and p53, as well as a *POLE* mutational profile analysis, for preoperative use.

The TCGA study was the product of several leading comprehensive cancer centers (the paper had nearly 250 contributors). While such studies are needed to bring stratification to a higher level, it is clear that extensive genetic, transcriptomic and proteomic (and other high-throughput) analysis of individual patients is limited to a few clinical environments, both from a technological as well as a financial perspective. For routine clinical purposes, a cheaper, more accessible approach is needed. The TMA-based immunohistochemistry used in study IV is one such solution. Although the results are not directly comparable with the

results of the panel presented by Talhouk et al. (2016), there are obvious similarities. In the present study, the primary aim was to evaluate prognostic features rather than categorize patients as in the aforementioned classification. Based on the descriptions of the TCGA subgroups, several similarities could be found between markers in the prognostic panel of study IV and the TCGA groups: p53 was associated with the copy-number high group and poor prognosis, whereas the copy-number low group was associated with increased hormone expression. However, ultramutated and hypermutated subgroups could not be recognized as such with the TMA-based prognostic panel from study IV.

A vast amount of work remains to be done in the field of predictive medicine to answer the question of how low-, intermediate- or high-risk patients should be treated to achieve optimal results. Further prospective studies are needed, and in such studies, it is essential that the data and high quality tissue material are systematically collected, and that studied variables (such as LVI) are systematically determined across research centers.

As reviewed above in Sections 2.6.3 and 6.3 there is variation in the cut-off values of several of the established prognostic biomarkers. Immunohistochemical staining results should preferably be assessed as continuous variables or several groups, regardless of predisposed cut-off values, to allow for the comprehensive evaluation of tissue biomarkers and determine optimal cut-off lines. Future studies are warranted to prospectively validate the prognostic panel presented in study IV, consisting of ASRGL1 and p53, preferably in a national or a Nordic setting.

7 SUMMARY AND CONCLUSION

Despite the intensive research work in the field of endometrial carcinoma, there is a substantial lack of clinically useful prognostic biomarkers. Based on the studies included in this thesis, the following conclusions can be made:

1) Apolipoprotein E was the most differentially expressed gene when poorly differentiated grade 3 EEC was compared in microarray analyses to well-differentiated grade 1 EEC. Increased *APOE* expression was related to grade 3 disease, and there was no significant change between grade 1 and grade 2 disease. Altered expression of many of the differentially expressed genes identified in study I have since been associated with changes in different malignancies.

2) PR was the only biomarker among clinicopathological and well-described tissue biomarkers (ER, PR, p53, Ki-67, PTEN, MLH1 and HER2) that had an independent prognostic role in EEC that was limited to the uterus.

3) The novel biomarker ASRGL1 was found to be an independent prognostic factor in two independent EEC cohorts, even when adjusted for well-described clinicopathological variables.

4) The employed machine-learning based strategy indicated that from the studied well-described (ER, PR, p53, Ki-67, MLH1 and HER2) and novel tissue biomarkers (L1CAM and ASRGL1), EEC patients can be stratified using p53 and ASRGL1 stainings into three risk groups with significantly different clinical behavior.

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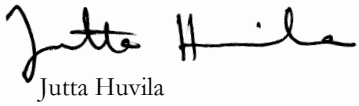
Turku, April 18th



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