Imperial College London

UNRAVELLING THE INTERPLAY BETWEEN MICROBIOME, INFLAMMATION, METABOLIC DYSREGULATION AND ENDOMETRIAL CARCINOGENESIS

THESIS DISSERTATION

by

Anita Semertzidou

A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

November 2021

Supervisors:

Professor Maria Kyrgiou

Dr David MacIntyre

Professor Julian Marchesi

Professor Phillip Bennett

Declaration of Originality

I declare that all work presented in this thesis is entirely my own unless stated otherwise. Published and unpublished data and articles, referred to throughout this thesis, are duly cited within the body of the text and referenced in the bibliography in accordance with College guidance.

Signature: Anita Semertzidou

Date: 22/11/2021

Copyright Declaration

Acknowledgements

A long yet enjoyable journey has come to an end.

I could have never imagined that the initial drive to tick a box in the academic achievements section would unlock so many doors in the way I think, the way I perceive the world and who I came to be. Besides the obvious benefits of completing a PhD as a tool for career progression, it is the underlying benefits scratching beneath the surface that hold the greatest meaning. The interaction with curiosity-driven scientists, who envisage the resolution of mankind's biggest biomedical problems has been a revelation to me, their passion infectious and their logic tremendously educational.

What this PhD journey represents to me could hardly be summarised in words but in brief, it has expanded my horizons to unparalleled levels, has taught me to organise, strategize, troubleshoot and be relentless in the pursuit of a goal, attributes that have been great assets in my life beyond science. Engaging in understanding how 'things work' and engineering ways to manipulate physiology for human benefit has given me a new perspective as a new-born scientist but also as a clinician, being able to see out of the box, understanding the limitations of current treatments offered to oncology patients and boosting my enthusiasm to explore new therapeutic avenues.

For all the aforementioned reasons and many more, I feel compelled to acknowledge the people who made this journey possible for me. First and foremost, I would like to thank **Professor Maria Kyrgiou** who trusted me to embark on a PhD adventure during the most challenging time in my personal history trapped in a non-sustainable form of life in Poole, Dorset. I am deeply grateful for dragging me out of the misery and giving me this amazing opportunity to evolve both personally and professionally.

Massive thanks to my co-supervisor, **Dr David MacIntyre**, the person who technically taught me everything I know about microbiome analysis but most importantly the person who taught me the most invaluable lesson of how to think scientifically. I now

realise why he started reading a paper by looking at the methods, while I always jumped to results ☺. I admire your passion for science and attention to detail without cutting any corners, which I think you have also instilled to me.

Many thanks to microbiome field expert, **Professor Julian Marchesi**, for always being available to answer my questions, promptly reading my manuscripts and expanding my understanding of the microbiome world. Also, **Professor Phillip Bennett** by providing an inspiring example of how a leader should be and how to manage a heterogenous group of people in an efficient and graceful way.

I also need to thank **Dr Ann Smith** for pre-processing of 16S rRNA gene sequencing data, **Richard Williams** and **Nadia Fernandes** for processing of genetic and DNA methylation data, **Professor Jan Brosens** and his student **Komal Makwana** for kindly teaching me how to grow organoids, generously offering their precious frozen organoids from benign patients and Prof's participation in my Early and Late Stage Assessment.

Special thanks to fellow clinicians and researchers, **Eilbhe Wheelan** and **Olivia Raglan**, the first people who showed me around and taught me the basics of patient recruitment. A huge thanks to my new-found sister **Emer O'Connor**, with whom we have followed parallel paths since the beginning, completing PhDs while working 24h shifts at Guy's Cancer Centre. Your support and mentorship on professional issues and beyond have been a catalyst to the way I have handled things on a number of occasions. Will miss our lengthy handovers over patients and life ☺

My **HCA family**, the best working environment I have ever experienced in my life. Despite the challenges of working while doing research, I could have never asked for a better 'working crew' that ended up being family. Thanks to **Dr Kamal Ahmed** for supporting aspiring hybrid clinician-researchers but also thanks to all nurses, chefs and staff from H2 ward.

My parents, Vasilis and Dora, whose boundless love and support throughout my life and emphasis on education have enabled me to work towards reaching my full potential. **My brother**, Theo, who unintentionally set the example as the big brother and opened the way abroad and into academia making my transition to the UK smoother and academic choices clearer.

Last but not least, I would like to thank **all patients** participating in the study. Their eagerness to take part to 'help others' as they said, has been as moving as motivating for me. I hope my findings will lay the groundwork for discoveries that could directly benefit them.

Lastly, I dedicate this work to research itself for its infinite power and to all those visionary, inquisitive minds that driven by natural curiosity unleash their scientific creativity to unravel nature's wonders, achieve the unachievable and give hope to the hopeless....

Publications, Presentations and Posters related to this thesis

Published

- ◊ **Semertzidou A**, Brosens JJ, McNeish I, Kyrgiou M. Organoid models in gynaecological oncology research. Cancer Treat Rev. 2020;90:102103.

Manuscripts in preparation

- ◊ **Semertzidou A,** Marchesi J, Smith A, Bennett P, Brosens JJ, McNeish I, MacIntyre D & Kyrgiou M. The role of genital tract microbiota continuum in endometrial malignancy.
- ◊ **Semertzidou A,** Grout- Smith H, Kalliala I, MacIntyre D, Gang A, Tsilidis K, Kyrgiou M. Diabetes and anti-diabetic interventions and the risk of gynaecological and obstetric outcomes: an umbrella review.

Other publications arising from work related to this thesis

- ◊ E. Whelan, I. Kalliala, **A. Semertzidou**, O. Raglan, S. Bowden, G. Markozannes, S. Cividini, K. Kechagias, I. McNeish, J. Marchesi, D. A. MacIntyre, P. R. Bennett, K. K. Tsilidis, M. Kyrgiou. Risk Factors for Ovarian Cancer: An umbrella review of the literature. AJOG. *(Under review)*
- ◊ Paraskevaidi M, Cameron SJS, Whelan E, Bowden S, Tzafetas M, Mitra A, **Semertzidou A**, Athanasiou A, Bennett PR, MacIntyre DA, Takats Z, Kyrgiou M. Laser-assisted rapid evaporative ionisation mass spectrometry (LA-REIMS) as a metabolomics platform in cervical cancer screening. EBioMedicine. 2020;60:103017.
- ◊ Kechagias KS, **Semertzidou A**, Athanasiou A, Paraskevaidi M, Kyrgiou M. Bisphenol-A and polycystic ovary syndrome: a review of the literature. Rev Environ Health. 2020;35(4):323-331.

Oral Presentations

International

- ◊ **ESGO 22nd European Gynaecological Oncology Congress of the European Society of Gynaecological Oncology**, 23-25 October 2021, Prague, Czech Republic. The role of genital tract microbiota continuum in endometrial malignancy.
- ◊ **ESGO 21st European Gynaecological Oncology Congress of the European Society of Gynaecological Oncology**, 2-5 November 2019, Athens, Greece. Oral presentation: Diabetes and gynaecological cancers: an umbrella review.

Regional

- ◊ **RCOG World Congress 2021,** 9-12 June. The role of genital microbiota continuum in endometrial malignancy.
- ◊ **British Gynaecological Cancer Society 2021**, 13-14 May. The role of genital tract microbiota in endometrial malignancy.

National

- ◊ **Annual National Conference, Greek Society for Colposcopy and Cervical Pathology 2020**, 30 Oct-1 Nov. Female genital tract microbiome in endometrial and ovarian carcinogenesis.

Poster Presentations

International

- ◊ **Annual International Meeting, International Society for Stem Cell Research**, 23-27 June 2020, Virtual. Human malignant and benign endometrial organoids recapitulate the phenotype and epigenetic signature of parent tissue.

Regional

- ◊ **RCOG World Congress 2021,** 9-12 June. Diabetes and the risk of gynaecological and obstetric outcomes: an umbrella review.
- ◊ **British Gynaecological Cancer Society 2021**, 13-14 May. Poster presentation: Diabetes and gynaecological cancers: an umbrella review.

Abstract

Background

Inflammatory and metabolic cues have long been associated with the pathogenesis of endometrial cancer. Increasing evidence implicates the reproductive tract microbiota with health and disease states, however its role in metabolic and inflammatory dysregulation in the context of endometrial oncogenesis is poorly described.

Aims

The overarching goal of this thesis was to explore diabetes and microbial dysbiosis as complicit factors in endometrial cancer. The first aim was to critically appraise the strength of epidemiological evidence for associations between diabetes and anti-diabetic interventions and the risk of endometrial cancer and other gynaecological or obstetric outcomes. The second aim was to investigate if genuine microbial signals from low bacterial biomass sites of the upper genital tract can be detected above background and if so, determine if they exist as a microbial continuum along the length of female genital tract and rectum of endometrial cancer patients and patients with benign intra- or extrauterine pathology. The third aim was to test the hypothesis that endometrial cancer patients harbour a distinctive microbial fingerprint in their genital tract and rectum compared to benign controls that contributes to endometrial cell proliferation and inflammation in a 3-dimensional endometrial organoid model. Lastly, this thesis aimed to validate benign and malignant endometrial organoids as tools for disease modelling by interrogating their phenotypic, genetic and epigenetic resemblance to parent tissue.

Results

An umbrella review of existing meta-analyses demonstrated suggestive evidence supporting a positive association between diabetes and endometrial cancer and improved survival in metformin users. Increased endometrial cancer mortality among diabetic patients was only supported by weak evidence. Metataxonomics-based

characterisation of microbiota and qPCR showed that a subset of benign and endometrial cancer patients harbour microbial communities distinguishable from background contaminants. A microbial continuum along the genital tract is most evident in benign patients compared to endometrial cancer patients, whereas vaginal microbiota is poorly correlated with rectal microbiota in both cohorts. Endometrial cancer is associated with reduced cervicovaginal and rectal bacterial load and compositionally characterised by *Lactobacillus* depletion, increased microbial diversity and enrichment of *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus* in the lower genital tract and endometrium. Microbiome-host interactions were studied using 3D endometrial organoid models, which were shown to be morphologically and (epi)genetically consistent with progenitor tissue. *L. crispatus* supernatant decreases endometrial cancer organoid viability at high concentrations and does not significantly affect inflammatory pathways in benign or endometrial cancer organoids as measured by cytokine secretion.

Discussion & Conclusion

Metabolic dysregulation and microbiota alterations are associated with endometrial cancer. *L. crispatus*, a female genital tract commensal, does not interfere with inflammatory signalling in endometrial organoids. Further demystifying which host pathways altered microbiota might influence could provide insights in the context of microbiota manipulation for clinical purposes.

Table of Contents

List of Abbreviations

Abdo	Abdominally
aCGH	array comparative genomic hybridization
AMSTAR	A measurement tool to assess systematic reviews
ASRM	American Society for Reproductive Medicine
Ben	Benign
BMI	Body mass index
BV	Bacterial vaginosis
CAH	Complex atypical hyperplasia
CCL4,5	C-C Motif Chemokine Ligand 4,5
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
COX-2	Cyclo-oxygenase 2
CRC	Colorectal cancer
CRT	Chemoradiotherapy
CS	Caesarean section
CSTs	Community state types
CTLA-4	Cytotoxic T-lymphocyte- associated antigen 4
Cx	Cervix
DCC	Dextran-coated charcoal
DEPC	Diethylpyrocarbonate
DM1,2	Diabetes mellitus 1,2
DMSO	Dimethyl sulfoxide
E2	Oestradiol
EC	Endometrial cancer
ECM	Extracellular matrix
EEC	Endometrioid endometrial cancer
EGF	Epidermal growth factor
EH	Endometrium higher
EL	Endometrium lower
eMSCs	Endometrial mesenchymal stem cells
EMT	Epithelial-mesenchymal transition
EndoH	Endometrium higher
EndoL	Endometrium lower
ER	Oestrogen receptor
ERK1/2	Extracellular signal-regulated kinases 1/2

FBS	Foetal bovine serum
FDR	False Discovery Rate
FFA	Free fatty acids
FGF10	Fibroblast growth factor 10
FGT	Female genital tract
FT	Fallopian tube
G-CSF	Granulocyte colony-stimulating factor
G1,2,3	Grade 1,2,3
GDM	Gestational diabetes mellitus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gr	Grade
HCA	Hierarchical clustering analysis
HGF	Hepatocyte growth factor
HPV	Human papillomavirus
HR	Hazard ratio
hrHPV	High risk human papillomavirus
HV	Higher vagina
IADPSG	International Association of the Diabetes and Pregnancy Study Groups
IBD	Inflammatory bowel disease
IDF	International Diabetes Federation
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
IQR	Interquartile range
IRAK	IL-1 receptor associated kinase
IRS-1	Insulin receptor substrate 1
ISGyP	International Society of Gynaecological Pathologists
Lap	Laparoscopically
LB	Lysogeny broth
LCC	Lactobacillus-conditioned
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LGA	Large for gestational age
Lgr5	Leucine-rich repeat containing G-protein-coupled receptor 5
LPS	Lipopolysaccharide
LV	Lower vagina
Mal	Malignant
MAPK	Mitogen Activated Protein Kinase

