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PROGNOSTIC FACTORS IN ENDOMETRIAL CANCER

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Prognostic Factors in Endometrial Cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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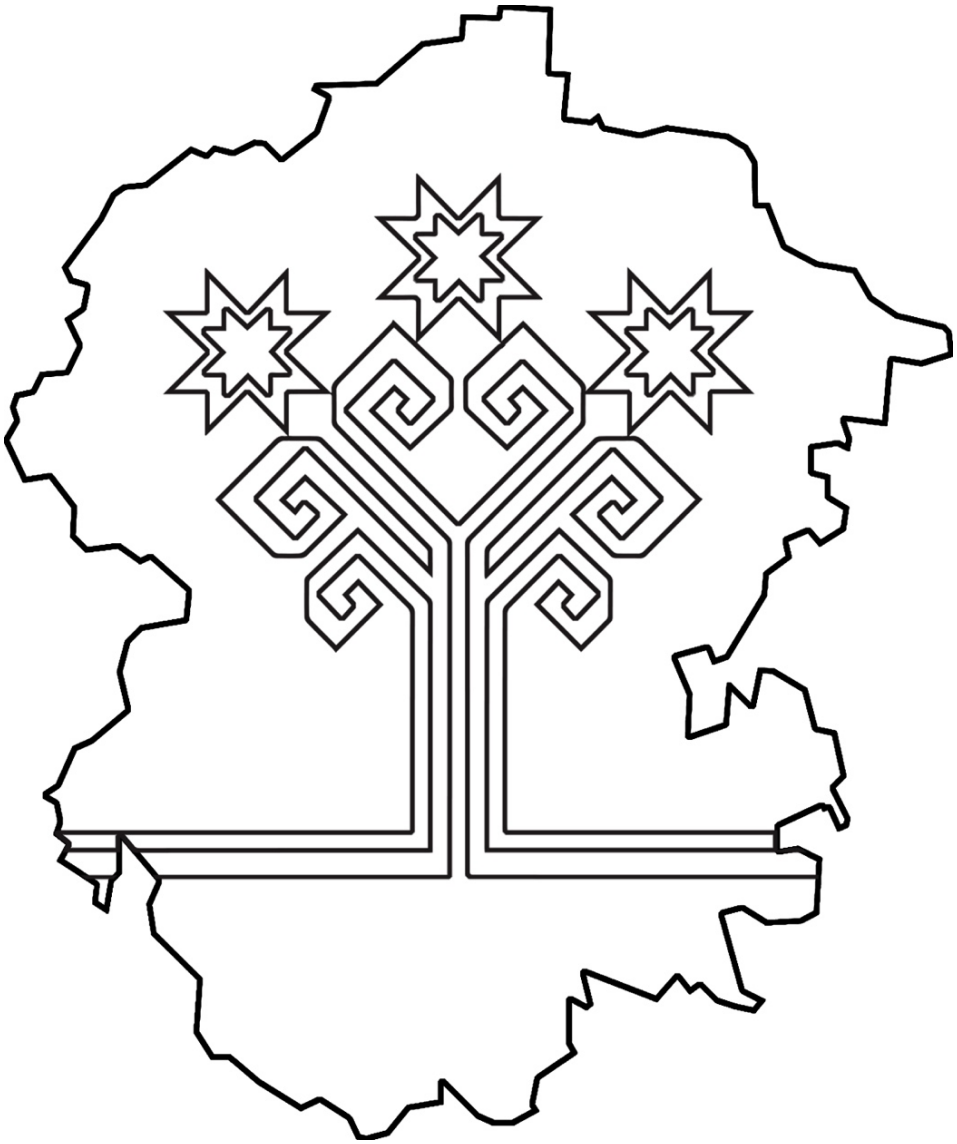
‘Мĕн акнă, вăл шăтать’

Chuvash proverb



My Motherland - Chuvash Republic

To my beloved grandmother, Zoia Razumova, whom I never had the chance to meet.

ABSTRACT

Endometrial cancer (EC) is the most common malignancy of the female reproductive system in Sweden. Patients with EC generally have a good prognosis, though some face recurrence and have a lower survival rate despite treatment. Current prognostic factors for EC are still not able to accurately reflect disease prognosis.

Therefore, the overall aim of the present project was to evaluate the impact of different, patient-specific factors on the prognosis of women with EC, with a special focus on the LRIG family of proteins, cadmium, dietary factors, daily routines, and the endometrial microbiome. Identifying these factors could pave the way for better determination of EC prognosis.

Study I analysed the role of LRIG1, LRIG2, and LRIG3 proteins on survival rates in women with EC. The analysis demonstrated that most patients had >50% positive cells for all three LRIG proteins. The LRIG1 protein score was high in most patients (97.3%). The percentage of LRIG3-positive cells was positively associated with better overall survival (P=0.019). There was no LRIG3 cell membrane staining in 30.4% of women who died, compared to 7.7% of EC survivors (P=0.01). Consequently, the results reveal a potential prognostic role of LRIG3, not LRIG1 and LRIG2, in EC.

Study II assessed the role of cadmium intake on the survival of 416 women with EC residing in Sweden. Median dietary cadmium intake was 13.1 μg/day. High dietary cadmium intake was associated with lower overall survival (P=0.05), but not with progression-free survival (P=0.348). Accordingly, high dietary cadmium intake seems to be an adverse prognostic factor in EC.

Study III evaluated the prognostic role of modifiable factors, such as dietary habits and daily routines, in EC using machine learning models. Among the 186 variables considered, consumption of sugar-sweetened beverages and fried potatoes increased the risk of EC recurrence and death, while physical activity decreased the risk of death. As a consequence, modification of specific dietary habits and daily routines might favourably impact EC prognosis.

Study IV investigated the role of endometrial microbiota in women with EC and compared it to the bacterial profile of women with benign conditions of the gynaecological tract (endometrial hyperplasia, EH or endometrial polyp, EP). Most women in the EC and EH/EP groups were postmenopausal and had a BMI above the normal range. The median age in the EC group was 10 years higher than that in the EH/EP group. The preliminary analysis showed that patients with EC have endometrial microbiota distinct from that of patients with EH/EP: *Atopobium* and *Porphyromonas* were present in patients with EC, while *Lactobacillus* was present in those with EH/EP. Thereby, endometrial microbiota might play a role in EC prognosis.

To conclude, this thesis provides additional knowledge on certain molecular and non-molecular factors that might play a role in EC, including its prognosis. Moreover, it emphasises the importance of continued investigation of other potential prognostic factors in EC.

LIST OF SCIENTIFIC PAPERS

I. RAZUMOVA Z, Oda H, Govorov I, Lundin E, Östensson E, Lindquist D, Mints M
The Prognostic Role of LRIG Proteins in Endometrial Cancer
Cancers (Basel). 2021;13(6):1361

II. RAZUMOVA Z, Govorov I, Östensson E, Mints M
Cadmium Intake as a Prognostic Factor in Endometrial Cancer: A Swedish Cohort-Based Study
Nutrition and Cancer. 2022;74(1):175-184

III. Wersäll OC, RAZUMOVA Z, Govorov I, Mints M.
Dietary Habits and Daily Routines as Prognostic Factors in Endometrial Cancer: A Machine Learning Approach
Nutrition and Cancer. Submitted

IV. Govorov I, RAZUMOVA Z, Andersson E, Mints M
Role of Endometrial Microbiota in Endometrial Cancer
In preparation

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LIST OF ABBREVIATIONS

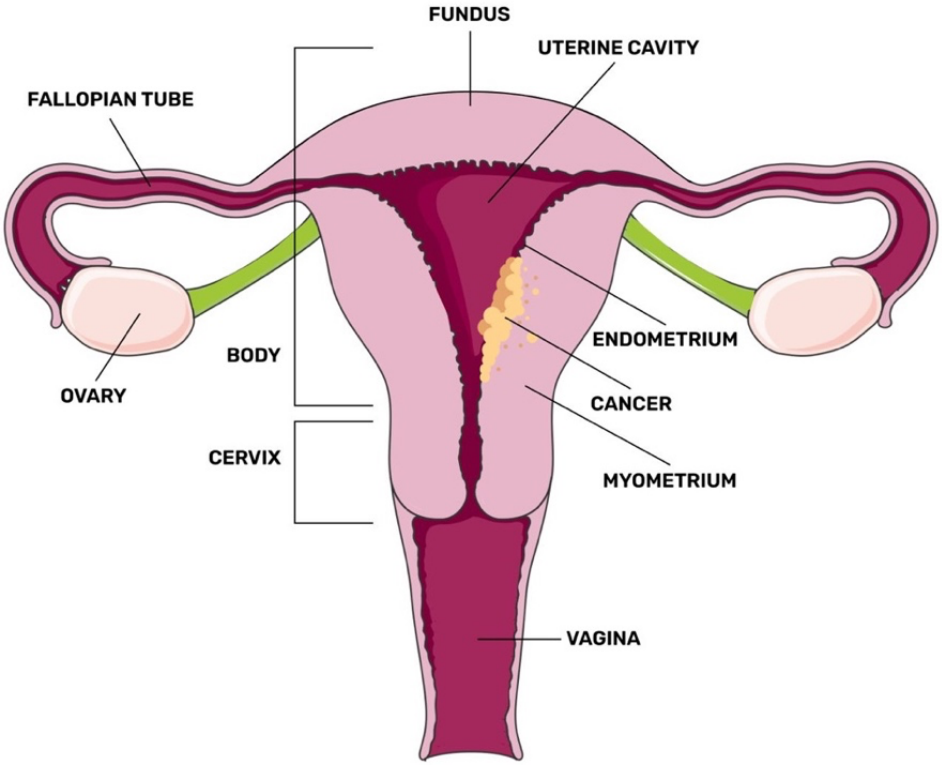
BMI	body mass index
DNA	deoxyribonucleic acid
EC	endometrial cancer
EH	endometrial hyperplasia
EP	endometrial polyp
IHC	immunohistochemistry
LRIG	leucine-rich repeats and immunoglobulin-like domains
LVSI	lymphovascular space invasion
MET	metabolic equivalent
MMR	mismatch repair
MMRd	mismatch repair deficient
MRI	magnetic resonance imaging
NSMP	no specific molecular profile
OS	overall survival
PFS	progression-free survival
POLE	DNA polymerase epsilon
RSF	Random Survival Forest
VIMP	variable important measures predictive
WHO	World Health Organisation
abn	abnormal expression
mut	mutation
wt	wild type

1 INTRODUCTION

1.1 ENDOMETRIAL CANCER

Endometrial cancer (EC) is a cancer of the inner layer of the uterus, which is called the endometrium (Figure 1).[1] The other two layers of the uterus are the myometrium, the thickest layer composed of smooth muscle fibres, and the serosa, the outer lining layer consisting of the visceral peritoneum. In Sweden, EC usually develops in postmenopausal women; median age at diagnosis is 71 years.[2]

Figure 1. Schematic illustration of a uterus with endometrial cancer.