MDS	Multidimensional scaling
MEK1/2	MAPK/ERK kinase
MMP-2	Matrix metalloproteinase 2
MMR	Mismatch repair
MSI	Microsatellite instability
mTORC1,2	mTOR complex 1,2
NA	Not attempted
NGS	Next generation sequencing
NICU	Neonatal intensive care unit
NK	Not known
NS	Non-significant
NSAIDs	Non-steroidal anti-inflammatory drugs
OR	Odds ratio
OS	Overall survival
OTUs	Operational taxonomic units
Ov	Ovary
P4	Progesterone
PARP	Poly adenosine diphosphate-ribose polymerase
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PD1	Programmed death 1
PDGM	Pregestational diabetes mellitus
PDK1	3-phosphoinositide-dependent protein kinase 1
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PR	Progesterone receptor
PSC	Pluripotent stem cell
qPCR	Quantitative PCR
RCTs	Randomised controlled trials
RFS	Recurrence-free survival
RIVF	Recurrent In Vitro Fertilisation failure
RM	Recurrent miscarriage
RR	Relative risk
RTK	Receptor tyrosine kinases
SCFA	Short-chain fatty acid
SEER	Surveillance, Epidemiology and End Results

SHBG	Sex-hormone-binding globulin
TAK1	Transforming growth factor B-associated kinase 1
TCGA	Cancer Genome Atlas
TEM	Transmission electron microscopy
TLR4	Toll-like receptor-4
TNF-α	Tumour necrosis factor alpha
TRAF6	TNF receptor-associated factor
TRP	Transient receptor potential
TSC2	Tuberous sclerosis complex 2
UGT	Upper genital tract
UMI	Unique molecular identifier
Vag	Vagina
VEGF	Vascular endothelial growth factor
WCRF CUP	World Cancer Research Fund Continuous Update Project
WHI	Women's Health Initiative
WHO	World Health Organisation
3D	3-dimensional

List of Tables

List of Figures

Figure 8.3: Proposed model of bacterial pro- and anti-tumorigenic effects through modulation of inflammatory, metabolic and cell survival pathways.

Figure 8.4: Next-generation female genital tract organoid cultures.

CHAPTER 1. Introduction

Content from this chapter was published as:

Semertzidou A, Brosens JJ, McNeish I, Kyrgiou M (2020). Organoid models in gynaecological oncology research. Cancer Treat Rev 90: 102103.

1.1 Endometrial Cancer

1.1.1 The epidemiologic burden of endometrial cancer

Endometrial cancer (EC) has a dominant place among gynaecological cancers and is the fourth most common malignancy in women after breast, lung and colorectal disease. Data from different sources in the UK and USA demonstrate a steady increase in incidence and mortality rates especially since the start of 21st century. From 1985 to the early 1990s, incidence rates for those aged 55 years and over remained unchanged, whereas for women aged 50-54 years rates declined. In contrast, from 1990s the incidence in both age groups has increased at an estimated 2.55% per annum (95% CI 2.13–2.98%) [1]. A UK population-based cancer registry analysing data from 6867 women with endometrial cancer between 1994 and 2006 further confirmed this trend showing an increase of endometrioid endometrial cancer from 12.0 per 100,000 (confidence interval (CI) 10.7-13.2) in 1994 to 16.3 per 100,000 (CI 14.9-17.7, P<0.001) in 2006, which was most marked in age groups 60-79 years (P<0.001). No change in other endometrial cancer subtypes was detected. Likewise, mortality rates in the UK declined from 41.3 per million women in 1985 until about 1999 (30.0 per million) and increased to 35.9 per million in 2008 [2]. More recent data spanning from 1990 to 2017 from the Surveillance, Epidemiology and End Results (SEER) Program have confirmed an increase in endometrial cancer incidence rates in both white and black women (Figure 1.1) [3].

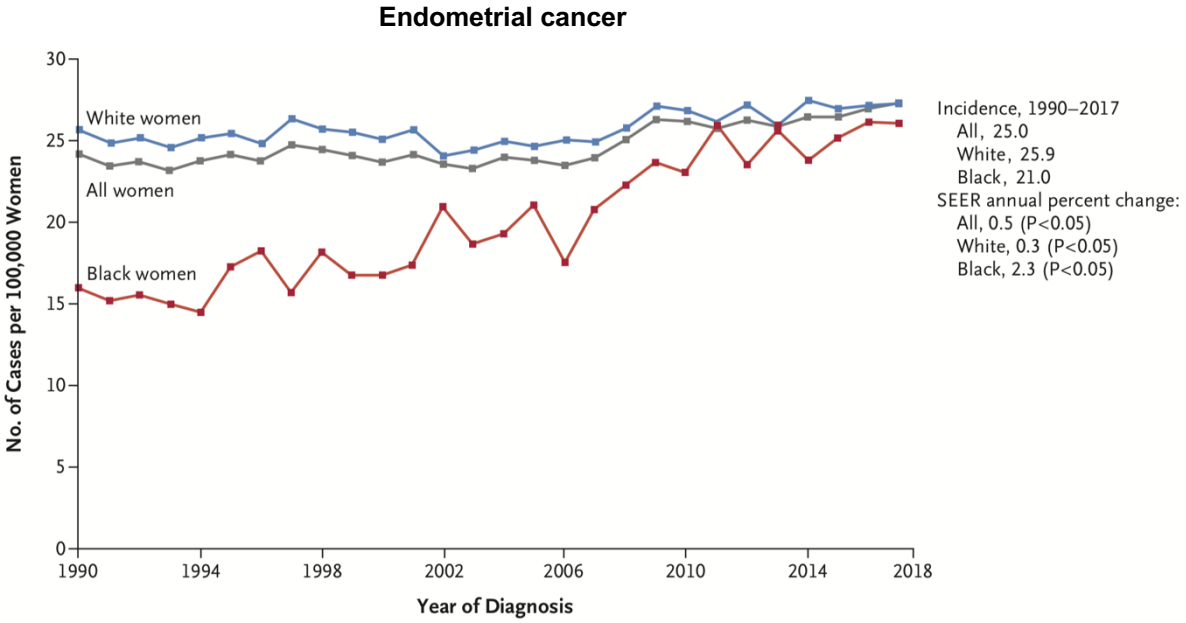


Figure 1.1. Endometrial cancer incidence. Age-adjusted incidence of endometrial cancer among white and black women based on Surveillance, Epidemiology and End Results (SEER) data from 1990 through 2017. The incidence refers to cases of endometrioid and non-endometrioid endometrial cancer, age-adjusted to the US standard population in 2000; sarcomas are excluded (adapted from Lu *et al.* [3], reproduced with permission from Copyright Massachusetts Medical Society).

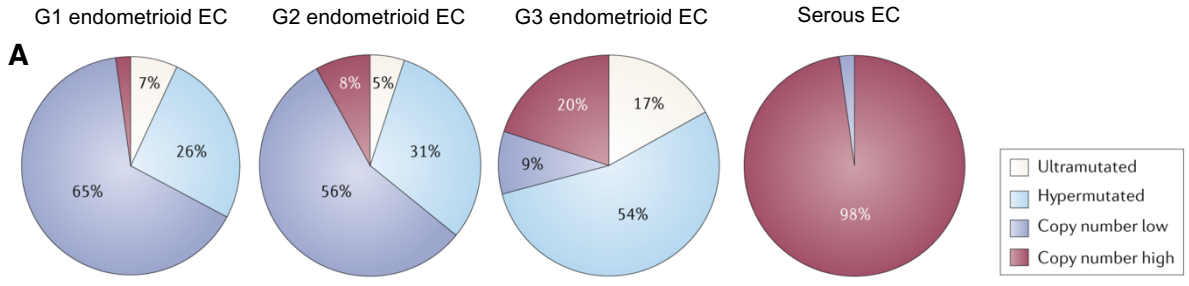
USA projections estimate an increase to 42.13 endometrial cancer cases per 100,000 by the year 2030, a 55% increase over 2010 endometrial cancer rates [4]. The notable rise in endometrial cancer incidence has occurred in concert with obesity and diabetes epidemics and other factors such as increased life expectancy, reduced rates of hysterectomies for benign indications, increased use of hormone replacement therapy and tamoxifen.

1.1.2 Classification schemes of endometrial cancer

In 1983 a landmark Gynecologic Oncology paper defined two types of endometrial cancer based on clinical and epidemiologic observations [5]. Type I (70-75% of cases) encompasses the endometrioid type, affects perimenopausal women, is estrogen-driven, low-grade and low-stage and is associated with metabolic syndrome and good prognosis. On a molecular level, *PTEN* and *KRAS* gene mutations as well as microsatellite instability have been associated with this type. Type II endometrial cancers (25-30% of cases) represent non-endometrioid types (serous, clear cell),

affect older postmenopausal women, are estrogen-independent, high-grade, clinically aggressive and associated with poor prognosis [6]. *TP53* mutations, higher expression/amplification of *HER-2/neu* protein and alterations in *p16* and *E-cadherin* genes are characteristic for non-endometrioid carcinomas [7 8].

The dualistic model of endometrial cancer, although still very popular and conceptually useful, has been criticised as being oversimplified and unable to capture the biologic heterogeneity of disease. The Cancer Genome Atlas (TCGA), which is the most comprehensive molecular study to date of 373 endometrial carcinomas, incorporated advances in next-generation sequencing, reverse-phase protein array and DNA methylation and defined four classes of endometrial cancer on the basis of genetic and epigenetic alterations: 1) POLE (ultramutated) 2) MSI – microsatellite instable (hypermutated) 3) copy number low (endometrioid) and 4) copy number high (serous like) tumours (Figure 1.2) [9-12].



B

	POLE (ultramutated)	MSI (hypermutated)	Copy-number low (endometrioid)	Copy-number high (serous-like)
Copy-number aberrations	Low	Low	Low	High
MSI/*MLH1* methylation	Mixed MSI high, low, stable	MSI high	MSI stable	MSI stable
Mutation rate	Very high (232×10^{-6} mutations/Mb)	High (18×10^{-6} mutations/Mb)	Low ($2{\cdot}9 \times 10^{-6}$ mutations/Mb)	Low ($2{\cdot}3 \times 10^{-6}$ mutations/Mb)
Genes commonly mutated (prevalence)	*POLE* (100%) *PTEN* (94%) *PIK3CA* (71%) *PIK3R1* (65%) *FBXW7* (82%) *ARID1A* (76%) *KRAS* (53%) *ARID5B* (47%)	*PTEN* (88%) *RPL22* (37%) *KRAS* (35%) *PIK3CA* (54%) *PIK3R1* (40%) *ARID1A* (37%)	*PTEN* (77%) *CTNNB1* (52%) *PIK3CA* (53%) *PIK3R1* (33%) *ARID1A* (42%)	*TP53* (92%) *PPP2R1A* (22%) *PIK3CA* (47%)

Figure 1.2. TCGA molecular classification of endometrial cancer. A. Pie charts showing the distribution (% of tumours) of low-, intermediate- and high- grade (G1, G2, G3 respectively) endometrioid EC and serous EC among TCGA molecular subgroups (adapted from Urick *et al.* [13], reproduced with permission from Springer Nature). **B.** Genomic characteristics of four TCGA classes of endometrial cancer (adapted from Murali *et al.* [10], Copyright License Number 5312400288146). Mb: Megabase, MSI: Microsatellite instability.

The ultramutated POLE subgroup was a novel category discovered by TCGA, which sparked interest because of its favourable prognosis despite the high mutation rate [14]. POLE encodes the major catalytic of the Polε (Polymerase Epsilon) DNA polymerase enzyme complex responsible for leading strand DNA replication. Its function ensures high fidelity of base incorporation and low replicative errors in daughter strand. MSI stems from defects in post-replicative DNA mismatch repair system resulting in changes in microsatellites (short, repeated sequences of DNA). Mismatch repair deficiencies can arise from i) inherited cancer syndromes (e.g., Lynch), ii) acquired/somatic mutations or iii) epigenetic events e.g., methylation of one of the genes involved in mismatch DNA repair, most commonly MLH1. The third molecular subgroup (copy number low) is genomically relatively stable, MMR-proficient, carries a moderate number of mutations, predominantly within the PI3K/Akt and Wnt signalling pathways and almost exclusively encompasses endometrioid tumours with oestrogen (ER) and progesterone receptor (PR) positivity. Copy number high tumours have the worst prognosis, include uterine serous tumours and ~25% of high-grade endometrioid tumours, demonstrate few DNA methylation changes, low oestrogen receptor/progesterone receptor levels, and frequent *TP53* mutations (92%) [12].