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1.2 INCIDENCE

In 2022, EC was estimated to account for about 7% of all cancers in women, with North America and Western Europe reporting the highest EC rates.[3,4] EC is also the most common tumour of the female genital tract in Sweden, where approximately 1400 women are diagnosed with EC every year.[5,6] The high prevalence of EC in these geographic regions

might be connected to high levels of the endometrioid subtype, as well as differences in patient characteristics.[7]

Cancer survival rates began to improve in the 1970s, but this was not the case for cancer of the uterine corpus or uterine cervix.[8] Although EC rates were increasing by 1-2% yearly, they have stabilised during the last few years.[9,10] EC mortality rates, which had been rising consistently since the 1990s, have finally begun to decline.[11]

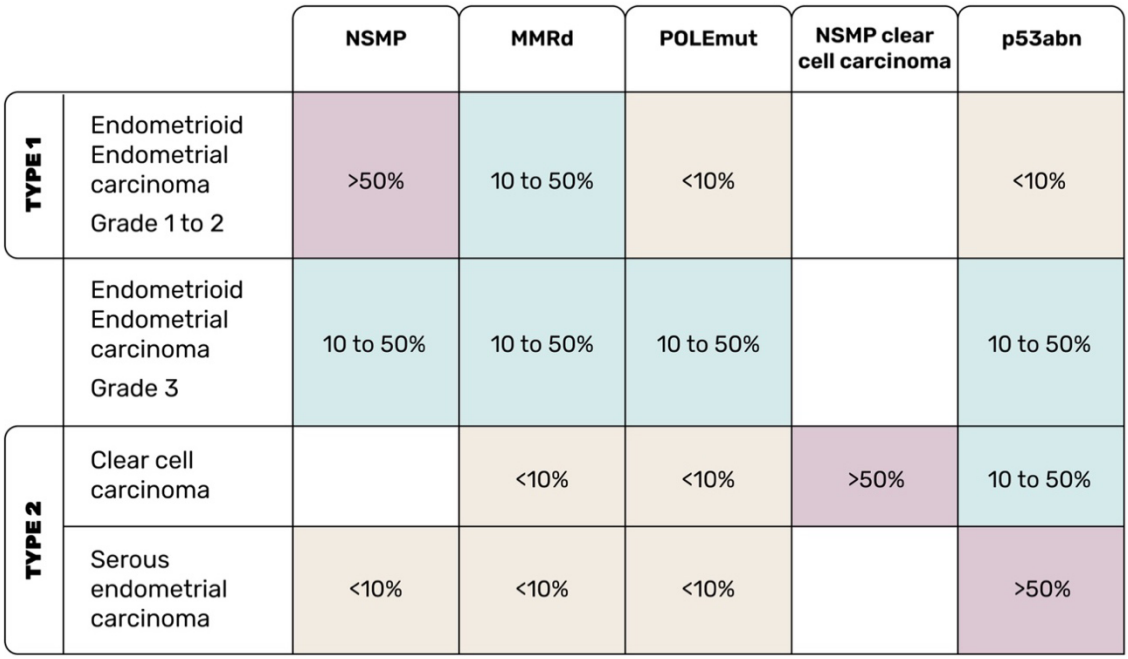
1.3 CLASSIFICATION

1.3.1 Histomorphologic Classification

EC is divided into two main histological subtypes, type 1 and type 2, based on a classification that was first described in 1983 by Dr Bokhman.[12] The incidence rates, tumour characteristics, and clinical characteristics of these types differ. Type 1 EC represents tumours with endometrioid histology and is responsible for about 80% of all EC cases.[13] It is often referred to as oestrogen-dependent; it is responsive to progestins, and intraepithelial neoplasm may be a precursor. Type 2 EC comprises tumours with non-endometrioid histology and is responsible for approximately 10-20% of all cases. Serous, clear cell, mucinous, squamous, transitional cell, mesonephric, and undifferentiated tumours belong to type 2.[13] These tumours have a worse prognosis, higher risk of metastasis, and a deeper myometrial invasion.

In 2020, the World Health Organisation (WHO) published the 5th edition of the Classification of Tumours of the Female Genital Tract, which emphasises integrating molecular classification in EC.[13] However, this molecular classification is still related to the traditional histomorphologic classification (Figure 2).[13]

Figure 2. Type 1 and 2 classification and relationship to histomorphologic and molecular endometrial cancer classification.



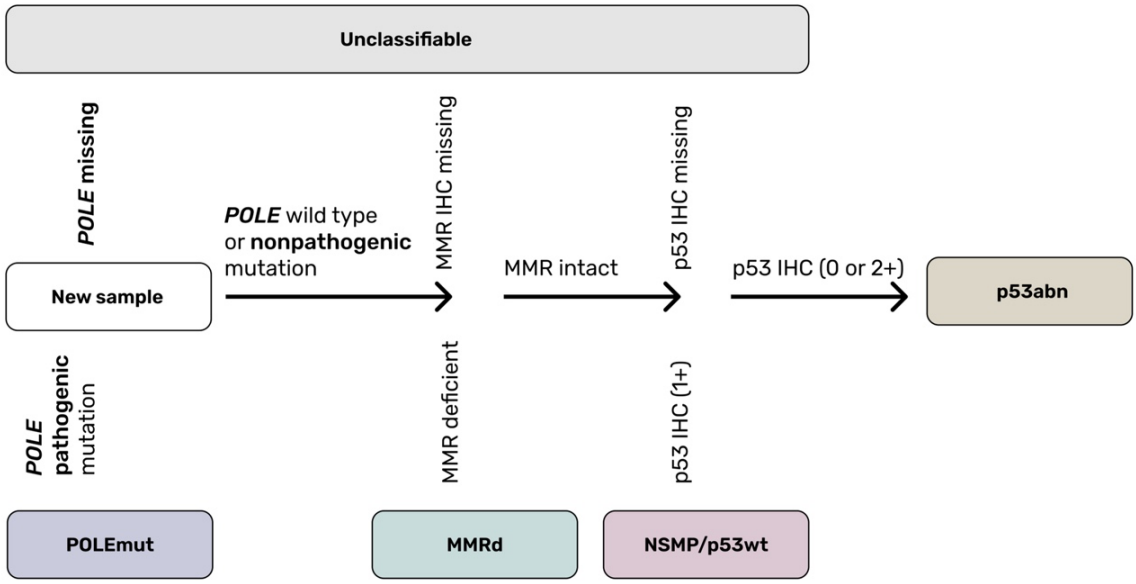
		NSMP	MMRd	POLEmut	NSMP clear cell carcinoma	p53abn
TYPE 1	Endometrioid Endometrial carcinoma Grade 1 to 2	>50%	10 to 50%	<10%		<10%
	Endometrioid Endometrial carcinoma Grade 3	10 to 50%	10 to 50%	10 to 50%		10 to 50%
TYPE 2	Clear cell carcinoma		<10%	<10%	>50%	10 to 50%
	Serous endometrial carcinoma	<10%	<10%	<10%		>50%

Modified from UpToDate Endometrial cancer: Pathology and classification by Huvila J, MD, PhD, McAlpine JN, MD, FACOG, FRCPSC, available from: URL: https://www.uptodate.com/contents/endometrial-cancer-pathology-and-classification) accessed 22 March 2022. NSMP: no specific molecular profile; MMRd: mismatch repair deficient; *POLE*: DNA polymerase epsilon; mut: mutation; abn: abnormal expression.

1.3.2 Molecular classification

The Cancer Genome Atlas has shifted the paradigm on EC classification by introducing a new molecular classification.[4] Although several EC risk groups had already been defined based on EC types with specific histological characteristics,[7] the molecular classification adds a new perspective and is currently being integrated into clinical practice.[7,14-19] At present, all diagnostic biopsies or material obtained during surgery in newly diagnosed EC should be classified using the new molecular classification as a matter of routine. The molecular classification is determined based on immunohistochemistry (IHC) analysis of p53, MSH6, and PMS2; and, most importantly, mutation analysis of the exonuclease domain of DNA polymerase epsilon (*POLE*) (Figure 3).[7,14,20,21] About 3% of all EC cases have two or more molecular characteristics and are therefore considered to be 'multiple-classifiers'.[22] Of note, histopathological typing is still an important tool for tumour classification and should still be done according to WHO standards.[13]

Figure 3. Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) molecular algorithm.[20-22]



POLE: DNA polymerase epsilon; mut: mutation; MMR: mismatch repair; IHC: immunohistochemistry; MMRd: mismatch repair deficient; NSMP: no specific molecular profile; wt: wild type; abn: abnormal expression; EC: endometrial cancer.