Given the poor inter-observer reproducibility in assigning histotype and grade to endometrial cancer by microscopic techniques, it has been argued that genomic-based classification might be more appropriate to classify endometrial tumours [15 16]. The usefulness of molecular classification in guiding treatment decisions has not been fully assessed and further validation is required before its integration into clinical practice. The guidelines published in 2019 by the International Society of Gynaecological Pathologists (ISGyP) conclude that mutational analysis of POLE and immunohistochemistry (IHC) analysis of p53 and MMR proteins (PMS-2 and MSH-6) are considered optional as a surrogate to classify tumours into the 4 TCGA groups, particularly for high-grade endometrioid and serous types [17]. All women who are diagnosed with EC should undergo systematic clinical screening for Lynch syndrome, based on personal/family history, and/or IHC/molecular screening for MSH-6 and PMS-2 IHC alone, with subsequent MSH-2 or MLH1 IHC, when indicated.

Lastly, as EC patients with MMR deficiency or MSI may benefit from immunotherapy, consideration should be given for MMR deficiency testing for women with EC who are candidates for immunotherapy [17].

1.1.3 Risk factors for endometrial cancer

Several factors have been recognised as contributing to the development of endometrial cancer. Non-modifiable risk factors include age, race, taller stature and genetic predisposition (e.g. Lynch syndrome), while modifiable factors are nulliparity, obesity, diabetes, hypertension, polycystic ovary syndrome (PCOS) and use of oestrogen-only unopposed hormone replacement therapy or tamoxifen [18]. Endometrial carcinoma risk is also positively associated with early age at menarche and older age at menopause. Conversely, parity, breastfeeding, coffee consumption, smoking, physical activity, oral contraceptive or metformin use and bariatric surgery have all been reported to confer a reduced risk [19].

Metabolic dysregulation brought about by the Western lifestyle adopted in many societies, involving high dietary intake and sedentary lifestyle, has been implicated in the observed steady increase in endometrial cancer incidence in recent years. A study by Liao comprising 23 cohort studies for endometrial cancer incidence in diabetic patients (Type 1 and 2) demonstrated an increased risk (RR 1.89, 95% CI 1.46–2.45) [20]. This is consistent with previous meta-analyses but should be interpreted cautiously, since most included cohort studies were not adjusted for BMI, which is a well-acknowledged risk factor for endometrial cancer [21 22]. In accordance with this, the Women's Health Initiative (WHI) study showed that individuals with higher insulin levels were more prone to develop endometrial cancer [23].

Pelvic inflammatory disease has also recently been associated with endometrial cancer risk. According to a 2015 nationwide population-based retrospective cohort study, in women with a history of pelvic inflammatory disease the risk of developing endometrial cancer is increased by 1.89-fold [24].

1.1.4 Molecular pathogenesis of endometrial cancer

1.1.4.1 Genetic mutations in endometrial cancer

Endometrial cancer arises in an environment of excess estrogen relative to progesterone [25-29]. Unopposed estrogen exerts a proliferative action on uterine epithelium leading to simple endometrial hyperplasia, which can further evolve to complex atypical hyperplasia (CAH), the precursor lesion of endometrial cancer, histologically marked by back-to-back glands, sparse intervening stroma and atypical nuclei [30]. On a molecular level, *PTEN* mutations have been detected in about 55% of patients with endometrial hyperplasia [31 32]. Genetically engineered mouse models of EC have shown that biallelic *Pten* loss leads to the development of CAH, whereas biallelic *Pten* loss together with mutational activation of *Pik3ca* results in progression of CAH to EC [33]. These findings clearly indicate that *PTEN* mutation is an early yet insufficient event in endometrial carcinogenesis and that multiple genetic hits are required for progression to cancer.

Table 1.1. Common genetic mutations in endometrial cancer (adapted from Murali *et al*. [10], Copyright License Number 5312400288146).

Genetic mutation	Type I	Type II
PTEN mutation	52–78%	1–11%
PIK3CA mutation	36–52%	24–42%
PIK3R1 mutation	21–43%	0–12%
KRAS mutation	15–43%	2–8%
ARID1A mutation	25–48%	6–11%
CTNNB1 mutation	23–24%	0–3%
TP53 mutation	9–12%	60–91%
HER2 amplification	0	27–44%
Microsatellite instability	28–40%	0–2%

Endometrial cancer is characterised by substantial PI3K pathway aberrations. This was initially highlighted by the finding of *PTEN* mutations in Cowden syndrome, an inherited autosomal-dominant cancer syndrome with a 5-10% lifetime risk of developing uterine cancer and was later affirmed in sporadic endometrial cancer cases [31 34-37]. The tumour suppressor gene *PTEN*, located on chromosome 10q23.3, is mutated in up to 80% of endometrioid tumours and represents the most common genetic alteration in endometrial cancer (Table 1.1) [31]. The second most common mutated component of the PI3K/AKT/mTOR pathway (52% of endometrioid cancers) is *PIK3CA*, which encodes the catalytic subunit of PI3K, p110a [38]. *PIK3CA* mutations coexist with *PTEN* mutations in 60% of cases [39]. *PIK3R1*, which codes for p85a, the regulatory subunit of PI3K, is mutated in 43% [40 41], while *KRAS* is also mutated in about 43% of cases [42]. Moreover, *Ctnnb1* (which encodes β-catenin) exon 3 deletion leads to endometrial tumorigenesis when combined with *Pten* loss and *Pik3ca* activation in mouse models [43]. Epigenetic silencing of *MLH1*, leading to mismatch-repair deficiency (MMR-D), is a frequent aberration in endometrioid EC that often independently co-occurs with *PTEN* inactivating mutations [12 44]. Endometrial tumorigenesis is accelerated in $Pten^{+/-}/Mlh1^{-/-}$ mice as compared with $Pten^{+/-}$ mice [45]. Mutational inactivation of the *ARID1A* tumour suppressor gene is another common aberration in EC. Immunohistochemical analysis of human CAHs have shown that endometrial glands with loss of both *ARID1A* and *PTEN* expression have higher proliferative indices than adjacent glands with loss of only *PTEN* [46].

In serous endometrial cancers, *PTEN* mutations are found in only 11% of cases, whereas *PI3KCA*, *PI3KR1* and *KRAS* mutations in 35%, 12% and 8% respectively [47]. *TP53* mutations predominate (80-90%) and seem to be an early event as found in serous endometrial intraepithelial carcinoma, which precedes serous cancer development [42 48]. Clear cell endometrial carcinomas are poorly characterised on genomic level but seem to share mutational features with other EC subtypes [49-52]. The majority of uterine carcinosarcomas are believed to originate from high grade endometrioid or serous EC after acquiring a sarcomatous component caused by epithelial-mesenchymal transition. The genomic alterations of carcinosarcomas

resemble those found in other types of EC [53-58]. Intriguingly, *PTEN* and *TP53* co-occur in carcinosarcomas [53 55 57], even though they are mutually exclusive in other EC subtypes (except grade 3 EEC) [12 49]. Taken together, this evidence suggests that the mutational landscape of endometrial cancer is doubtlessly complex and there are still plentiful research avenues to be explored.

1.1.4.2 Proliferation pathways - PI3K signalling

The phosphoinositide 3-kinase (PI3K) pathway regulates key aspects of metabolism, cellular growth and survival and has emerged as a significant component of endometrial cancer biology [59-63]. PI3K is a heterodimeric enzyme consisting of a catalytic subunit (p110) and a regulatory subunit (p85). Upon stimulation of receptor tyrosine kinases (RTK), PI3K phosphorylates the lipid phosphatidylinositol 4,5-biphosphate (PIP2), creating phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn recruits Akt (also known as protein kinase B, PKB) to the membrane [64]. At the membrane, Akt is phosphorylated and partially activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1), while phosphorylation of Akt by mTOR complex 2 (mTORC2) leads to full enzymatic activity [65]. Akt phosphorylates and inhibits tuberous sclerosis complex 2 (TSC2) within the multiprotein TSC1/TSC2 complex. TSC complex is known to indirectly inhibit mTOR complex 1 (mTORC1) [66 67]. Hence, PI3K-AKT signalling activates mTORC1, a key regulator of various metabolic processes. PTEN (phosphatase and tensin homolog), a negative regulator of the PI3K pathway, is a tyrosine phosphatase that hydrolyses PIP3 back to PIP2 and deactivates the signalling cascade [68 69]. The PI3K pathway cross-talks with other signalling pathways including the RAS–ERK pathway [70]. Ras recruits the serine/threonine protein kinase Raf, which activates MEK1/2 (MAPK/ERK kinase) and subsequently ERK1/2 (extracellular signal- regulated kinases 1/2) [71]. Activated ERK1/2 phosphorylates several substrates and regulates different transcription factors impacting on gene expression [72].

1.1.4.3 Inflammatory pathways - NF-κB signalling

The concept of interplay between inflammation and cancer has long been recognised. In 1863, Virchow hypothesized that the origin of cancer was at sites of tissue injury, where ensuing inflammation caused uncontrolled cell proliferation. Epidemiologic studies have established robust relationships between chronic inflammation and certain types of cancer. The most prominent example is that of inflammatory bowel disease predisposing to colorectal cancer [73]. In endometrium, menstruation itself mimics an inflammatory process with continuous cell turnover. The inflammatory endometrial niche is more pronounced in obese patients since obesity is considered to promote chronic low-grade inflammation in cells [74].

The EPIC study, a large prospective study with ten participating countries, has demonstrated an increased risk of endometrial cancer among women with high TNFα levels, a major pro-inflammatory cytokine, and its two soluble receptors (sTNFR1 and sTNFR2) after adjustment for various confounders [75]. Another study showed that polymorphisms in inflammation pathway genes *FABP1, CXCL3, IL6, MSR1,* and *MMP9* are associated with endometrial cancer risk [76]. Mechanistically, studies using malignant endometrial epithelial cells have shown that NF-κB activates COX-2 (cyclo-oxygenase 2), which in turn increases production of PGE2 (prostaglandin E2) both in malignant and adjacent endometrial stromal cells [77]. Enhanced production of NF-κB and COX-2 is encountered in many cancers including endometrial as well as in proliferating endometrium and endometrial hyperplasia [78-80]. Intriguingly, a decrease in NF-κB expression is associated with enhanced apoptosis in low grade endometrial carcinomas [78]. Furthermore, aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) seem to exert anti-proliferative effects in a dose-dependent manner in endometrial, colon and other types of cancer [81-83].

In obese patients, it is believed that increased free fatty acids (FFA) and lipopolysaccharide (LPS) levels activate Toll-like receptor-4 (TLR4), which dimerizes, and recruits downstream adaptor molecules such as myeloid differentiation protein 88 (MyD88)/MyD88 adapter-like protein (MAL) to mount an inflammatory response [84].

The activated MyD88/MAL activates IL-1 receptor associated kinase (IRAK) [84 85], TNF receptor-associated factor (TRAF6) [86], transforming growth factor B-associated kinase 1 (TAK1) [87], and JNK [88] and IKK complexes [89]. The activated IKK complex phosphorylates IκBa leading to its polyubiquitination and ultimately proteasomal degradation and NF-κB release. NF-κB translocates to the nucleus thereafter, activating pro-inflammatory molecule expression [89].

1.1.4.4 Metabolic pathways - IGF signalling

Metabolic dysregulation caused by obesity and/or diabetes has been intensively investigated as a contributing factor to endometrial tumorigenesis. The underlying mechanisms are still to be fully elucidated but the current hypothesis suggests that insulin resistance causes hyperinsulinaemia that activates the insulin-like growth factor-1 (IGF-1) signal transduction pathway [90]. IGFs and insulin, that act through the PI3K/mTOR pathway, stimulate cell proliferation and inhibit apoptosis [90-96]. Insulin also downregulates expression of IGF-binding proteins 1 and 2, thus increasing circulating levels of IGF-1 and 2 [97]. Animal studies have corroborated epidemiologic links of metabolic derangements with endometrial cancer. Maternal obesogenic diet induces endometrial hyperplasia, an early hallmark of endometrial cancer, in a diethylstilbestrol mouse model [98]**.** Insulin-deficient (diabetic) animals have shown that insulin promotes tumour growth and development in xenograft models [99-104], while inactivation of the IGF-1 receptor decelerates tumour growth [96 105]. Insulin also impacts on endometrial cancer development by reducing concentrations of sex-hormone-binding globulin (SHBG) in the blood, increasing the bioavailability of oestrogens [106].

Historically, the link between inflammation and metabolic dysregulation was first suggested by the finding that TNF-α overproduction by adipocytes in obese patients induces insulin resistance [107]. Exposure of cells to TNF-α or elevated levels of free fatty acids, both abundant in obese patients, stimulates inhibitory phosphorylation of serine residues of insulin receptor substrate-1 (IRS-1) [108 109]. This phosphorylation reduces both tyrosine phosphorylation of IRS-1 in response to insulin and the ability

of IRS-1 to associate with the insulin receptor thereby inhibiting downstream signalling and insulin action [108] [110] [111]. The IRS family of proteins, which encompasses 4 members (IRS1-4), is the substrate of the insulin and IGF-1 receptors [112]. Under normal conditions, tyrosine-phosphorylated IRS acts a scaffold to organise and mediate signalling complexes. The signal is propagated via two main branches: the PI3K-Akt-mTOR, responsible for glucose uptake, metabolism and cell growth and the Ras-ERK pathway that controls cell proliferation and differentiation [113] [114]. Although tyrosine phosphorylation of IRS is an integral part of insulin signal transduction, multi-site phosphorylation of serine/threonine of IRS-1 can regulate insulin signalling both positively and negatively. Increased IR serine phosphorylation has been observed in insulin-resistant states, both in rodents and in humans [113] [114]. The role of pro-inflammatory cytokines in metabolic dysregulation has been extensively investigated (reviewed in Hotamisligil [115]). It remains unclear whether inflammation in diabetes occurs in the context of obesity, a well-known initiator of chronic inflammation, since this theory would be insufficient to describe lean patients with diabetes.