1.4 RISK FACTORS

Non-genetic features and medical conditions are well-studied in relation to EC. Hypertension, diabetes, and other diseases connected to redundant oestrogen levels, such as early menarche, late menopause, and high body mass index (BMI), are well-known risk factors for type 1 EC.[23] Due to the limited epidemiologic data, risk factors for type 2 EC are less known.

1.4.1 Hypertension

Hypertension seems to be associated with an increased risk of EC. The mechanism of influence has not been well-explored, but it might be connected to inhibition of apoptosis and promotion of cellular aging.

1.4.2 Diabetes mellitus

A meta-analysis found that diabetes mellitus increases the risk of EC, even after adjustment for obesity and physical activity. It was found that women with diabetes type 1 had a three-fold risk of EC, and women with diabetes type 2 had a two-fold risk. However, there were limitations, since the majority of the studies included did not distinguish between diabetes type 1 and 2.[24]

1.4.3 Hormones

Different hormones affect the risk of EC differently. Studies have found connections between the risk of EC and use of combined oral contraceptives, hormone replacement therapy, menarche, menopause, and parity.[25-27] Combined oral contraceptives decrease the long-term risk of EC. Indeed, combined oral contraceptives contain both oestrogen and progesterone, and progesterone may protect against EC by opposing oestrogen and down-regulation of the DNA synthesis in the endometrium.[25] Hormone replacement therapy usually includes oestrogen and progesterone in different regimens; a high dose of progesterone reduces the risk of EC, whereas a low dose can increased the risk.[28] Early menarche, late menopause, and nulliparity increase the risk of EC. Advanced age at last birth, not high number of births, has been reported to decrease the risk of EC.[27]

1.4.4 Obesity

Obesity (BMI $\geq$30kg/m^2) is the biggest risk factor for EC, and possibly the most important preventable risk factor for cancer.[29,30] More than 40% of EC cases are related to obesity. It has been hypothesised that adipose tissue contributes to a peripheral up-regulation of bio-available oestrogen, resulting in EC.[29]

1.5 PREOPERATIVE AND INTRAOPERATIVE WORK-UP

Abnormal uterine bleeding is the most common and significant symptom of EC and is found in up to 90% of cases.[31,32] Age and risk factors should always be considered before arriving at a diagnosis of EC. Abnormal cervical cytology is sometimes present in patients with EC, including the presence of endometrial or atypical glandular cells, or even adenocarcinoma.[33]

After physical and pelvic examination, blind endometrial biopsy is considered to be the most important primary diagnostic procedure, and is highly efficient when more than half of the endometrium is affected.[23] The sensitivity of the method is more than 90%.[34] Hysteroscopy with targeted biopsy is a more efficient way to identify lesions.

Magnetic resonance imaging (MRI) is crucial in evaluating myometrial invasion, cervical involvement, and lymph node metastasis.[35-76] Transvaginal ultrasound is also used to detect myometrial invasion in EC and has a performance rate similar to that of MRI.[38,43,50,77-82] However, in the case of preoperative use of transvaginal ultrasound, it should be performed by an experienced sonographer.[78] Positron emission tomography scanning is another sound method for preoperative assessment of lymph node metastasis in EC, but it has moderate sensitivity, likely because it takes few cancer cells to induce high levels of ^{18}F-fluoro-2-deoxy-D-glucose metabolism.[83-94] Computer tomography of the thorax, abdomen, and pelvis might also be used in evaluating ovarian, lymph node, peritoneal, or other metastatic forms of EC.[95,96]

Frozen EC sections are now considered to be less useful in clinical practice due to low reproducibility, correspondence with paraffin-embedded biopsies, and increased use of sentinel lymph node biopsies. Moreover, inappropriate fixation of the frozen material can lead to incorrect lymphovascular space invasion (LVSI) assessment.[7]

1.6 MANAGEMENT

1.6.1 Early-stage disease

All women with EC who are considered operable should undergo total hysterectomy with bilateral salpingo-oophorectomy without vaginal cuff resection, preferably using a minimally invasive surgical approach. Sentinel lymph node biopsy should be done in high-intermediate-risk/high-risk EC but might be not performed in those with low-risk/intermediate-risk disease.[7]

The European guidelines for the management of patients with EC strongly advocate for the use of their prognostic risk groups as an essential decision-making tool regarding adjuvant treatment (Figure 4).[7]

Figure 4. Definition of prognostic risk groups.[7]

Risk group	Molecular classification unknown	Molecular classification known*†
Low	► Stage IA endometrioid + low-grade‡ + LVSI negative or focal	► Stage I–II ***POLEmut*** endometrial carcinoma, no residual disease ► Stage IA **MMRd/NSMP** endometrioid carcinoma + low-grade‡ + LVSI negative or focal
Intermediate	► Stage IB endometrioid + low-grade‡ + LVSI negative or focal ► Stage IA endometrioid + high-grade‡ + LVSI negative or focal ► Stage IA non-endometrioid (serous, clear cell, undifferentiared carcinoma, carcinosarcoma, mixed) without myometrial invasion	► Stage IB **MMRd/NSMP** endometrioid carcinoma + low-grade‡ + LVSI negative or focal ► Stage IA **MMRd/NSMP** endometrioid carcinoma + high-grade‡ + LVSI negative or focal ► Stage IA **p53abn** and/or non-endometrioid (serous, clear cell, undifferentiated carcinoma, carcinosarcoma, mixed) without myometrial invasion
High–intermediate	► Stage I endometrioid + substantial LVSI regardless of grade and depth of invasion ► Stage IB endometrioid high-grade‡ regardless of LVSI status ► Stage II	► Stage I **MMRd/NSMP** endometrioid carcinoma + substantial LVSI regardless of grade and depth of invasion ► Stage IB **MMRd/NSMP** endometrioid carcinoma high-grade‡ regardless of LVSI status ► Stage II **MMRd/NSMP** endometrioid carcinoma
High	► Stage III–IVA with no residual disease ► Stage I–IVA non-endometrioid (serous, clear cell, undifferentiated carcinoma, carcinosarcoma, mixed) with myometrial invasion, and with no residual disease	► Stage III–IVA **MMRd/NSMP** endometrioid carcinoma with no residual disease ► Stage I–IVA **p53abn** endometrial carcinoma with myometrial invasion, with no residual disease ► Stage I–IVA **NSMP/MMRd** serous, undifferentiated carcinoma, carcinosarcoma with myometrial invasion, with no residual disease
Advanced metastatic	► Stage III–IVA with residual disease ► Stage IVB	► Stage III–IVA with residual disease of any molecular type ► Stage IVB of any molecular type

*For stage III–IVA *POLE*mut endometrial carcinoma and stage I–IVA MMRd or NSMP clear cell carcinoma with myometrial invasion, insufficient data are available to allocate these patients to a prognostic risk group in the molecular classification. Prospective registries are recommended.
†See text on how to assign double classifiers (eg, patients with both *POLE*mut and p53abn should be managed as *POLE*mut).
‡According to the binary FIGO grading, grade 1 and grade 2 carcinomas are considered as low-grade and grade 3 carcinomas are considered as high-grade.
LVSI, lymphovascular space invasion; MMRd, mismatch repair deficient; NSMP, non-specific molecular profile; p53abn, p53 abnormal; *POLE*mut, polymerase-mutated.

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1.6.2 Advanced disease

Stage III and IV EC are defined as advanced disease. In this case, a multidisciplinary approach is essential to increase the quality of care. Debulking surgery is necessary in cases where complete resection is possible, and the patient has an acceptable clinical profile. Neoadjuvant systematic therapy, external beam radiation therapy, and intrauterine brachytherapy might be also potential treatment options.[7]

1.6.3 Recurrent disease

Women with recurrent disease, including peritoneal and lymph node recurrence, are considered to be operable only if complete macroscopic resection of the tumour with acceptable morbidity is possible. Hormonal therapy, immunotherapy, chemotherapy, and radiotherapy can also be considered postoperatively based on the specific patient's profile.[7,

1.7 PROGNOSTIC FACTORS

Stage is a known prognostic factor in EC; indeed, survival rates differ dramatically between stages.[97] For example, more than 90% of patients with stage I EC are still alive 5 years after diagnosis, compared to around 20% of those with stage IV EC.[97] Involvement of the lower segment of the uterus, even in otherwise low-risk EC, might also increase the risk of lymph node metastasis.[98] Furthermore, the presence of LVSI has been shown to be a bad prognostic factor in a cohort of more than 1000 women with EC.[99] Distant recurrence was more common in women with diffuse LVSI than those with focal or no LVSI.

Despite the rapidly increasing data pool on the prognostic role of different EC characteristics, including molecular ones, knowledge gaps still exist. For example, the predictive role of specific proteins and metals, dietary habits and daily routines, and endometrioid microbiota in EC has not been well studied.

1.7.1 LRIG proteins

The LRIG family of integral surface proteins contains leucin-rich repeats, immunoglobulin-like domains, a transmembrane glycoprotein, and a cytoplasmic tail.[100] Interestingly, LRIG genes do not universally serve as tumour-suppressors or tumour-promoters of LRIG proteins. The LRIG1 protein is also called Lig-1 (3p14) and was first described 20 years ago. The protein is often absent in human cancers. Its expression is low in tumours of the skin, cervix, bladder, and lung, but it is upregulated in astrocytoma, prostate cancer, and lung carcinoid tumours.[101] LRIG2 (1p13) is similar to LRIG1 in terms of domain organisation. LRIG2 expression has been found in all human tissues. In contrast, in glioblastoma cells, LRIG3 (12q13) was set as a potent tumour suppressor.[102,103]

1.7.2 Cadmium

Cadmium is a cancerogenic heavy metal which is found in the environment.[104] Food items like vegetables and cereals are the most common source of cadmium in Sweden, because of significant cadmium concentrations in soil and the high consumption of these items.[105] Cadmium concentrations in food and consumption levels of these foods contribute to total dietary cadmium intake.[106] Tobacco use is another source of metal intake due to the high levels in tobacco products.[104,107]

Absorption of cadmium is only about 5%, but it is stored in the kidneys and liver and has a biological half-life of up to 30 years.[104] Low iron levels lead to higher cadmium absorption; therefore, cadmium levels are often high in women.[108] The metal also affects kidneys and bone, has mutagenic effects, and increases oestrogen receptor a-mediated cell proliferation.[109-113] Therefore, it has an oestrogenic effect: it increases uterus weight, hyperplasia, hypertrophy of endometrial lining, etc.[114]

1.7.3 Dietary factors

A healthy diet can help one maintain health, but a poor diet has been associated with some cancers. Many epidemiological studies have explored dietary habits and specific nutrients in EC. However, no studies have evaluated the influence of food and its specific components on EC outcomes.

Swedish consumers are commonly considered to be health-oriented, socially conscious, and environmentally responsible. The limitations of existing studies are connected to the difficulties in distinguishing which factors are the most influential on EC outcome. A cohort study of Swedish women found no connections between certain types of food and EC.[115]

Fried or roasted starch-rich foods contain high levels of acrylamide, which is considered 'probably carcinogenic' to humans.[116,117] In addition, beverages with high levels of sugar increase insulin levels, which inhibit the production of sex hormone-binding globulin, which binds to circulating sex steroids. This phenomenon is known to be related to type 1 EC.[29,118,119] Even though the relationship between many food items and EC is well studied, the prognostic role of these items remains unclear.

1.7.4 Daily routines

Because of the various connections between lifestyle factors and EC, many studies have investigated whether lifestyle could have an impact on EC prognosis. In the USA, lifestyle factors in relation to EC were studied at three different hospitals. The results showed that obesity, but not dietary habits or physical activity, was an important factor influencing cancer stage. However, age was found to be a major confounder; therefore, no definite conclusions could be drawn.[120]

As previously mentioned, diabetes increases the risk of EC, and those with diabetes may also have a higher risk of mortality from EC. [24,121] However one study on the prognosis of diabetic women with EC who were taking Metformin, found that patients using Metformin had a higher EC survival rate, even compared to women without diabetes.[122] This led to further studies investigating whether Metformin could have a role in the treatment of EC. One study found that Metformin reduced the risk of recurrence in obese women with type 1 EC.[123]

Obesity is a negative prognostic factor for women with EC, and has been suggested to increase the risk of death in all types of cancer, with an estimated one in five cancer deaths in women caused by obesity, and the risk of death being much higher in women with a BMI ≥40 than those with a BMI <25.[124] Another study examining survival in relation to BMI in patients with EC found that higher BMI was associated with a higher risk of mortality from EC, whereas other studies have shown no connection between BMI and survival in stage II EC.[125,126]

The relationship between parity and EC prognosis has not been investigated. One study assessed the relationship between parity and EC subtype, and found that number of children had no effect on EC incidence, but seemed to be associated with age at EC diagnosis.[127]

1.7.5 Endometrial microbiota

The uterine cavity was long considered to be sterile, but it is now known that vaginal microbiota play an essential role in the female reproductive system.[128] Recent studies have suggested that the upper parts of the reproductive tract, which were also considered sterile, such as the uterine cavity, fallopian tubes, and the recto-uterine hole, may contain resident microorganisms.[129,130] The researchers concluded this due to the development of advanced genome sequencing methods, which detect bacterial genomes in zones with a low microbial burden. Microorganisms found in those organs are not inactive; instead, they interact with the surrounding tissues and produce cytokines, signalling molecules, and enzymes.[131]

Infection is recognised as a major cause of cancer worldwide, with approximately 16% of cancer cases can be attributed to infection.[132,133] Dysbiosis of the endometrial microbiota, together with a rise in pro-inflammatory cytokines, had also been reported in patients with EC.[134] Apparently, *Micrococcus* was more abundant in patients with EC, compared to those with benign lesions. In contrast, *Pseudoramibacter Eubacterium, Rhodobacter, Vogesella,*

Bilophila, Rheinheimera, and *Megamonas* were increased in women with benign lesions. In short, diminished microbial diversity was observed in patients with EC.

2 AIMS

The overall aim of the project was to evaluate the impact of different factors on the prognosis of women with EC, with a special focus on the LRIG family of proteins, cadmium, dietary factors, daily routines, and the endometrial microbiome. We intended to identify patient-specific prognostic factors for EC and explore them.

Study I

To analyse the role of LRIG1, LRIG2, and LRIG3 proteins on survival rates in women with EC.

Study II

To assess the prognostic role of cadmium intake in women with EC residing in Sweden.

Study III

To evaluate the prognostic role of modifiable factors, such as dietary habits and daily routines, in women with EC, using novel machine learning models.

Study IV

To investigate the role of endometrial microbiota in women with EC and compare it to the bacterial profile of women with benign conditions of the gynaecological tract.

3 MATERIALS AND METHODS

3.1 STUDY I

3.1.1 Study sample

One hundred consecutive women who had surgical treatment due to EC at the Karolinska University Hospital (Solna, Sweden) between 2007 and 2012 were eligible to participate in Study I. Twenty-five were excluded due to insufficient biological material, leaving 75 women in the final analytical sample.

3.1.2 Biological samples

EC biopsies were obtained during surgery, dehydrated, and embedded in paraffin using validated protocols. Biopsies were evaluated by a pathologist after undergoing staining with haematoxylin and eosin.

3.1.3 Immunohistochemistry

The expression of LRIG1, LRIG2, and LRIG3 proteins were assessed using an IHC method with polyclonal antibodies. All stained material was also independently evaluated by two senior pathologists using a light microscope. These pathologists had no knowledge of disease outcome in the study sample. Staining intensity was analysed using a four-grade semi-quantitative scale (no staining, weak, moderate, or strong intensity) and then grouped into no/weak and moderate/strong in statistical analyses. Percentage of positive cells was categorised as at or above median (referred as high) or below median (referred as low), to create relatively equal groups.

3.1.4 Medical records

All medical records were assessed electronically through Take Care, an e-health software used in Stockholm County. Detailed medical history and data on histopathological analysis were taken from the system following all local regulations.

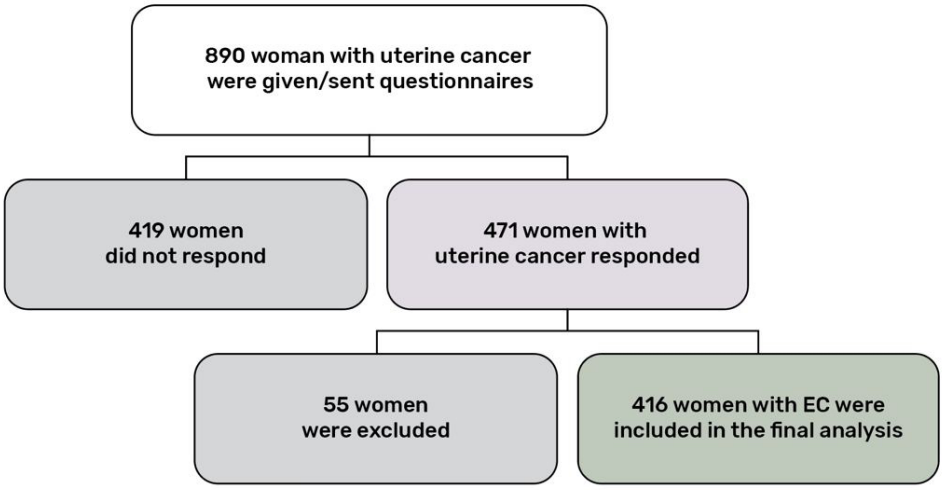
3.2 STUDIES II AND III

3.2.1 Cohort

Eight hundred ninety women treated for EC at the Karolinska University Hospital (Solna, Sweden) were invited to participate in Studies II and III; 471 (53%) agreed to join and provided written informed consent.

Patients treated outside Stockholm County, patients diagnosed with cancer other than EC, patients admitted due to EC recurrence after the study began, and patients who did not complete the questionnaires correctly, were excluded from the final analysis. As a result, 416 women were included in the final analytical sample (Figure 5).

Figure 5. Flowchart of the patient cohort.



EC: endometrial cancer.

All women in the study sample underwent hysterectomy following the collection of EC samples, which were evaluated by a pathologist in accordance with standard protocols using IHC. According to local and international guidelines, some patients received adjuvant treatment following surgery. All data on diagnostics, treatment, and follow-up were collected from the Take Care system.

3.2.2 Questionnaires

Two questionnaires created by Terry et al. (Institute of Environmental Medicine, Karolinska Institutet) and based on internationally accepted and validated questionnaires, were used to collect data. Both questionnaires and an invitation letter were sent to all women included in

the study. The first questionnaire covered dietary habits in the form of a food frequency questionnaire, as well as daily activities, and consisted of eight different modules. The second questionnaire covered a range of questions on physical parameters, health-related issues, etc. Dietary habits and other lifestyle aspects like alcohol consumption and smoking were analysed.

3.2.3 Assessment of cadmium intake

Cadmium intake from food (i.e., dietary cadmium intake, μg cadmium per day) was calculated for each patient individually, as frequency of consumption of different food items × average daily consumption supplied by the Nutritional Epidemiology Unit at Karolinska Institutet. Cadmium intake from the air and water are statistically insignificant and therefore were not taken into account in our calculations.[135]

The cadmium levels in the food items mentioned in the questionnaire were extracted from the most recent reports of the National Food Agency (Uppsala, Sweden).[105,136-145] If several concentrations were presents for one food item, the concentrations were averaged. Reports from Denmark had to be used for several food items.[146]

Cadmium intake from tobacco products was analysed separately, calculated by multiplying cigarette/snuff, mean number of cigarettes/snuff patches used, and mean number of years of tobacco use.[107,147]

3.3 STUDY IV

3.3.1 Study sample

Sixty women admitted to the Almazov National Medical Research Centre (St. Petersburg, Russian Federation) between June 2020 and December 2021 with abnormal postmenopausal uterine bleeding or endometrial thickening, validated by transvaginal ultrasound, were invited to participate in Study IV.