Human microbiota have recently been implicated in the regulation of host functions. Whether the female genital tract and rectal microbiome is intersecting with the molecular mechanisms driving endometrial cancer warrants further investigation and falls within the scope of this thesis.

1.2 The Human Microbiome

The concept of human “microbiota” was defined by Joshua Lederberg as ‘‘the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space’’ [116]. The human body is inhabited by approximately the same number of bacteria as human cells [117]. Therefore, the completion and publication of the Human Genome Project in 2001 was succeeded by a vigorous investigation of the human microbiota giving rise to the concept of “metagenomics”, the study of collective microbial genomes [118] [119]. The Human Microbiome Project, which was

launched in 2008, comprised two phases with separate aims: the first phase sought to determine a core microbiome in five major anatomic sites known for their microbial abundance: nasal passages, oral cavity, skin, gastrointestinal tract and vagina, while the second phase (Integrative Human Microbiome Project) aimed to monitor dynamic microbial changes in pregnancy and preterm birth, inflammatory bowel disease and prediabetes [120]. The Human Microbiome Project generated a staggering 14.23 terabytes of data that are now publicly available and accelerated research interest in the role of human microbiota in health and disease. Evidence has shown that the human microbiome is not a static entity but subject to changes dictated by diet, lifestyle, hygiene and use of drugs [121].

The development of metagenomics was preceded by advances in high-throughput sequencing technologies, like 16S rRNA gene profiling and metagenomic shotgun sequencing, that enabled identification of 20-40% of microbes that were considered uncultivable [118]. Research emphasis was put on defining a "healthy microbiome" investigating not only the diversity but also the abundance and evenness of bacterial load. Nevertheless, decoding the microbial genetic make-up is probably insufficient to establish causal relationships for disease occurrence. The concept of "functional microbiome" arose marking an era where microbial gene expression (meta-transcriptome and meta-proteome) and metabolism (meta-metabonome) are equally or more important than microbial community structure [122].

1.2.1 Female genital tract microbiome in health, metabolic disease and gynaecological (pre)cancer

1.2.1.1 Cervicovaginal microbiome

Until relatively recently, the composition of the vaginal microbiome had been described using only culture and microscopy techniques which enabled a relationship between *Lactobacillus* species colonisation and health to be acknowledged [123] [124]. In infancy, the vaginal microbiota is thought to be dominated by a mixture of aerobic and anaerobic bacteria including *Prevotella*, *Enterobacteriaceae*, *Streptococcus* and

Staphylococcus species, while increased oestrogen levels at puberty are accompanied by a marked increase in *Lactobacillus* species [125]. In 2010, Ravel et al. provided the first sequencing-based characterisation of the vaginal microbiome in reproductive-age, healthy women (n=394) [126]. They showed that the vaginal microbiome profile could be classified into five community state types (CSTs) I, II, III, IV, and V. Four out of five of these CSTs were dominated by one or more species of *Lactobacillus*; CST I, which occurred in 26.2% of the women sampled, was dominated by *L. crispatus*, whereas CST II (6.3%), III (34.1%), and V (5.3%) were dominated by *L. gasseri*, *L. iners*, and *L. jensenii*, respectively. The most diverse communities were those of CST IV (27%) and included primarily anaerobic bacteria such as *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia*, and *Mobiluncus*. Differences in ethnic groups were also detected with CST I noted to be most prevalent in white women (45.4%), and CST III most prevalent in Asian women (42.7%). Black (40.4%) and Hispanic (38.1%) women had a higher prevalence of CST IV. These data suggest that although *Lactobacillus* spp. is indeed characteristic of healthy, asymptomatic women of reproductive age, there is not a single healthy microbiome composition shared by all women, reinforcing the idea of functional redundancy, whereby different bacteria carry out the same metabolic functions.

In most women, cervical microbial colonisation compositionally reflects that of the vagina but tends to have lower biomass, with *Lactobacillus* and *Gardnerella* being the most prevalent taxa [127]. A wealth of longitudinal and cross-sectional studies have investigated the relationship between microbiota composition, HPV (human papillomavirus) infection and cervical carcinogenesis. A 2019 network meta-analysis concluded that non-*Lactobacillus* or *L. iners*-dominant cervicovaginal microbiota are associated with 3-5 times higher risk of being infected with any HPV type and 2-3 times higher risk of high-risk HPV acquisition and cervical dysplasia/cancer compared with *L. crispatus* [128]. Increased HPV clearance of untreated cervical intraepithelial neoplasia 2 (CIN2) at 12 months has been reported in women with *Lactobacillus* dominance at baseline, whereas HPV persistence has been observed in

Lactobacillus-depleted, high-diversity microbiota enriched with anaerobes such as *Megasphaera* spp., *Prevotella timonensis* and *Gardnerella vaginalis* [129-131]. Surgical treatment for CIN2/3 has been shown to be accompanied by a decrease of microbial diversity, *L. crispatus* increase, even though *L. iners* remained the most prevalent species with unchanged abundance post-treatment [132-135].

Cervicovaginal microbiota have also been associated with ovarian and endometrial cancer. *Lactobacillus* depletion (<50%) has been observed in ovarian cancer patients aged 50 years or older (61% vs 59% of healthy controls), younger than 50 years (53% vs 29% of healthy controls), while women harbouring BRCA1 mutations have been shown more likely to be *Lactobacillus*-depleted than age-matched wild-type controls (OR 2.79 [95% CI 1.25-6.68]; p=0.012) [136]. Another study reported that genital chlamydia infections increase the probability of developing ovarian cancer, which was found to be 90% greater in women with the highest, compared with the lowest (optical density, >or =0.40 vs. <0.10) levels of chlamydia-EB antibodies [137]. In a recent comparison of the vaginal microbiota of 36 women with endometrial cancer and 69 healthy post-menopausal women, relative abundance of *Lactobacillus* and *Bifidobacterium* were significantly higher in the healthy group, while the cancer group was enriched for 16 phylogroups associated with bacterial vaginosis (BV), including *Sneathia*, *Prevotella*, *Peptoniphilus*, *Fusobacterium*, *Anaerococcus*, *Dialister*, *Moryella* and *Peptostreptococcus* [138].

Associations between anti-cancer treatment and lower genital tract microbiota composition have also been explored. Post-radiotherapy, women with endometrial and cervical cancer demonstrate higher relative abundance of *Mobiluncus, Atopobium* and *Prevotella* spp. and reduced relative abundance of *Lactobacillus*, *Gardnerella* and *Peptostreptococcus* spp. [139]. In a study of ovarian cancer patients, platinum resistance was positively associated with detection of vaginal *Escherichia* (>20% relative abundance), *L. iners* was associated with little, or no, gross residual disease, while other *Lactobacillus* species were dominant in women with >1 cm gross residual disease [140].

1.2.1.2 Microbiome of the upper genital tract

The microbiome of the upper genital tract (UGT) is less well characterized, with the long-standing view of its sterility only recently being questioned. The sterile womb dogma, coined by French paediatrician Henry Tissier in 1900, particularly began to be questioned with data generated from molecular based approaches, including next generation sequencing. For example, a recent study described a continuum of microbiota along the female genital tract and reported that endometrium is less dominated by *Lactobacillus* compared to vagina and other bacteria such as *Pseudomonas, Acinetobacter, Vagococcus* and *Sphingobium* can be detected [141]. Mitchell *et al.* studied the colonization of the upper genital tract by 12 vaginal bacterial species in 58 non-pregnant women undergoing hysterectomy for benign indications [142]. Using quantitative PCR (qPCR), 95% of women had UGT colonization with at least one species (n = 52). The most common species were *L. iners* (45% UGT, 61% vagina), *Prevotella* spp. (33% UGT, 76% vagina) and *L. crispatus* (33% UGT, 56% vagina). As expected, median quantities of bacteria in the UGT were lower than vaginal levels. Another study compared the vaginal and endometrial microbiota of asymptomatic and fertile nonpregnant women using next generation sequencing (NGS) of the 16S rRNA gene [143]. Results further confirmed previous findings demonstrating uterine colonization in 100% of samples, with *Lactobacillus* being most prevalent followed by *Gardnerella*, *Prevotella*, *Atopobium*, and *Sneathia*. In approximately one fifth of the women analysed, the bacterial composition identified in the endometrium varied greatly from that in the vagina, hinting that vaginal ascension may not be the only route of uterine colonization.

Associations between the reproductive tract microbiota and several other poor reproductive health outcomes have been described, including implantation failure [144], recurrent pregnancy loss [144], preterm labour [145] [146], chorioamnionitis [147] [148] and preeclampsia [149-151]. In benign gynaecology, changes in microbiota have been found in chronic endometritis [152-154] and endometriosis [155-157]. In contrast, data linking microbiota to malignant gynaecology is scarce. In the only existing study to date, bacterial load along the female genital tract in 17 patients with endometrial cancer and

4 with endometrial hyperplasia was assessed and compared to 10 benign controls [158]. Characterisation of microbiota using 16S rRNA amplicon sequencing revealed enrichment of several phyla such as Firmicutes (*Anaerostipes*, *Dialister*, *Peptoniphilus*, *Ruminococcus*, and *Anaerotruncus*), Spirochaetes (*Treponema*), Actinobacteria (*Atopobium*), Bacteroidetes (*Bacteroides* and *Porphyromonas*), and Proteobacteria (*Arthrospira*) in the reproductive tract of patients with endometrial hyperplasia and cancer.

Several recent studies have also purportedly identified a fallopian tube and ovarian microbiome. Signature operational taxonomic units (OTUs) for the fallopian tube include *Pseudomonas*, *Erysipelothrix*, *Facklamia* [141], *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria* and *Fusobacteria* [159]. The presence of bacteria in the ovary has been suggested by immunohistochemistry findings of bacterial LPS in ovarian cancer and noncancerous tissue. The composition of the ovarian microbiome was found to differ between cancer and healthy individuals with the former demonstrating a trend toward reduced richness, Shannon/Simpson and evenness index [159 160]. A study comparing the microbiota of 25 ovarian cancer samples with 25 normal distal fallopian tube samples observed an increase of the *Proteobacteria/Firmicutes* ratio, an enrichment of *Acinetobacter*, *Sphingomonas* and *Methylobacterium* and concurrent depletion of *Acidobacteria* and *Lactococcus* [159]. Another study compared the bacterial, viral and fungal composition of ovarian cancer samples with matched and non-matched ovarian control samples. An enrichment of 10 viral families was observed in the cancer samples, including Parapox, Pox, Myxoma and HPV (16/18 and low risk) viruses and a predominance of *Proteobacteria* (52%) followed by *Firmicutes* (22%) [161]. Cancerous ovarian tissue has also been reported to contain detectable levels of *Chlamydia* [161 162], *Mycoplasma* [163] and CMV [161], however other studies have failed to validate these findings [164]. Other less studied sample types in ovarian cancer include the peritoneal microbiome, which has been reported to have decreased bacterial diversity compared to healthy controls [165], whereas the serum microbiome of 166 ovarian cancer patients versus 76 patients with benign ovarian tumours showed no difference in α- or β-diversity and high relative

abundances of the genus *Acinetobacter* [166]. The observation that many of the microbes identified in the UGT have been recognised as contaminants in other studies highlights the importance of inclusion of negative controls in any microbiome study.

1.2.1.3 Gut microbiome

The genomic component of the gut microbiome is estimated to be 99% of bacterial origin, encompassing 1,000- 1,150 bacterial species and a gene set that is 150 times larger than that of the human genome [167]. Compared to other body sites, it is characterised by high diversity and consists mainly of obligate anaerobes, which outnumber facultative anaerobes and obligate aerobes by 2-3 times [168]. *Firmicutes* and *Bacteroidetes* make up 90% of gut microbiota, while *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* are encountered in lower quantities [169-172]. Even though each individual harbours at least 160 species that are largely shared [167], intra- and interindividual variability does exist and is determined by several factors, including mode of delivery at birth (C-section vs vaginal birth) [170 173], breastfeeding, nutritional status (lean vs obese), age [170 174], diet, pathological conditions (e.g. diabetes, inflammatory bowel disease) and pharmacological factors (pre-, probiotics, antibiotics) [168-170]. Hence, a description of a "core healthy gut microbiome" at high taxonomic resolution has proven almost impossible to achieve.