3.3.2 Biological samples

Biological samples were collected in the operating theatre. A swab from the upper vaginal tract was taken followed by routine preoperative vaginal preparation. Samples from the endometrial cavity were taken using the Tao brush Endometrial SamplerTM (Cook Medical, Bloomington, Indiana, USA), a single-use tool covered by a sheath, which protects the specimen from cross-contamination from the cervix and vagina.[148] All samples were placed in sterile Falcon tubes and stored at -80^{O}C until final histopathological examination.

3.3.3 Next-generation sequencing

Reversible-terminator sequencing-by-synthesis was utilised on the MiSeq System, Illumina's next-generation sequencing instrument. It uses reversible-terminator sequencing-by-synthesis technology to provide end-to-end sequencing solutions. Onboard cluster generation, amplification, genomic DNA sequencing, and data analysis – including base calling, alignment, and variant calling in a single run – was used. Sequencing facility and bioinformatics staff processed deidentified data amplicon sequence variants, which are better than convenient operational taxonomic units.[149]

3.4 STATISTICAL ANALYSIS

3.4.1 Study I

Central tendency was reported using median and interquartile range due to a mostly non-normal distribution. Pearson $\chi 2$ or Fisher's exact test were used to identify differences within categorical data, and the Mann Whitney U test/Kruskal-Wallis test was used to compare the distribution of continuous variables. The time-to-event was set at EC recurrence, all-cause death, or 1st of April 2020, whichever occurred first. Progression-free survival (PFS) was defined as the period between EC diagnosis and recurrence, and overall survival (OS) as the period between EC diagnosis and all-cause death. Kaplan-Meier curves were used to demonstrate the probability of the event over time, and the log-rank test was used to compare survival distribution. Variables that differed significantly in the univariate analysis were included in the Cox regression multivariate analysis. The significance level was set at 0.05. IBM SPSS 27.0 (IBM Corp., Armonk, NY, USA) and RStudio 1.3.1093 were used, running on Mac OS.

3.4.2 Study II

The cohort was described using descriptive statistics. Cox proportional hazard models were used for the statistical analyses. All women were considered at risk of recurrence or death from the time of EC diagnosis. The time-to-event was set at EC recurrence, all-cause death, or 1st of February 2019, whichever occurred first.

Crude and adjusted hazard ratios were estimated by tertiles of daily dietary cadmium intake, with the lowest tertile being the reference group. All individual characteristics were assessed as potential effect modifiers by including them into the model and running a Wald test. The significance level was set at 0.05. IBM SPSS 27.0 (IBM Corp., Armonk, NY, USA) and RStudio 1.3.1093 were used, running on Mac OS.

3.4.3 Study III

Median and interquartile range were used to describe central tendency due to the predominantly non-normal distribution of the continuous variables. The Mann-Whitney U test was used to compare continuous variables between unpaired samples, while the $\chi 2$ test was used for categorical variables. A Cox proportional hazards model was run to evaluate the association between recurrence or survival and several predictor variables.

Random Survival Forest (RSF, an extension of Breiman's learning method for right-censored data), was used for time-to-event (recurrence or death) analysis.[150] ForestSRC and the ggRandomForest packages were utilised for additional visualisation of the data.[151,152] The RandomForestSRC package handled missing values using *adaptive tree imputation*. The significance level was set at 0.05. RStudio 1.2 and Anaconda were used, running on Mac OS.[153,154]

3.4.4 Study IV

Due to predominantly non-normal distribution, we performed univariate analyses using the non-parametric tests. The Mann-Whitney-Wilcoxon test was used to compare the distribution of quantitative variables, while Fisher's exact test was used for qualitative parameters.

After demultiplexing and splitting the samples into individual fastq files, paired-end sequencing data was processed using the dada2 and phyloseq packages, since they infer amplicon sequence variants instead of less accurate, conventional, operational taxonomic units.[155,156]

3.5 ETHICAL APPROVAL

The studies were conducted according to the guidelines of the Declaration of Helsinki. Participation was voluntary, and all participants received comprehensive information about the study and a signed written informed consent form. We anonymised all patient data following data collection.

Studies I, II, and III were approved by the ethical review board at Karolinska Institutet (protocol codes 2006/649, 20th of June and 2010/1536-31/2, 22nd of August 2011). Study IV was approved by the ethical review board at Almazov National Medical Research Centre (protocol code #0306-20, 15th of June 2020).

4 RESULTS

4.1 STUDY I

Median age in our final analytical sample (n=75) was 68.0 years. The women were generally overweight, with a mean BMI of 27.1 kg/m. Thirty-two received radiotherapy, and 22 received chemotherapy as an adjuvant treatment. Fourteen out of 51 women with type 1 EC, and 23 out of 24 women with type 2 EC had adjuvant treatment.

Among over 630 person-years of follow-up, 14 patients had recurrence and 23 died. Median age at diagnosis was higher in patients who died of EC (P=0.003), but BMI and parity were not. Around 50% of the patients used hormone replacement therapy, and only about 10% had diabetes. Recurrence was associated with grade (P=0.306, P=0.008), stage (P=0.538, P<0.0001), and depth of invasion (P=0.301, P=0.009). Mortality rate was correlated with grade and stage, but not with depth of invasion (P=0.080, P=0.497). Expression of progesterone receptors and oestrogen receptors was higher in patients without recurrence and in those who died.

LRIG1 protein score was high in most patients (97.3%). LRIG3 was underexpressed in type 2 EC (Table 1).

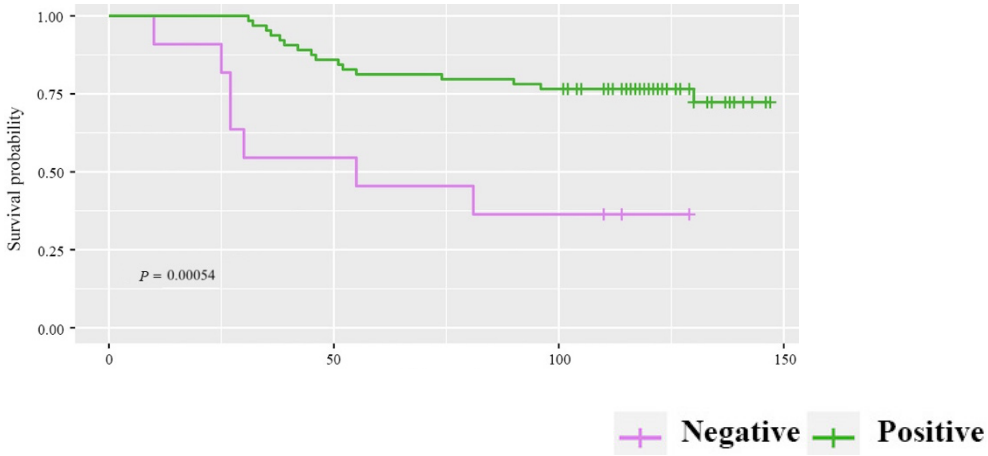
Table 1. LRIG proteins immunohistochemical staining (n=75).

Protein	Protein score*	N (%)	Type 1	Type 2
LRIG1	0	0	0	0
	1	0	0	0
	2	2 (2.7)	1 (2)	1 (4.2)
	3	73 (97.3)	50 (98)	23 (95.8)
LRIG2	0	1 (1.3)	1 (2)	0
	1	34 (45.3)	24 (47.1)	10 (41.7)
	2	22 (29.3)	13 (25.5)	9 (37.5)
	3	18 (24.0)	13 (25.5)	5 (20.8)
LRIG3	0	4 (5.3)	1 (2)	3 (12.5)
	1	29 (38.7)	16 (31.4)	13 (54.2)
	2	25 (33.3)	18 (35.3)	7 (29.2)
	3	17 (22.7)	16 (31.4)	1 (4.2)

*Protein score: 0 = 0%, 1 = 1-25%, 2 = 26-50%, 3 >50% of positive cells.

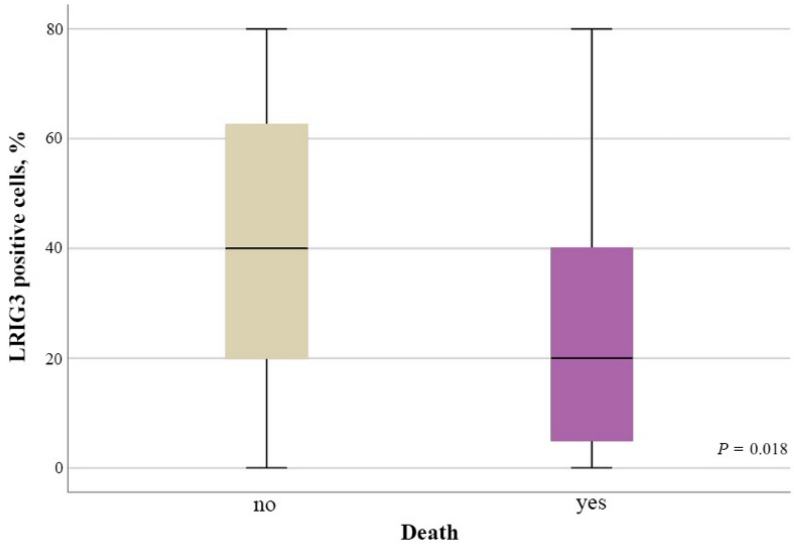
There was no LRIG3 cell membrane staining in 30.4% of women who died, compared to 7.7% of EC survivors (*P*=0.01) (Figure 6). LRIG3 cell membrane staining was correlated with a lower mortality risk (*P*=0.01), which remained after adjustment for age and BMI (*P*=0.003). Conversely, LRIG3 staining in the nucleus and cytoplasm was relatively equal.