Aberrant gut microbial profiles have been described in metabolic disease with functional sequelae. Obesity-associated microbiota include an increased *Firmicutes/Bacteroidetes* ratio [175-177] and an abundance of short-chain fatty acid (SCFA) producers, such as *Eubacterium ventriosum* and *Roseburia intestinalis* [178]. Diabetes and pre-diabetes also induce alterations in gut microbiota exhibiting a loss of butyrate-producing taxa, a decrease in abundance of *Akkermansia muciniphila* and an increase in abundance of bacteria with pro-inflammatory potentials [179 180]. Animal studies have shown that germ-free mice on a high-fat diet gain less weight than their control counterparts and do not develop insulin resistance [181]. Furthermore, transplantation of intestinal microbiota from either conventionally raised mice or ob/ob

germ-free mice to germ-free mice leads to increased adiposity and insulin resistance [182].

The mechanistic details of the interrelationship between gut microbiota and metabolic derangement have only recently started to unfold. It is postulated that LPS signalling via TLR4 leads to inflammation and insulin resistance. LPS is elevated in obese rodents and humans and can increase intestinal permeability by reducing expression of tight junction proteins [183-185]. This allows for LPS translocation, binding to TLR4 receptors that are present in all cell types and downstream effects on metabolic, inflammation and survival pathways.

There is limited evidence to date describing the gut microbiota in gynaecological malignancies before, during and after anti-cancer treatment. Cervical cancer-associated gut microbiota in patients with no previous treatment have been reported to display higher proportions of the *Proteobacteria* phylum and seven genera, *Escherichia,Shigella*, *Roseburia*, *Pseudomonas*, *Lachnoclostridium*,*Dorea* and*Succi nivibrio* [186]. Similarly, *Proteobacteria* and *Firmicutes* were reportedly increased in the stool of 10 Lynch syndrome patients with ovarian cancer, whereas *Bacteroides* were depleted [187]. Two studies including 58 [188] and 35 [189] women with cervical, vaginal or vulvar cancer followed up gut microbial changes over the course of chemoradiotherapy (CRT) and concluded that gut microbiome diversity continuously decreases during CRT, which also correlates with radiation toxicity identifying patients at high risk [189]. Of note, diversity was found to be restored at week 12 post-CRT in 60% of patients [188]. An increase in *Proteobacteria* [188] and *Fusobacterium* [190] and a decrease in *Clostridiales* [188 189] and *Firmicutes* [190] was observed post-radiotherapy. Another study assessed the response to chemoradiotherapy and survival in cervical cancer patients based on their faecal microbial composition. Short-term survivor samples were significantly enriched in *Porphyromonas*, *Porphyromonadaceae, and Dialister*, whereas long-term survivor samples were significantly enriched in *Escherichia, Shigella, Enterobacteriaceae, and Enterobacteriales*. *Pasteurellales, Haemophilus, and Veillonella* were recognised as independent predictors for

recurrence-free survival (RFS) and overall survival (OS) [191]. Radiation-associated morbidity has also been associated with baseline microbiota. An increased abundance of *Bacteroides, Dialister, Veillonella, Sutterella, Finegoldia*, and *Peptococcaceae* and a decreased abundance of *Clostridium XI/ XVIII, Faecalibacterium, Oscillibacter, Parabacteroides, Prevotella,* and unclassified bacteria at baseline were correlated with diarrhoea post-radiotherapy [189 192]. Following radiotherapy, radiation enteritis was marked by higher abundance of *Proteobacteria* and *Gammaproteobacteria* and lower abundance of *Bacteroides [186]*. In ovarian cancer, platinum resistance was associated with lower phylogenetic diversity than platinum-sensitive patients [140].

1.2.2 Genital - Gut microbiome interactions

Evidence suggests that the gut might serve as a reservoir of microbes that seed the vagina and lower reproductive tract. Collection of paired vaginal-rectal swabs from 132 pregnant women has showed that 44% of bacterial species were shared between the two distal mucosal sites, with *L. crispatus*, *L. gasseri* and *L. jensenii* being the most frequently isolated *Lactobacillus* species in rectum, while *L. iners* was rarely recoverable [193]. These findings are in agreement with a cross-sectional study of 531 non-pregnant females, which used culture methods to identify microbes [194]. Relative to *L. crispatus*, the rectum was more commonly the sole site of *L. gasseri* colonization [195]. Intriguingly, co-colonization of the vagina and rectum with *Lactobacillus* species correlated with the lowest prevalence of BV [194].

The gut is also potentially able to influence genital microbiota via oestrogen-driven mechanisms. *Lactobacilli* proliferation is considered to be supported by oestrogen-induced glycogen production in the vaginal epithelium [196]. The “oestrobolome” defined as “the aggregate of enteric bacterial genes whose products are capable of metabolizing oestrogens” [197] has led to the concept of oestrogen-mediated gut-vagina axis [198]. Several gut microbes (e.g. *Bacteroides, Bifidobacterium, Lactobacillus, Escherichia, Prevotella*) have the ability to deconjugate hepatically conjugated oestrogens through the action of β-glucuronidase and β-glucosidase and more specifically they convert estrone-3- and estradiol-17-glucuronides to the aglycones

estrone and oestradiol, thus increasing free oestrogen, which is reabsorbed in blood circulation [199-202]. Unbound oestrogen is biologically active and exerts its action on the genital tract by thickening the epithelium and increasing mucus and glycogen production but could also have a deleterious effect on hormone-driven cancers. Paradoxically, most *Lactobacillus* species cannot metabolise glycogen [203 204]; α-amylase-like activity which allows glycogen depolymerization has been detected in vaginal secretions [205 206], but whether α-amylase is produced by the human host or vaginal bacterial communities still remains unclear. Overall, it can be postulated that perturbations of oestrogen-metabolizing bacteria in the gut could affect genital tract colonisation by lactobacilli.

1.2.3 Microbiome function in cancer and cancer therapy

A delicate balance between protective commensal microbes and harmful pathogens might intersect with carcinogenic pathways either by suppressing or activating them. However, the role of microbes in causing and sustaining cancer remains largely obscure. Microorganisms are implicated in 20% of human malignancies but few microbes directly cause cancer with most of them being complicit factors [207 208]. Notable examples are *Helicobacter pylori* and gastric cancer [209 210], hepatitis B/C and hepatocellular carcinoma [211] and HPV virus and cervical cancer [212]. Recent evidence suggests that bona fide oncomicrobes that integrate oncogenes into host genomes are rare and it is the global changes in microbiome and host-microbiome interactions that may drive carcinogenesis [213]. On the other hand, several health-promoting indigenous bacteria have been shown to have anti-cancer properties [214].

1.2.3.1 Tumour-promoting microbiome

Experiments in germ-free animals have demonstrated carcinogenic effects of microbiota in various organs, including the skin, colon, liver, breast and lungs [215-222]. Similarly, antibiotic-induced eradication of the intestinal microbiota in mice reduces the risk of cancer in the liver and the colon, as does the depletion of specific microbes in humans [223-227]. Mechanistically, experiments using LPS, a TLR4 agonist, support a

relationship between the microbiome and hepatocellular and pancreatic cancer [228 229], while gut microbiota have been reported to switch p53 from tumour-suppressive to oncogenic suggesting that the functional outcome of a mutation could also be microbiome-dependent [230]. Furthermore, CRC-associated dysbiosis may promote colon carcinogenesis via epigenetic dysregulation [231].

The proposed mechanisms of microbiota-driven carcinogenesis can be summarized as follows: 1) barrier breach, 2) TLR activation, 3) microbial-derived genotoxins and 4) bacterial-derived shifts in local metabolism (Figure 1.3) [232]. In more detail, a key component of the harmonious symbiosis between host and microbiome is the anatomical barrier that separates them in the form of an epithelial lining with or without additional features such as a mucous layer or secretion of antibacterial peptides. Breach of this barrier can lead to invasion of potentially hazardous bacteria into circulation and dissemination to distant organs. The microbiome itself represents a functional barrier by competing with non-indigenous bacterial species for energy resources, thus suppressing thrive of pathogens.

TLRs play a pivotal role in innate immunity by recognizing microbe-associated molecular patterns (MAMPs) and activating both inflammatory and survival signalling pathways. TLR4, a receptor for the Gram-negative bacterial cell wall component LPS, is a tumour-promoter in the colon, liver, pancreas and skin [228 229 233 234]. TLR4 -deficient mice exhibit anti-tumour properties, whereas mice expressing constitutively activated epithelial-derived TLR4 have increased tumourigenesis [233-235]. Downstream effect of TLR4 activation includes signal transduction via the nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways [234 236].

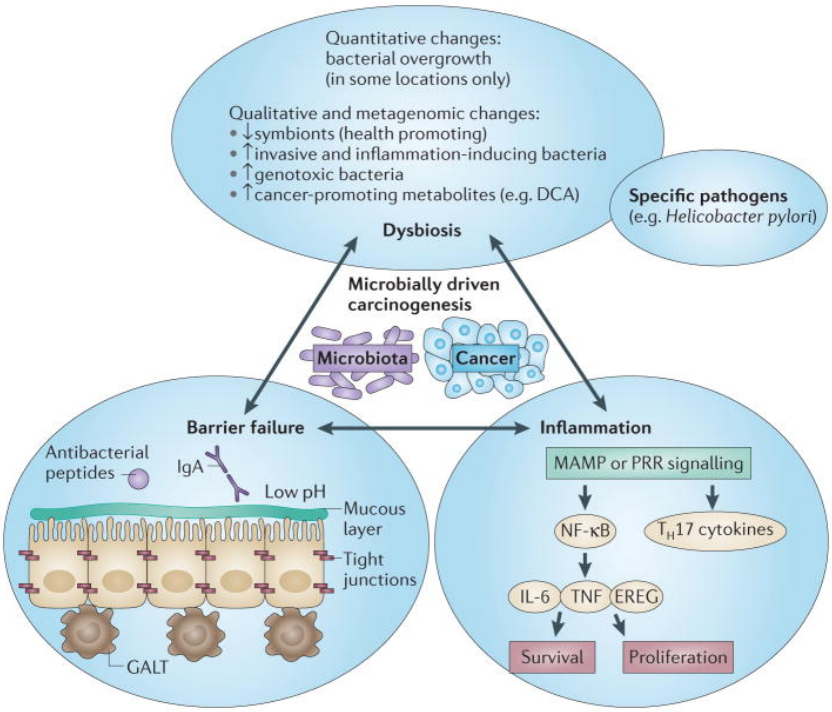


Figure 1.3. Host-microbiota crosstalk and potential contributions to cancer development. Homeostasis is maintained by a eubiotic environment that suppresses overgrowth of pathobionts, creates a mucosal antibacterial barrier and regulates inflammatory pathways. Disruption of the microbial equilibrium manifested as bacterial quantitative and qualitative changes could lead to a failing barrier and inflammation mediated by pattern recognition receptors (PRRs) and downstream cytokines impacting on survival and proliferation pathways. DCA: deoxycholic acid, EREG: epiregulin, IgA: immunoglobulin A, GALT: gut-associated lymphoid tissue, IL-6: interleukin-6, MAMP: microorganism-associated molecular pattern, NF-κB: nuclear factor-kappa B, TH17: T helper 17, TNF: tumour necrosis factor (adapted from Schwabe *et al*. [232], reproduced with permission from Springer Nature).

Microbial-derived genotoxins encompass toxins like colibactin, *B. fragilis* toxin, cytolethal distending toxin and cytotoxic necrotizing factor 1 that compromise DNA repair and cause chromosome instability [237-239]. Bacterial-derived metabolism refers to the metabolic processes carried out by microbes and evidence suggests that this can have a tumour-promoting or anti-tumour effect. Bacteria are involved in nutrient, bile acid and xenobiotic metabolism as well as in biosynthesis of vitamins and isoprenoids [240 241]. Microbial carbohydrate fermentation generates short-chain fatty acids that exert a protective effect by maintaining integrity of the epithelial barrier and inhibiting NF-κB activation in host immune cells [242]. Hormonal metabolism including oestrogens and testosterone is also influenced by bacterial species. For example,

microbiota produce β-glucuronidase that deconjugates oestrogens, increasing their active, unbound circulating levels which in turn can bind to oestrogen receptors and upregulate oestrogen-dependent processes including cancer [197].

1.2.3.2 Anti-tumour microbiome

Commensal microbes can exert beneficial actions and maintain host health and homeostasis. A eubiotic environment promotes immune maturation and development [243], mitigates inflammation [244], confers colonisation resistance against pathogens [244], modulates host metabolism [244], regulates post-translational modifications of the host proteome [245] and affects drug metabolism [246]. Faecal microbiota transplantation from wild mice to laboratory mice has been shown to reduce inflammation and improve resistance against mutagen/inflammation-induced colorectal tumorigenesis demonstrating the protective mechanisms of wild mice microbiota [247].

Substantial evidence suggests that *Lactobacillus* species have anti-inflammatory and anti-cancer properties. For example, lactic acid, produced by lactobacilli, decreases IL-6, IL-8, MIP-3a, TNF-a and RANTES production and increases release of anti-inflammatory IL-10 in human cervicovaginal epithelial cells [248]. Specific *Lactobacillus* species and their metabolites have also been shown to induce cell apoptosis *in vitro* and increase chemosensitivity in cancer cell models [249-251]. Microbes also converge with metabolic pathways as illustrated by a study showing that microbiota regulate type 1 diabetes through Toll-like receptors [252]. Moreover, host insulin signalling can be impaired by specific microbiota that affect the phosphorylation of the insulin receptor, insulin receptor substrate (IRS) and Akt [253] [254]. The positive effect of *Lactobacillus* on metabolism has been illustrated through modulation of glycaemic responses reducing susceptibility to type 2 diabetes and increasing efficacy of obesity treatments [255] [256].

Disruption of the microbial equilibrium induces immune dysregulation that may promote mutagenesis and affect responses to drug treatment [213] [257]. The immunomodulatory potential of the microbiome in cancer has recently been

highlighted by studies that have demonstrated divergent responses to immunotherapy based on intestinal microbial composition [258]. Malignant cells are able to exploit immune checkpoints on T cells, which under normal conditions protect healthy cells from an immune response, as a way to evade immune detection and elimination [259]. Checkpoint inhibitors, anti-CTLA-4 (cytotoxic T-lymphocyte- associated antigen 4) and anti-PD1 (anti-programmed death 1), cancel immune evasion and enable T cells to exert their cytotoxic action against tumour cells with high mutational burden [260]. Animal studies have shown that the efficacy of immuno-therapeutics is diminished in germ-free mice [261] [262], while transplantation of healthy human faecal microbiota or administration of *Bifidobacterium* [261] or *Bacteroides fragilis* [262] in particular, into rodent models increases response to anti-PD-1 immunotherapy by stimulating anti-cancer/anti-bacterial $CD8^+$ T cell response [263-265]. In humans, epidemiological studies have shown that repeated exposure to antibiotics predisposes to development of certain cancers [266], whilst prior antibiotic treatment compromises response to immune checkpoint inhibitor therapy in patients with cancer and reduces overall survival [267]. Interestingly, studies have demonstrated that checkpoint blockade of T cells, that stimulates their action against tumour cells, is influenced by the intestinal microbiome [258]. *L. acidophilus*, for example, has been shown to enhance anti-tumour immunity when combined with CTLA-4 blocking immunotherapy in a mouse colon cancer model [262].

Immune checkpoint inhibitors for endometrial cancer are currently used in advanced and recurrent cases as mono- or combination therapy with chemotherapeutics or PARP/kinase inhibitors in Phase II trials. Response rates have been modest, with the highest rates observed in MMR (mismatch repair) deficient tumours [268]. MMR deficiency/MSI (microsatellite instability) high and PD-L1 (programmed death-ligand 1) positivity have been proposed as predictive biomarkers of response [269] [270] and a high mutational burden appears to be a common denominator in responsive tumours, possibly attributable to their high immunogenicity that makes them amenable to immunomodulation [271]. Studies to explore possible enhancement of

immunotherapeutic efficacy in gynaecological cancers following manipulation of commensal microbiota are still lacking.

1.3 Endometrial Organoids as Cell Models of Gynaecological Disease

To date, gynaecological research has employed a wide array of techniques to address discipline-related queries, including oncogenesis, with varying success. The widespread use of primary 2D cultures has been hindered by their limited expansion and short lifespan. This led to the development of cell lines that can be cultured indefinitely owing to their immortalization, which rescues them from cellular senescence [272]. Their major limitation, however, is that, by definition, they deviate from normal cell physiology, accumulate mutations and undergo cross-contaminations over time, thus compromising reliability of results and necessitating regular authentication of origin [273-275]. On the other hand, animal models exhibit functional completeness given that they represent multiorgan living organisms and recapitulate the complex, inter-organ signalling networks more accurately than cell cultures. However, animal use is accompanied by many disadvantages, such as increased technical skill need, cost, time, decreased scalability and ethical considerations about animal welfare. To circumvent limitations of existing modelling platforms in gynaecological research, a scientific shift to 3D organoid models has recently been undertaken. Organoids are emerging as a promising tool for gynaecological basic and applied science. One of the biggest advantages is that unlike cell monolayers, they demonstrate the fundamental component of spatial organization and cell polarity that is seminal in achieving structural and functional tissue fidelity [276]. A key characteristic of organoid cultures that differentiates them from primary cell monolayers is the preservation of a constant stem cell repository that allows long-term expansion through manipulation of biophysical and biochemical cues.

Two fundamental features of organoid cultures are the extracellular matrix (ECM) and culturing media enriched in growth factors, hormones and signalling molecules. ECM

represents the non-cellular constituent of organs providing not only the structural scaffolding for epithelium, stroma and vessels but also the biochemical/biomechanical signals that cells composing each tissue require for their survival [277 278]. A number of ECM biomaterials have been used in 3D FGT organoid cultures so far, including decellularized tissues (e.g. EHS matrix- Matrigel) [279-286], natural biopolymers (e.g. collagen, hyaluronic acid) [287] and synthetic polymers (e.g. PEG, PGA) [288 289]. The culturing media are pivotal determinants of cell culture prosperity and stemness-differentiation cell fates. In this respect, the WNT/β-catenin signalling pathway and its ligands RSPONDIN-1 and WNT3a have a central role since a solid body of evidence has clearly demonstrated their ability to modulate normal and cancer stem cell self-renewal driving physiological as well as carcinogenic processes [290]. Clevers and Sato have provided significant insight in WNT requirements in small intestine and colon organoid models showing that “mini-guts” can be formed from single Leucine-rich repeat containing G-protein-coupled receptor 5 (Lgr5) stem cells, localized in crypts, when Rspondin-1, EGF (epidermal growth factor), Noggin (BMP inhibitor), nicotinamide and p38 inhibitor were concurrently supplemented to the medium [291]. Conversely, most pluripotent stem cell (PSC)-derived organoids seem to depend on an intrinsic program of self-organisation with minimal reliance on exogenous supplementation of WNT components and growth factors [292].

1.3.1 Establishment and growth dynamics of endometrial organoid cultures

Benign uterine organoids have been derived from a variety of sources in humans and animals and derivation efficiency seems to be source- and cycle phase-dependent. Mouse endometrium yields higher numbers of organoids during the oestrous phase and in the early reproductive period [279], while in humans high formation rates have been reported from non-pregnant secretory endometrium (100%) and decidual tissue (96-100%) when cultured over 7-14 days [280 281]. Formation ability is not affected by menstrual cycle, menopausal status and benign/premalignant pathology, as indicated by successful derivation from proliferative, atrophic, ectopic and hyperplastic endometrium [279 280 282]. Of note, organoids derived from different ASRM (American Society for Reproductive Medicine) grade endometriotic lesions self-organise within

the same timeframe (7-14 days) as healthy endometrium, but their formation efficiency and yield are considerably lower [282]. Other sources of uterine organoids include human uterine leiomyoma and myometrium [293 294], human endometrial mesenchymal stem cells (eMSCs) [295] and bovine endometrium/fibroblast co-cultures [296]. Proliferation and growth potential of benign uterine organoids vary, with a subset prospering in culture whereas the rest undergoing growth arrest and apoptosis [280]. This observation could be attributed in part to the stem/progenitor cell Lgr5+ cargo that appears to be confined to the tips of endometrial glands after birth enabling long-term expansion (>2 passages) only in $Lgr5^{high}$ cell populations in mouse-derived endometrial organoids [297]. Following fragmentation, single eutopic and ectopic endometrial cells are able to self-organise, proliferate and expand in size demonstrating their clonogenic ability [279 282]. Dense cell aggregates, however, demonstrate higher proliferation and growth capability than single cells, which is further accelerated by oestradiol (E2) but not progesterone (P4) [279]. Passage frequency is dictated by cell density and is normally undertaken every 4-7 days in 1:4-1:6 ratio with the most sustainable cultures surviving for over 3 years and demonstrating successful revival post-cryopreservation [281]. Organoids from endometriotic lesions and hyperplastic endometrium demonstrate cellular proliferation and longevity for 4-6 months [282], whilst leiomyoma spheroids exhibit low proliferation potential [293].

Histological type and grade of endometrial cancer do not affect organoid formation ability; reported efficiency rates, however, do vary among studies (20-100%) [282 298-300], which is in accordance with the low formation rates reported for malignant organoids derived from prostate and oesophageal cancer [301 302]. Malignant endometrial organoids form within 12h-20d, but unlike their healthy/benign counterparts their longevity is compromised with only a subset (20-62%) expanding long-term (>2 weeks) reaching a size of >100µm [282 298 299]. Interestingly, the highest sustainability rates of malignant organoids were observed in serous adenocarcinoma (92%) [299].

1.3.2 Biologic properties of endometrial organoids

An ideal research model should reflect the phenotypical and genetic features, receptors, signalling transduction pathways and gene expression patterns of the precursor tissue, ideally with no or minimal aberrations over time.

Human benign eutopic and ectopic endometrial organoids capture parent tissue features at morphological, genomic and transcriptomic level. The glandular architecture is preserved with a columnar epithelial lining, basal nuclei and microvilli directed towards a central lumen containing hyaline, cellular debris and a few degenerating viable cells [279-281]. Tissue-specific molecular and cell polarity markers are maintained along with cellular bi-potency as illustrated by the presence of both secretory and ciliated cells [279-281]. Interestingly, basal expression of oestrogen and progesterone receptors is low in endometrial organoids but significantly enhanced when treated with ovarian hormones [280], while menstrual phase-dependent histological changes and markers also reflect those observed *in vivo* [279].

Endometrial organoids maintain the parental gland genetic make-up, the *MSH2* and *MSH6* mutations of hyperplastic endometrial tissue from Lynch syndrome patients, while fallopian tube (FT) organoids exhibit gene expression patterns of the precursor luteal phase FT epithelium [280 282 303]. The finding that chromosomal stability is maintained from early (P2-4) until late passage (P8-15) in endometrial organoids suggests that organoids can be used as a reliable tool for disease modelling in the long haul [280]. Hormone responsiveness during the menstrual cycle has been showcased in both endometrial and FT organoids [279 280 285]. In endometrial organoids, high $Ki67^{+}$ proliferation was noted after E2 treatment driving cilia formation, secretory features following P4 administration and cell apoptosis after hormone withdrawal [279-281]. When media were enriched with chorionic gonadotropin, human placental lactogen, and cAMP to mimic early pregnancy events, endometrial organoids differentiated into a decidual-like phenotype [280].

The morphological heterogeneity of malignant organoids is reflective of the histological type and grade variations of gynaecological cancer. Low-stage and -grade endometrial cancer organoids exhibit a highly discernible lumen with surrounding cells resembling a gland, whereas high-grade and -stage endometrial lesions generate dense cell clusters and cribriform structures lacking a lumen [282 300]. Endometrial serous adenocarcinoma organoids, on the other hand, demonstrate small buddings that mirror the native tissue papillary structure [299]. Histological concordance of malignant endometrial organoids with primary tissue including pleiomorphic nuclei, disorganised epithelium and breach of basement membrane has been noted along with preservation of tissue-specific molecular and proliferation (Ki67) markers [280 298].

Malignant endometrial organoids have been shown to maintain the genetic and mutational fingerprint of original tissue and display disease-specific gene expression. In endometrial cancer organoids, loss of function mutations in *PTEN*, microsatellite instability, and somatic mutations in *PIK3CA*, *TP53*, *ARID1A*, *POLE* and *FAT1* are preserved [282], while 31 out of 34 somatic single nucleotide variants (S-SNVs) were found to be shared between organoid cultures and parental macroscopically visible lesions of the same tumour [300]. At a transcriptomic level, malignant endometrial organoids exhibited high expression of *WNT*, *IGF1*, *IGF1R*, *STAT3*, *VEGF* and epithelial-mesenchymal transition (EMT) genes, consistent with previous findings on endometrial cancer tissue [282].

1.3.3 Applications of endometrial organoids in gynaecological oncology research

Female genital tract organoids are expected to answer fundamental questions on female genital tract developmental biology and organogenesis as well as on health and disease processes. This undeniably goes hand in hand with a tremendous potential in all three directions of applied biomedical research: drug testing, gene editing and transplantation *in vivo*. Most importantly, organoids pave the way for

personalised medicine since patient-derived cells can be used for the whole spectrum of their applications (Figure 1.4).