Figure 6. LRIG3-positive staining of the cell membrane in women who died and in endometrial cancer survivors.



Percentage of LRIG3-positive cells was positively associated with better OS (*P*=0.019), and negatively associated with ploidy (*P*=0.016) (Figure 7). This percentage was also lower in high-grade EC (*P*=0.009).

Figure 7. Percentage of LRIG3-positive cells in women who died and in endometrial cancer survivors.

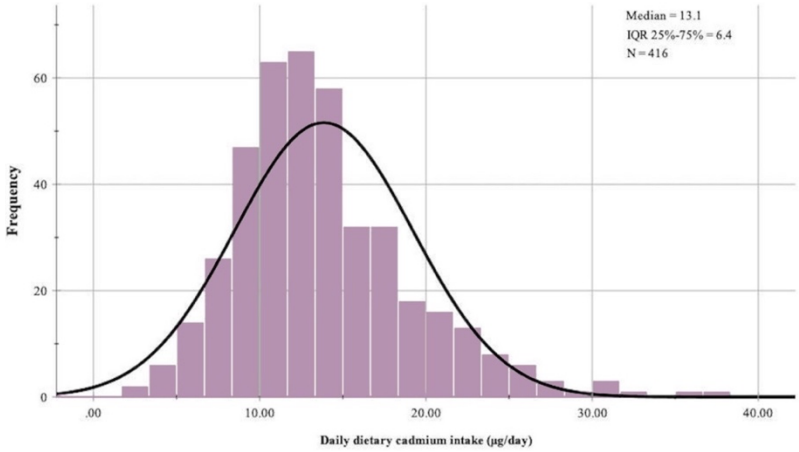


No association between LRIG1- and LRIG2-positive cells and PFS or OS was observed.

4.2 STUDY II

In the sample of 416 women with EC, median dietary cadmium intake was 13.1 µg/day, with a respective median dietary cadmium intake per week of 1.3 µg/kg of body weight (Figure 8). Interestingly, grain products and vegetables were the most critical source of cadmium, accounting for about 75% of total intake.

Figure 8. Dietary cadmium intake in the study sample (µg/day).



Median age at EC diagnosis and BMI were 67.0 years and 26.0 kg/m^2, respectively. The most common tumour type was early-stage diploid endometrioid carcinoma. Current and former users of tobacco products had a similar median cadmium exposure (Table 2).

Table 2. Cadmium intake from tobacco (µg/day).

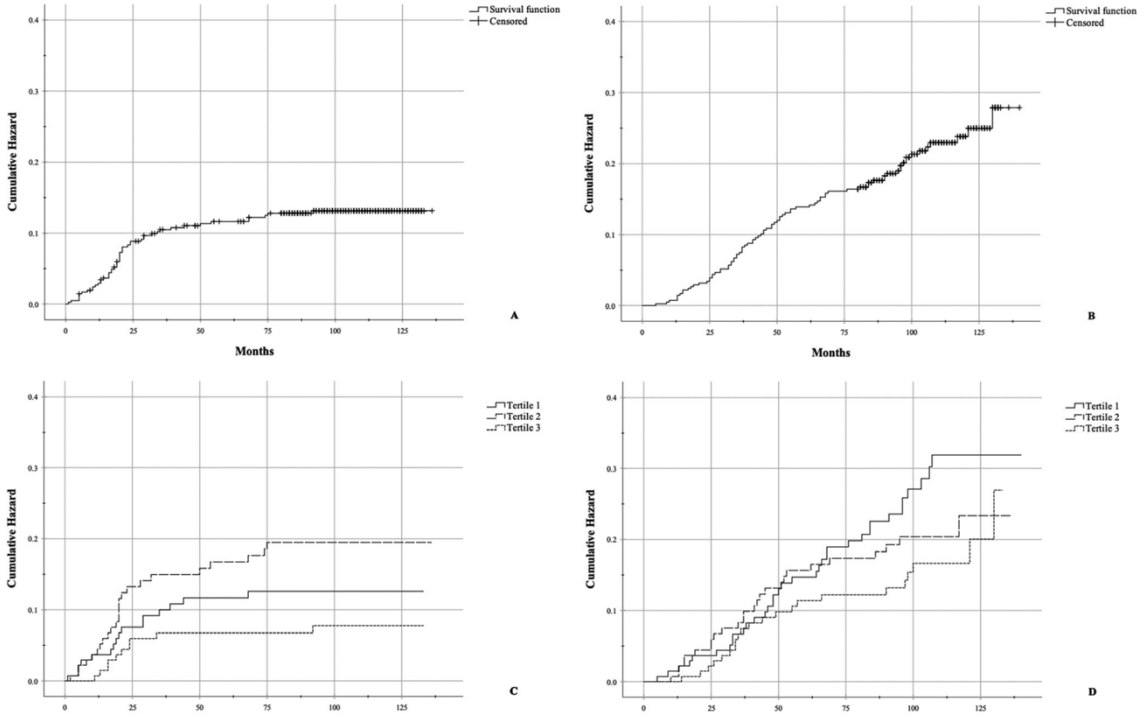
Type of tobacco	Use	Number of women	Daily cadmium intake (mg/day)
Cigarettes	Current	16	13.1
	Former	158	12.7
Snuff	Current	8	13.3
	Former	3	n.a.*

*Snuff intake was not reported in the group, n.a.: not applicable.

Median follow-up was more than 8 years. Dietary cadmium intake was correlated with worse OS (P=0.05) (Figure 9). Notably, physical activity reduced the probability of death (P<0.0001). At the same time, dietary cadmium intake was not correlated with PFS (P=0.348), but PFS events varied across tertiles (12.9%, 18.1%, and 7.9%). Patients with a

dietary cadmium intake corresponding to tertile 2 had more PFS events than those in tertiles 1 and 3 (P=0.031).

Figure 9. Progression free survival (A and C) and overall survival (B and D) in the whole cohort (A and B) and in each tertile of median dietary cadmium intake (μg/day).



Patients with type 2 EC had a lower PFS (P<0.0001) and more advanced-stage disease (P<0.0001). Tobacco users (former and current) did not have better or worse PFS and OS (P=0.945, P=0.363).

Educational level, use of tobacco products, BMI, leisure time, and physical activity had no significant influence with regard to tertiles of dietary cadmium intake.

4.3 STUDY III

By the end of follow-up, 54 women had recurrence (13.0%, median time-to-recurrence: 20 months) and 83 had died (20.0%, median OS: 45 months). As expected, the higher PFS and OS rates were associated with advanced stage, non-endometrioid histological type, poor differentiation, and aneuploidy. Median age was higher among women who had recurrence or died.

The PFS model included all 186 variables. Recurrence and all-cause death were indicated as the major outcomes, while time-to-recurrence and OS were labelled as time-to-event characteristics. Death and OS period were not analysed in the model with recurrence labelled as the outcome (and *vice versa*), to ensure that the model was not skewed towards these significant variables. We then calculated variable important measures predictive (VIMP) and minimal depth for all variables and ranked the top 10 in descending order.

Several of the variables had high rankings according to both VIMP and minimal depth. For recurrence, these included stage, ploidy, tumour type, prunes, and fried potatoes; while for survival they included age, stage, tumour type, ploidy, sugar-sweetened beverages, physical activity, and age at cessation of alcohol intake. As age and tumour features have a well-described modifying effect on PFS and OS, something that was observed also in the exploratory analysis, it was decided to concentrate on the variables related to nutrition, alcohol intake, and physical activity.

The Cox proportional hazards model revealed that prune consumption was not connected with EC recurrence or death rates, but each additional serving of fried potatoes increased both of these risks. These hazards persisted after adjustment for BMI, age, and smoking status. An additional serving of sugar-sweetened beverages increased the risk of death, which persisted after adjustment for confounding variables. In contrast, physical activity decreased the risk of death. More precisely, the risk of death was reduced by 7.3%, with each additional unit of metabolic equivalent (MET/day) of physical activity. The beneficial effect of physical activity persisted even after adjustment for age and BMI, but not stage (Table 3).

Table 3. Hazard ratios (HRs) and 95% confidence intervals (CIs) of recurrence or death according to selected dietary habits and physical activity.

		Unadjusted		Adjusted*	
Covariate		**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Fried potatoes	Death	8.62 (2.22-33.56)	0.002	5.13 (1.35-19.57)	0.017
	Recurrence	6.00 (1.06-34.01)	0.043	4.55 (0.88-23.48)	0.07
Sugar-sweetened beverages	Death	3.26 (1.83-5.80)	<0.0001	2.20 (1.13-4.27)	0.02
	Recurrence	2.24 (0.98-5.12)	0.055	1.99 (0.85-4.64)	0.113
Physical activity	Death	0.93 (0.89-0.96)	<0.0001	0.95 (0.91-0.99)	0.022
	Recurrence	0.97 (0.92-1.10)	0.183	0.98 (0.92-1.03)	0.441

* for patient age and body mass index.

Survival decreased from never drinkers to cessation at age 71-99 years, while eventful, continuous drinkers had a better prognosis. The amount of alcohol consumed did not significantly modulate the risk of EC recurrence or death (P=0.527 and P=0.137, respectively).

Consumption of fried potatoes was associated with that of bologna sausage and bacon (P<0.001 and P<0.001, respectively). At the same time, consumption of sugar-sweetened beverages was correlated with that of jam and white bread (P<0.001 and P<0.001, respectively).

4.4 STUDY IV

Of the 54 patients included in the final analysis, 27 had EC at final histopathological examination, while the other half had benign lesions (endometrial hyperplasia, EH; endometrial polyp, EP). Most women in the EC group and the EH/EP group were postmenopausal and had a BMI above the normal range. The median age in the EC group was 10 years higher than in the EH/EP group (Table 4).

Table 4. Background characteristics of the study population.