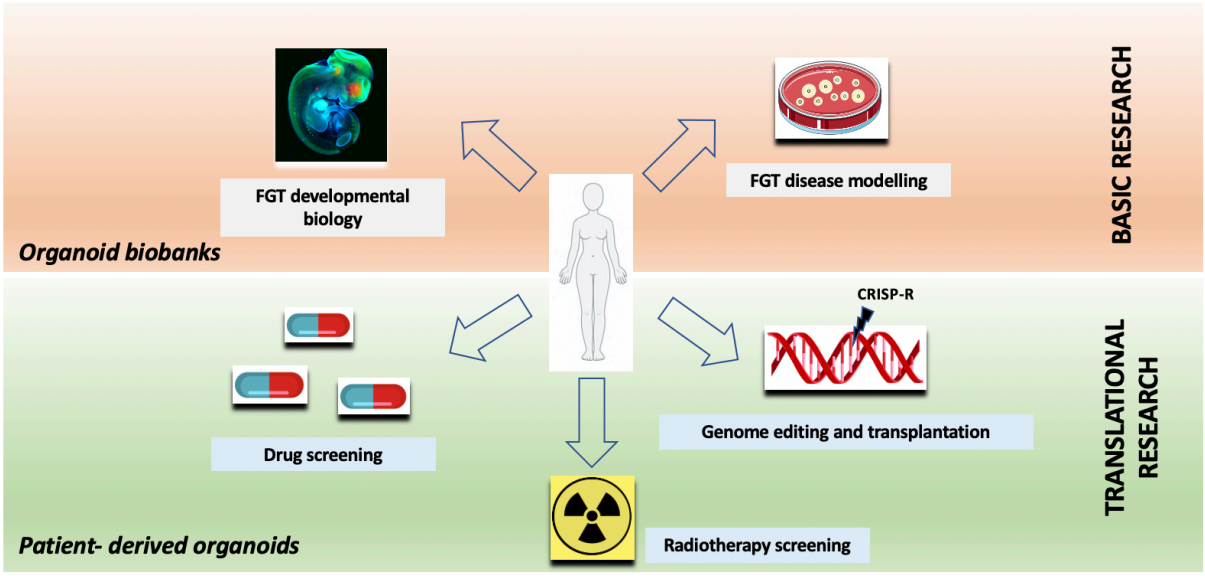


Figure 1.4. Applications of FGT organoids. Organoid biobanks can be established and used in basic science to answer fundamental questions on female genital tract developmental biology and organogenesis as well as on health and disease processes. In applied biomedical research, patient-derived organoids can be used for drug/radiotherapy testing, gene editing and *in vivo* transplantation enabling personalised medicine and maximising therapeutic efficacy (Adapted from Semertzidou *et al.* [304], permission to reproduce not required as the author of this article).

1.3.3.1 Modelling endometrial carcinogenesis and drug discovery

FGT organoids provide a valuable platform to investigate the mechanistic processes *ex vivo* leading to uncontrollable cell proliferation/metastasis and guide new drug discovery attempts. Genome editing of organoids and tumourigenic potential in xenografted models has already been explored in the context of gynaecological malignancy. Sporadic endometrial cancer is characterised by genomic aberrations in PIK3CA, PIK3R1, PTEN, KRAS, FGFR2, ARID1A (BAF250a) and CTNNB1 (β-catenin) genes as well as epigenetic silencing of MLH1 leading to microsatellite instability [305]. Knockdown of *E-cadherin* and *PTEN* expression in endometrial organoids results in disturbed cell polarity, loss of glandular configuration and increased proliferation [306]. Subcutaneous xenotransplantation of malignant uterine

organoids in mice leads to the development of proliferative lesions that retain the histological, molecular traits, and invasive and metastatic potential of the primary tumour [282 299]. High-grade endometrial cancer organoids injected in murine uterine horn generate highly invasive tumours with peritoneal metastases, while low-grade endometrial cancer organoids create localised lesions with lower proliferation [282]. Endometrial organoids also express the mechanosensitive channel PIEZO1 and transient receptor potential (TRP) channels from early (P2) until late (P8) passage demonstrating an enhanced response in premalignant and malignant organoids, that is proportional to the invasiveness of the tumour [282 307]. Of note, TRPV4 (together with TRPM4, TRPM7 and TRPC6) inhibitors significantly reduce endometrial cancer organoid formation efficiency and proliferation potential [282].

1.3.3.2 Drug and radiosensitivity screening in endometrial cancer

Chemo- and radiotherapy resistance is a common, frustrating, yet unpredictable occurrence in a significant number of gynaecological cancer patients, who often undergo excessively toxic treatments with no or minimal therapeutic benefit. It is therefore a clinical and research priority to construct a reliable model that can predict patient responsiveness and enable patient-tailored treatment strategies. Owing to their close morphological and genetic resemblance to progenitor tissue, malignant endometrial organoids are being increasingly used as preclinical models for drug screening and radiosensitivity assays. Several chemotherapeutic agents (paclitaxel, 5-fluorouracil, carboplatin, doxorubicin) and everolimus (mTOR inhibitor) have been tested on endometrial cancer organoids displaying patient-specific responses [282]. STAT3 transcription factor inhibitor BBI608 and paclitaxel appear to significantly arrest organoid growth in most samples, while tyrosine kinase inhibitors and fulvestrant inhibit growth in a subset of cultures [298]. Surprisingly, cisplatin, megestrol acetate, medroxyprogesterone acetate, levonorgestrel and mifepristone had no effect on organoid growth [298]. A study assessing the efficacy of 79 drug agents showed that everolimus has a cytostatic effect on a subset of endometrial cancer spheroids, while the survivin inhibitor YM155 is cytotoxic in non-endometrioid tumours [299].

1.4 Hypothesis & Aims

1.4.1 Rationale

Endometrial cancer is the fourth most common malignancy in women after breast, lung and colorectal disease. Several risk factors have been documented in literature as contributing to the development of endometrial cancer and only a fraction of cases is attributable to inherited genetic predisposition.

Epidemiological, *in vitro* and animal studies have pinpointed metabolic diseases, like obesity and diabetes, to play a pivotal role in endometrial oncogenesis and these are commonly encountered in women afflicted by the disease. Despite the abundance of observational and interventional studies looking into the association of diabetes and anti-diabetic treatments with endometrial cancer, the validity of evidence necessitates thorough scrutiny given the disparities in methodological quality of individual studies. Underpowered studies or bias in the analysis and reporting of findings can lead to erroneous results that overestimate the observed magnitudes of effect. In this regard, umbrella reviews represent the most robust, systematic approach to critically assess the totality of evidence provided by systematic reviews and meta-analyses against clearly defined statistical criteria and test the validity of reported estimates to navigate future healthcare policies.

Recent epidemiological studies have identified strong correlations between pelvic inflammatory disease, which is caused by bacteria, and endometrial cancer [24]. A small number of 16S rRNA gene sequencing studies have identified bacterial compositions in the female genital tract that associate with endometrial oncogenesis, and which can also be observed in obesity and post-menopause bio-ecosystems [158 308 309]. Vaginal high microbial diversity and *Lactobacillus* depletion have been documented in literature to accompany gynaecological precancerous and cancer states, as in the case of cervical dysplasia, cervical and ovarian cancer [136 310]. Collectively, these data suggest a potentially significant but enigmatic contribution of microbiota to endometrial cancer pathogenesis.

However, studies that interrogate the presence of commensal or pathogenic microbiota in the upper genital tract are more sparse owing to the anatomic inaccessibility of female internal organs and proneness to contamination. The low bacterial biomass of the upper genital tract means sample collection must be done under sterile conditions and integrate appropriate technical controls to account for air, equipment and reagent contamination that can be subsequently accounted for in downstream analyses. To date, however, few studies have done this.

Functional microbiome analysis to establish causative relationships is paramount to discover potential for clinical utility. Reliable cell models are required to investigate the role of oncobiome in endometrial carcinogenesis. Primary monolayer endometrial cultures have considerable limitations, e.g., short lifespan, lack of structural complexity, while endometrial cell lines despite their many advantages, e.g., cost-effectiveness, unlimited supply, indefinite propagation in culture, they undergo immortalisation processes and acquire mutations over time due to long-term use, which renders them overall less physiologically relevant. Organoids, on the other hand, are increasingly being used as complex, multi-dimensional, multi-cellular, biomimetic structures resembling entire organs and have now been derived from a variety of tissues, including endometrial. Representing a novel and promising technology in the study of endometrial disease, endometrial organoids, especially those derived from endometrial cancer, have only recently been described and therefore necessitate validation to confirm their morphological and (epi)genetic resemblance to primary tissue.

Unrestrained cell proliferation in endometrial cancer is thought to be triggered by a state of low-grade, chronic inflammation in obese patients. Excess adipose tissue releases free fatty acids and pro-inflammatory cytokines, such as TNF-α, and together with increased LPS circulating levels, secondary to loss of integrity of tight junctions in the gut epithelium of obese patients, an inflammatory cycle is maintained [311 312]. Further to this, evidence has shown that *Lactobacillus* has anti-inflammatory and pro-

apoptotic properties [313-315], suggesting a potential intersection of genital tract commensals with carcinogenic pathways.

1.4.2 Hypothesis

The hypotheses of the work described in this thesis are as follows:

i) Diabetes is an important determinant in endometrial cancer pathogenesis.

ii) The upper reproductive tract carries a microbial fingerprint above background contamination and forms a continuum with the lower genital tract. Moreover, I hypothesise that there is a distinctive microbial signature in the genital tract and rectum of endometrial cancer patients, which differs from benign controls.

iii) Endometrial organoids recapitulate the phenotypic and (epi)genetic features of parent tissue and therefore can be reliably used for disease modelling.

iv) *L. crispatus* has a protective, anti-proliferative, anti-inflammatory effect on endometrial organoids.

1.4.3 Aims

This thesis aimed to investigate the association of metabolic disease and dysbiotic microbiota with endometrial cancer, validate human endometrial organoids as modelling tools and explore the role of female genital tract microbiome in endometrial carcinogenesis. In particular, aims include:

1. To conduct an umbrella review investigating the strength and validity of existing epidemiological evidence for associations between diabetes and anti-diabetic interventions and the risk of endometrial cancer and other gynaecological or obstetric outcomes.
2. To interrogate the presence of a genuine microbial signature above background noise in low bacterial biomass sites (endometrium, fallopian tubes, ovaries) and assess the correlation of lower genital tract (vagina, cervix) microbiota with upper genital tract and rectum.

3. To characterise and compare the female genital tract and rectal microbiota in women with and without endometrial cancer and explore differences in bacterial composition according to disease characteristics (histological type, grade).
4. **a)** To develop an *in vitro* organoid culture model of human benign and malignant endometrium and compare their biologic characteristics.

   b) To assess the preservation of morphological/molecular features, genetic mutations and epigenetic signatures in endometrial cancer organoids in relation to progenitor tissue.
5. To investigate the mechanistic interplay between endometrial microbiota and endometrial carcinogenesis by evaluating the impact of *L. crispatus* supernatant on endometrial organoid proliferation and inflammation.

CHAPTER 2. Materials & Methods

2.1 Materials

Table 2.1. List of materials and reagents used

Experimental process	Reagent/Kit/ Equipment	Manufacturer	Catalogue no
General	Ethanol	VWR	64-17-5
	Phosphate buffered saline (PBS)	In house	-
	Tris/Borate/EDTA (TBE)	In house	-
	DMSO	Sigma	D2650
	Sterile distilled water	Gibco	15230071
	0.5ml Microcentrifuge tubes	Starlab	S1605-0000
	1.5ml Microcentrifuge tubes	Starlab	S1615-5500
	2ml Microcenntrifuge tubes	Starlab	S1620-2700
	15mL Sterile Containers	VWR	21008-216
	50mL Sterile Containers	VWR	21008-242
	10µL Sterile Filter Tip	Starlab	S1121-3810
	20µL Sterile Filter Tip	Starlab	S1120-1810
	200µL Sterile Filter Tip	Starlab	S1120-8810
	1000µL Sterile Filter Tip	Starlab	S1126-7810
Sample collection	Swab plastic liquid amies	VWR	710-0438
	Sterile swab snappable	VWR	115-8271
	Pipelle biopsy MARK II	Eurosurgical	111020100
	Speculum	Pelican	400103
16S rRNA gene extraction	QIAamp DNA mini kit (250)	Qiagen	51306
	Lysozyme	Sigma	L6876
	Lysostaphin	Sigma	L9043
	Mutanolysin	Sigma	M9901
	TE (10mM Tris, 1mM EDTA at pH 7.4)	In house	-
	Triton	Sigma	X100
	Tissue Lyser LT	Qiagen	85600
	0.1mm zircona/silica beads	BioSpec	11079101z
	5mL syringes	Greiner Bio-One	SYR5LL
PCR	AmpliTaq Gold DNA Polymerase	ThermoFisher Scientific	N8080241
	Deoxynucleotide mix (dNTP) 10mM	Sigma	D7295
	Nuclease-free water	Sigma	436912C