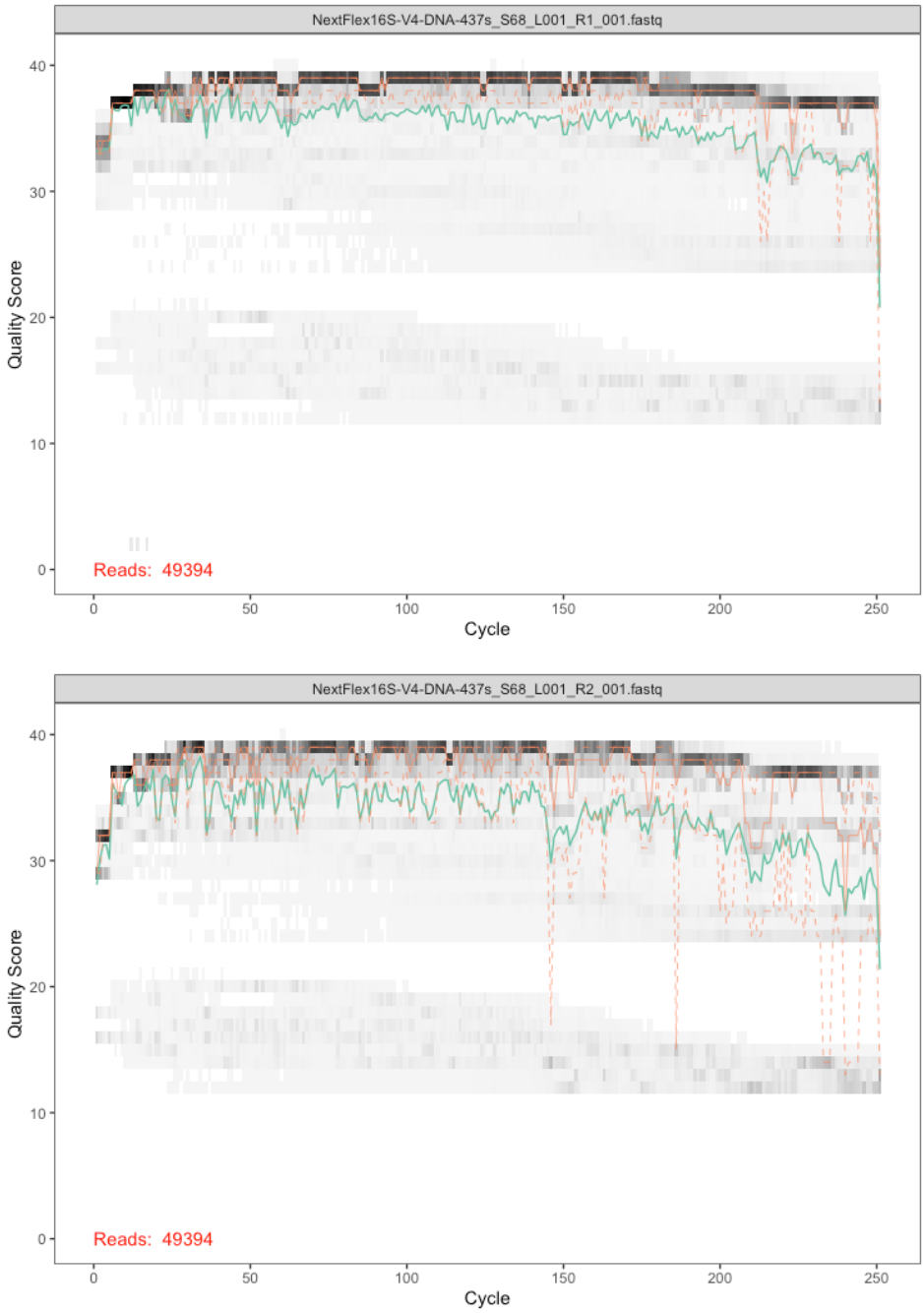
Characteristic	EC group (n=28)	EH/EP group (n=28)	*P*-value
Age (years)	65.00 (59.00-69.00)	55.00 (45.50-60.00)	0.002
Body mass index	32.80 (27.45-39.50)	29.00 (25.25-32.70)	0.06
Menopause (%)	88.89	59.26	0.01
Number of pregnancies	2.00 (2.00-4.00)	3.00 (1.00-4.00)	0.89
Number of childbirths	1.00 (1.00-2.00)	1.00 (0.5-2.00)	0.07

EC: endometrial cancer; EH: endometrial hyperplasia; EP: endometrial polyp.

The vast majority of tumours were endometrioid; only one was serous. Stage and grade were assessed by senior pathologists according to the classification of the International Federation of Gynaecology and Obstetrics, and were as follows: IA – 14, IB – 6, II – 3, IIIA – 1, IIIC1 – 1, IIIC2 – 1, IVB – 1; grade 1 – 1, grade 2 – 6, grade 3 – 10.

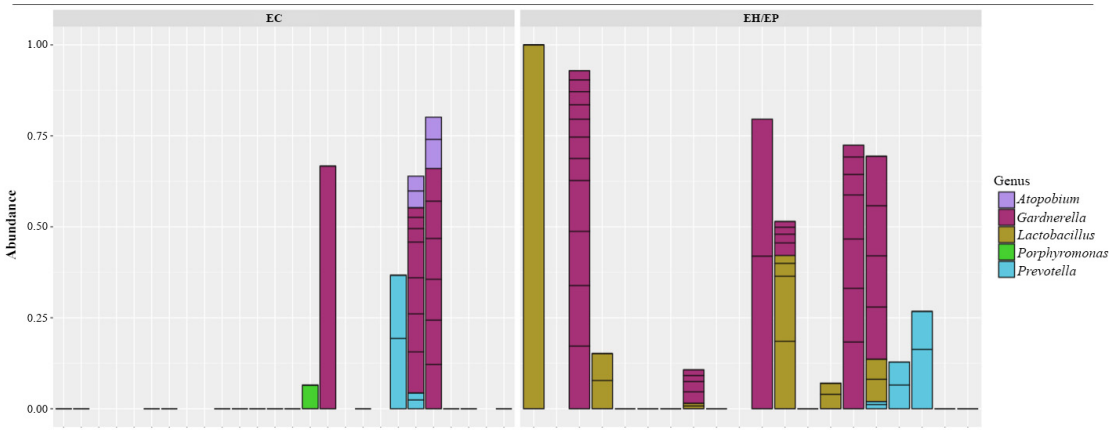
The inspection of the read quality profiles were initiated to adjust trimming parameters (Figure 10).

Figure 10. Quality profiles for forward and reverse reads.



After filtering and trimming, the core sample inference algorithm was applied, and the taxonomy was assigned using the Silva database.[157] Rickettsiales/mitochondria was filtered out, since it is highly probable, according to Silva, that these are mitochondrial sequences, not bacteria. The preliminary analysis showed that patients with EC have endometrial microbiota distinct from that of patients with EH/EP: *Atopobium* and *Porphyromonas* was present in patients with EC, while *Lactobacillus* was present in those with EH/EP (Figure 11).

Figure 11. Microbial composition in the two groups.



EC: endometrial cancer; EH: endometrial hyperplasia; EP: endometrial polyp.

5 DISCUSSION

5.1 STUDY I

Study I aimed to add new information on the molecular features of EC by analysing the expression of LRIG proteins as well as their prognostic role. Most patients in the cohort had a protein score of 3 for LRIG1. There was also a positive association between the number of LRIG3-positive cells and better OS, and an association between the number of LRIG3-positive cells and reduced morality risk. Low LRIG3 expression was associated with aneuploidy and higher-grade tumours. No correlation was found between expression of the two proteins and PFS or OS.

The LRIG proteins are responsible for signalling growth factor, and are found in many tissues in human body.[101-103,158] Notably, their respective genes are often deleted in human cancers.[159] LRIG1 and LRIG3 act as tumour suppressors, but LRIG2 acts as a tumour promoter.[160-166] The prognostic role of these proteins in a number of gynaecological tumours has already been published, but no information was available on their role in EC at the time of Study I.[162,165,167-170]

Although LRIG1 is a tumour suppressor, we observed no association with survival rates in our cohort. It is assumed that a bigger cohort might bring some new information, considering the notably high expression and the intensity of the protein we found in Study I.

LRIG2 was found to be significantly downregulated in endometrial adenocarcinoma cell lines.[171] However, no correlation between its expression and EC prognosis was found in our study. This might be partly due to a different cellular composition and tumour microenvironment, as well as our sample size.

LRIG3 expression played a positive prognostic role in EC according to the results of Study I. This might be due to cell cycle arrest and the induction of apoptosis.[172,173] Moreover, it was reported that LRIG3 has a negative control of the ERK pathway, which is often upregulated in human tumors.[166,174,175]

LRIG3 cell membrane expression was related to a lower mortality risk. This might be important, as existing knowledge points to the dominant expression of LRIG3 in cytosol, even though it is a transmembrane glycoprotein.[100]

The long follow-up time was a strength of this study, but the semi-quantitative analysis of the IHC samples is a potential source of bias.

5.2 STUDY II

Study II aimed to assess the role of oestrogen-mimicking metals on survival rates of patients with EC by specifically analysing the prognostic role of cadmium. High dietary cadmium intake was positively correlated with lower death events, but not recurrence, in women with EC. Median dietary cadmium intake was 13.1 µg/day and 1.3 µg/day of body weight, which is in accordance with previously published data from the same region.bonatti[105,106]

The Panel on Contaminants in the Food Chain has proposed a bearable cadmium intake of 2.5 µg/day of body weight, which is in accordance with our results and indicates that women in the cohort benefited from well-organised consumer protections.[176] According to Swedish law, women are generally not considered a vulnerable group in cadmium intake.[177] Hence, the study's results could be extrapolated to regions with regulations similar to those in Sweden. The prognostic role of the cadmium in hormone-related malignancies like EC in regions with high cadmium exposure needs to be investigated.

The most highest dietary cadmium intake was observed for cereals and vegetables, as described by the European Food Safety Authority.[176] Interestingly, high cadmium intake is mainly connected to food items that are usually considered healthy, but we found that this high intake resulted in a higher risk of death due to EC. It would be interesting to perform similar studies in areas with lower cadmium pollution.

Patients with type 2 EC and advanced-stage disease had lower survival rates. At the same time, vigorous physical activity was correlated with higher survival rates, in agreement with previous studies that report the beneficial role of regular physical activity in patients with different types of cancer.[178,179]

The present study is the first to explore the prognostic role of cadmium in patients with EC. Previous studies have looked at cadmium as a risk factor. A meta-analysis of eight studies assessed the association between dietary cadmium intake and overall cancer risk, and found no correlation.[180] However, subgroup analyses (study design, geographic location, and cancer type) revealed a positive association between dietary cadmium intake and cancer risk, especially in hormone-associated tumours, in studies from high-income countries. Another

study described no correlation between dietary cadmium intake and epithelial ovarian cancer risk.[181] This might be due to aetiological factors in ovarian cancer, which does not include oestrogen. As a result, it might still be important in EC.[182-184]

The strengths of the study are the sample size, consideration of individual clinical and pathological parameters, and a specially formed database on dietary cadmium intake based on the most recent data. Another study strength is the long-term follow-up, with a median of 102.0 months. Limitations include the retrospective nature of the study and potential errors due to self-reporting. Moreover, current guidelines recommend that metal levels be assessed in blood and urine, but none of those samples was used to validate cadmium levels in our study. Nevertheless, Julin et al. has validated the reliability between dietary cadmium intake and cadmium concentrations in urine and blood.[185-187]

5.3 STUDY III

Study III aimed to evaluate the prognostic role of dietary habits and daily routines in patients with EC using RSF, a machine learning approach. Consumption of fried potatoes and sugar-sweetened beverages, which may indicate specific dietary habits, were associated with a higher risk of increased survival rated in women with EC. At the same time, physical activity decreased the risk of death. Therefore, dietary habits and daily routines seem to impact the risk of recurrence and death in patients with EC.

Many studies have focused on different food items as potential risk factors for EC recurrence. However, this study focused on women who had already received or were undergoing treatment for EC. Therefore, the effects might be different from those described in other studies. It is essential to highlight that the results should be interpreted with caution, since each variable is an integral part of a complex system of dietary habits and daily activities. Mutually correlating elements can be combined into clusters that reflect an individual's existing nutritional preferences, which was also confirmed in the study. As a result, it is essential to look at the results more holistically and consider the existing relationships.

Starch-rich food items, especially when they are fried or roasted, have high acrylamide concentrations, and acrylamide has been known to be potentially carcinogenic since the 1990s.[116,117] The negative effect of acrylamide, vinyl monomer, was mostly explored in animal models. However, no observational studies have shown a direct causal link between

dietary acrylamide and human malignancy. The United States Food and Drug Administration demonstrated that acrylamide is present in many products, but it has slightly decreased in food items.[188] In Sweden, the average acrylamide intake is 25-40 μg/day per person and does not reach the indicated toxic level.[189]

Acrylamide may influence endocrine processes in the human body even in minor doses.[190-192] For example, glycidamide, acrylamide's metabolite, was reported to cause uterine adenocarcinoma in rats.[193] The monomer is also associated with high oestradiol levels in perimenopausal and postmenopausal women.[194] Moreover, acrylamide intake is associated with a slightly higher risk of EC, which was linear and more prominent in never smokers.[189] In this study, the risk or death remained even after adjusting for smoking status. No association was found between boiled and mashed potatoes, which is in line with the fact that those cooking methods have lower levels of acrylamide.

The intake of sugar-sweetened beverages was associated with a higher risk of death in women with EC. However, with their lower sugar levels or artificial sweeteners, light soda drinks did not affect survival rates. This might be because light soda drinks cause a rise in insulin that might exert antiapoptotic and mitogenic properties by increasing blood levels of insulin growth factor-1, which can inhibit the production of sex hormone-binding globulin, which binds to circulating sex steroids.[29,118,119,195-198] Lack of sex hormone-binding globulin increases bioactive free oestrogen levels, promoting endometrium proliferation.[119] The intake of sugar-sweetened beverages was also found to be positively associated with a higher risk of type 1 EC in 23,039 postmenopausal women.[199] Therefore, the results of Study III on the intake of sugar-sweetened beverages agree with already available data. However, it is essential to highlight that the study designs and sugar levels in sugar-sweetened beverages might differ. Moreover, OS analyses in this study included women who died from any cause. Besides, the intake of sugar-sweetened beverages might cause other conditions like metabolic syndrome and increase mortality but in a different way.

Physical activity is well-known to decrease the risk of many tumours, including EC.[208] For example, two studies reported that active women have 20-40% lower risk of EC than non-active women.[200,201] In this study, physical activity decreased the risk of death, but not recurrence. This might be due to normalisation of the production of insulin, lower levels of inflammatory markers and reactive oxygen species, immune system modulation, and weight reduction.[202-204]

No conclusion on how the cessation of alcohol intake affects survival rates in women with EC could be made, since alcohol intake distribution wasn't equal in the cohort, and therefore, no definitive effect of alcohol cessation on EC survival rates could be described. However, the evaluation of average alcohol intake did not show any difference in the outcomes. More alcohol-related parameters should be set to evaluate any hidden patterns.

The study strengths include a relatively large cohort, extended follow-up, and a high number of evaluated variables. But even though RSF is an effective method to analyse high-dimensional datasets with limited survival data, some confounding that was not measured might have influenced the results. At the same time, studies that aim to conduct a comprehensive assessment of dietary habits and daily routines must inevitably be conducted using extensive questionnaires, the intricacy and length of which could contribute to a relatively high drop-out rate. Furthermore, the severity of EC might influence a patient's desire to participate.[205] The retrospective study of dietary habits also carries the internal risk of recall bias. Patients did not have any time limit to complete the questionnaires, and the level of missing data was low. Some inaccuracies might also stem from the self-reported nature of the information. However, to compensate for that, we utilised a machine learning approach, which is more capable of handling multiple, interconnected variables and providing a holistic view of dietary patterns.

5.4 STUDY IV

Study IV aimed to investigate the role of endometrial microbiota in women with EC and compare it to the bacterial profile of women with benign conditions of the gynaecological tract. Most women in the EC and EH/EP groups were postmenopausal and had a BMI above the normal range. The median age in the EC group was 10 years higher than in the EH/EP group, and the vast majority of EC were endometrioid. The preliminary analysis showed that patients with EC have endometrial microbiota distinct from that of patients with EH/EP: *Atopobium* and *Porphyromonas* were present in patients with EC, while *Lactobacillus* was present in those with EH/EP.

The vaginal tract, mainly its lower parts, is well known for its active microbiota, with Lactobacilli being the most representative. At present, vaginal microbiota is a significant

factor in different diseases of the female reproductive system, like cervical cancer. On the other hand, the uterus was considered sterile until next-generation sequencing was used to discover the presence of endometrial microbiota different from those in the vagina.[206] For example, the number of bacteria in the uterus is many times lower than in the vagina and can grow at a mildly high pH.[207]

In this study, *Atopobium* and *Porphyromonas* demonstrated to be present in EC. These bacteria might influence endometrial cells, resulting in decreased production of proinflammatory cytokines and chemokines, which could provoke the development of inflammatory-associated diseases.[208] The joint presence of *Atopobium vaginae* and *Porphyromonas* species have already been shown to be significant in EC, but the results of Study IV have strengthened this hypothesis.[209] However, the causality between endometrial microbiota and EC prognosis is not yet well understood. In addition, exploration of inter-bacterium interaction in the uterine cavity bacterium-host interaction might produce novel, clinically relevant information on the endometrial microenvironment.

The strength of the study is its prospective nature and the use of intraoperative biological material. However, the study doesn't have an extended follow-up, and because it is a pilot study, the cohort was not very large.

6 CONCLUSIONS

→ Levels of LRIG3 protein seem to have a prognostic role in women with EC.

→ The role of LRIG1 and LRIG2 in EC is currently unclear.

→ High dietary cadmium intake seems to be associated with poor outcomes in women with EC, which questions well-established knowledge on the healthiness of a vegetarian diet.

→ Consumption of sugar-sweetened beverages and fried potatoes increases the risk of EC recurrence and death. But the cooking method itself might influence the risk of EC.

→ Physical activity positively affects survival rates in women with EC.

→ RSF is a proper method to explore survival rates in multivariable datasets.

→ Endometrial microbiota in patients with EC is different from those with EH/EP. *Atopobium* and *Porphyromonas* are the main component of endometrial microbiota in patients with EC, whereas *Lactobacillus* is more common in those with EH/EP.

7 FUTURE PERSPECTIVES

- The semi-quantitative nature of the LRIG proteins in IHC samples in Study I is a potential source of bias. Therefore, further investigation of these proteins in a larger cohort using software-based methods to analyse IHC expression might be beneficial. Further analysis of similar proteins is possible, but not considered to be of interest, taking into account the already well-studied molecular features of EC and based on the new molecular classification.

- Investigating the role of cadmium in EC prospectively, with simultaneous assessment of its levels in blood and urine, might increase the reliability of the results presented in Study II. In addition, the prognostic role of other oestrogen-mimicking metals might be of interest to explore.

- Further assessment of the role of dietary factors and daily activities in EC prognosis using prospectively collected data can compensate for the potential bias in Study III and bring additional insight to the topic of interest.

- Longer follow-up of the patients in Study IV will be conducted to reveal the prognostic role of endometrial microbiota in patient with EC compared to those with benign diseases of the uterus. Moreover, a larger cohort of women with EC might be used to correlate the bacterial landscape in EC, with stratification according to the new molecular classification.

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