	SYBR® Safe DNA gel stain	ThermoFisher Scientific	S33102
	6x Blue/Orange Loading Dye	Promega	G1881
	16s rRNA forward & reverse primers	ThermoFisher Scientific	Custom
	$MgCl_2$ 25µM	ThermoFisher Scientific	R0971
	Agarose	ThermoFisher Scientific	16500500
	StepOnePlus Real-Time PCR system	Thermo Fisher Scientific	4376600
Real-time PCR	BactQUANT 16S rRNA primers	Sigma	Custom
	BactQUANT probe (FAM labeled)	Thermo Fisher Scientific	4316034
	Lyophilized Genomic *E.Coli* DNA Strain B	Sigma	D4889
	Platinum Supermix UDG (including ROX)	Thermo Fisher Scientific	11730-017
	Microbial DNA-free water	Quiagen	338132
	96-well semi-skirted plate	Starlab	E1403-7700
	Optical disposable adhesive	Thermo Fisher Scientific	4311971
	StepOnePlus Real-Time PCR system	Thermo Fisher Scientific	4376600
Organoid cultures +treatments	RPMI-1640	Gibco/Life Technologies	21875-034
	Advanced DMEM/F12 medium	Life Technologies	12634010
	Collagenase from *Clostridium histolyticum*	Sigma-Aldrich	C9891
	DNAse I from bovine pancreas	Roche Diagnostics	11284932001
	Matrigel Growth Factor-Reduced	Scientific Laboratory Supplies	354230 356231
	Cell Recovery solution	Corning	354253
	Antibiotic/antimycotic	Thermo Fisher Scientific, Gibco	15240-062
	N2 supplement	Life Technologies	17502048
	B27 supplement minus vitamin A	Life Technologies	12587010
	N-Acetyl-L-cysteine	Sigma	A9165
	L-glutamine	Sigma	25030-024
	Nicotinamide	Sigma	N0636
	Recombinant human EGF	Peprotech	AF-100-15

	Recombinant human FGF-10	Peprotech	100-26
	Recombinant human HGF	Peprotech	100-39
	Recombinant human NOGGIN	Peprotech	120-10C
	Recombinant human Rspondin-1	Peprotech	120-38
	ALK-4, -5, -7 inhibitor, A83-01	Sigma	SML0788
	Y-27632	Abcam	Ab120129
	Charcoal	Sigma-Aldrich	C9157
	Dextran 70	Sigma-Aldrich	1179741
	Fetal Bovine Serum (FBS)	Thermo Fisher Scientific Gibco	10500-064
	LPS O111:B4 from *E.coli*	Sigma	L2630
	145mm petri dishes	Greiner Bio-One	639161
	No.22 scalpels, disposable, sterile	Swann Morton	0508
	Cell strainers, 40µm, disposable, sterile	Scientific Laboratory Supplies	352340
	48-well flat-bottom cell culture plate	Costar	3548
	96-well flat-bottom cell culture plate	Costar	3596
	Ibidi 8-well chamber	Thistle Scientific	IB-80826
	CryoTube vials 1.8mL	VWR	479-6843
	Cell culture humidified CO2 incubator , 37°C, 5% CO2		
	IncuCyte S3	Sartorius	4647
	Cupric sulfate pentahydrate cell culture	Sigma	C8027
Confocal microscopy	Formaldehyde solution 4%	Sigma	100496
	Bovine Serum Albumin (BSA)	Sigma	A3059
	Triton-X	Fisher Scientific	10102913
	Primary/Secondary Antibodies	*See Methods*	-
	SP5 Confocal Microscope	Leica Microsystems	-
DNA/RNA/miRNA extraction	AllPrep DNA/RNA/miRNA Universal kit	Qiagen	80224
	β-mercaptoethanol 14.3 M	Sigma	M6250
	Isopropanol	Sigma	563935
	QIAshredder homogenizer	Qiagen	79656
DNA quantification	Nanodrop spectrophotometer	Thermo Scientific	ND2000
	Qubit™ dsDNA BR Assay Kit	Thermo Fisher Scientific	Q32850

Organoid viability	CellTiter-Glo 3D Cell Viability Assay	Promega	G9681
	96-well opaque-walled plates	Greiner Bio-One	655075
	Tryptan blue	Thermo Fisher Scientific	T10282
	PHERAstar FS plate reader	BMG LABTECH	-
Magnetic Luminex Cytokine assay	Luminex Human Premixed Multi-Analyte Kit	R&D Systems	LXSAH
	Luminex MAGPIX Analyzer	R&D Systems	MAGPIX-XPON4.1-CEIVD
Protein quantification	Quick Start Bradford Protein Assay kit 2	Bio-rad	5000202
	Quick Start Bovine Serum Albumin Std Set	Bio-rad	5000207
Software	Excel	Microsoft	Version 16.54
	PRISM	GraphPad	Version 8.4.0
	Statistical Analysis of Metagenomic Profiles (STAMP)	http://kiwi.cs.dal.ca/Software/STAMP	
	ClustVis	https://biit.cs.ut.ee/clustvis/	
	MicrobiomeAnalyst	https://www.microbiomeanalyst.ca	

2.2 Methods for umbrella review

2.2.1 Search strategy and selection criteria

A literature search on the association of diabetes with adverse gynaecological and obstetric outcomes was performed in PubMed, MEDLINE, Embase and the Cochrane Database of Systematic Reviews from inception to March 2020 for systematic reviews and meta-analyses published in English. Diabetes encompassed diabetes type 1, type 2 and gestational diabetes. The search algorithm did not include diabetes as part of metabolic syndrome. I further searched for meta-analyses of randomised or non-randomised controlled trials that investigated the impact of anti-diabetic regimens on gynaecological and obstetric outcomes. The search algorithm can be found in Table 2.2. Additionally, the references of eligible articles were manually searched to identify articles missed by the initial search terms and any unpublished data. The protocol for

this umbrella review was registered with Open Science Framework (OSF) (Registration DOI: https://doi.org/10.17605/OSF.IO/9G6AB)

Table 2.2. Search algorithm for umbrella review

PubMed	
Exposure terms	diabe* OR diabetes
	AND
Primary outcome terms	endometrial neoplasm OR malign* OR cancer* OR carcinoma* OR tumor* OR tumour* OR endometr* OR corpus uteri OR uterine OR Ovarian Neoplasms OR Ovar* AND (cancer* OR carcinoma* OR neoplasm* OR tumor* OR tumour* OR adenocarcinoma* OR Endometrioid carcinoma* OR cystadenoma* OR cystadenocarcinoma* OR adenoma*) OR Androblastom* OR arrhenoblastoma* OR sertoli leydig OR Brenner OR granulosa cell tumor* OR granulosa cell tumour* OR luteoma* OR luteinoma OR Cervical neoplasms OR Cervi* AND (cancer* OR carcinoma* OR neoplasm* OR tumor* OR tumour* OR adenocarcinoma* OR squamous cell carcinoma* OR carcinoma OR carcinosarcoma*) OR CIN OR cervical intraepithelial neoplasia OR cervical dysplasia OR cervical precancer* OR Pregnancy OR Pregnant Women OR pregnan* OR Parturition OR parturi* OR gestation* OR Gravidity OR gravid* OR maternal* OR puerperium OR puerperi* OR postpartum period OR postpartum* OR pregnancy complications OR pregnancy outcome OR Polycystic ovary syndrome OR polycystic ovar* OR pco OR pcos OR pcod OR fertility OR infertility OR Abortion, Spontaneous OR miscarriage OR extrauterine pregnancy OR ectopic pregnancy OR abortion, induced OR termination of pregnancy OR molar pregnancy OR IVF OR ICSI OR insemination OR assisted reproduction OR pelvic floor disorders OR urinary incontinence OR fecal incontinence OR pelvic organ prolapse OR uterine prolapse OR vaginal prolapse OR pelvic floor defect OR pelvic floor* OR vaginal wall* OR Reproductive Techniques OR Genital Disease, Female OR Pelvic Floor Diseases OR menorrhagia OR metrorragia OR menstrual cycle OR menstrual disorder* OR heavy menstrual bleeding OR menstrual bleeding OR menstrual pain OR menstrual cycle* OR menopause OR postmenopaus* OR premenopaus* OR climacteric OR contracept* OR Norpregnanes OR Contraceptive Agents OR Contraceptive Agents [Pharmacological Action] OR Contraceptive Devices OR Contraception OR iud OR Intrauterine Devices OR emergency contraception OR Contraception, Postcoital OR nuvaring OR Desogestrel OR Contraceptive Agents, Female OR hormonal patch

	OR ortho evra OR Norgestrel OR Contraceptive Devices, Female OR Congenital abnormalities OR congenital AND abnormalit* OR defect* OR deformit* OR birth AND abnormalit* OR defect* OR deformit*
	AND
Species	NOT animal AND human
	AND
Review terms	systematic review OR meta-analysis OR metaanalysis
Cochrane Database of Systematic Reviews	
Exposure terms	diabe* OR diabetes
	AND
Primary outcome terms	obstetrics OR gynaecology OR endometrial OR cervical OR ovari OR birth OR maternal OR gestati OR pregnancy OR fertility OR menstrual OR polycystic OR hrt OR contracepti

2.2.2 Inclusion and exclusion criteria

Systematic reviews and meta-analyses of observational and randomised/non-randomised controlled trials studies conducted in humans that investigated the association of diabetes and anti-diabetic interventions with gynaecological and obstetric outcomes that were published in English were included in the study. Meta-analyses where exposure was hyperglycaemia, impaired glucose tolerance, glycaemic index/load or diabetes as part of polycystic ovary syndrome (PCOS) and outcomes that were not pertinent to obstetrics and gynaecology (eg. non-gynaecological cancer incl. breast cancer, childhood outcomes in children born to diabetic mothers) were excluded. Additionally, single-arm meta-analyses that lacked a comparison arm, meta-analyses assessing diabetes as an outcome and meta-analyses with study-specific data (number of incident events, number of study population or person years, relative risks and 95% confidence intervals) missing from original papers and which could not be retrieved after contacting authors were excluded.

In cases where more than one meta-analysis existed for the same exposure-outcome association, the meta-analysis with the largest number of cohort studies was selected. In a sensitivity analysis, I further assessed whether there were any differences in the summary findings when the same exposure-outcome association was assessed in more than one meta-analysis.

2.2.3 Data Extraction

For each eligible systematic review or meta-analysis, primary extraction of data was performed, including the name of first author, the journal and year of publication, the exposure (diabetes or anti-diabetic intervention), the exposure contrast (e.g. DM1 vs DM2), the outcome (e.g. endometrial cancer incidence/survival), the overall number of studies in a meta-analysis, the number of cohorts, the number of case-control and other types of studies, the summary relative risk (RR) or odds ratio (OR) and the 95% confidence interval (CI) for each meta-analysis. For duplicate meta-analyses sharing the same exposure, exposure contrast and outcome, the meta-analysis with the largest number of included cohort studies was selected for main analysis provided there were no study-specific data missing that could not be retrieved from primary studies. During secondary data extraction, each individual study per meta-analysis was used to extract the study name, first author and publication year, epidemiological design (cohort, case-control), number of cases and controls in case-control studies (or total population in cohort studies), maximally adjusted relative risk (e.g. odds ratio in case-control studies, risk ratio, hazard ratio or standardized incidence and mortality ratio in cohort studies) and the 95% confidence intervals. Two reviewers independently performed the literature search, assessed eligibility for inclusion and extracted the data. Disagreements were resolved, and consensus reached by discussion with a third investigator.

2.2.4 Evaluation of the strength of evidence by grading criteria

The robustness of evidence of the reported associations was assessed on the basis of previously described criteria [316-318], which form four grades of descending strength:

strong, highly suggestive, suggestive and weak (see Figure 2.1 and previous publications [19 319 320]). An association was deemed as strong when the P value of the random effects model was $<10^{-6}$, the meta-analysis included >1000 cases/exposed women, the P value of the largest included study was <0.05, heterogeneity between studies as measured with I^2 statistic was <50%, the meta-analysis did not show evidence of small study effects, the 95% prediction interval excluded the null value and there was no evidence of excess significance bias. An association was considered as highly suggestive if it presented a P value of $<10^{-6}$ in random effects model, included >1000 cases/exposed women, and the P value of the largest study in the meta-analysis was <0.05. Suggestive associations presented a P value of $<10^{-3}$ in random effects model and included >1000 cases/exposed women. P<0.05 in random effects models indicated weak associations.

2.2.5 Evaluation of methodological quality of included meta-analyses

To assess the methodological quality of included systematic reviews, I used the AMSTAR 2 tool, which uses 16 measures to classify systematic reviews into high, moderate, low or critically low quality [321]. The ever-increasing number of published systematic reviews necessitates stringent scrutiny of the methodology used to distinguish high-quality, bias-free reviews and assist healthcare decision-makers. The original version of AMSTAR (A MeaSurement Tool to Assess systematic Reviews), published in 2007, represented a concise critical appraisal tool that enabled the detailed assessment of the way systematic reviews were planned and conducted [322 323]. The revised AMSTAR 2 instrument retains 10 core domains from the original tool, while adaptations pertain to more simplified response categories, better alignment with PICO (population, intervention, control group, outcome) framework and a more extensive coverage of duplicate study selection and risk of bias for randomised and non-randomised studies (based on the Cochrane risk of bias instruments for randomised and non-randomised (ROBINS-I) studies) [324 325]. Other new domains include consideration for possible causes and significance of heterogeneity, justification of selection of study designs and consideration of excluded studies [321]. The revised tool also acknowledges seven domains as critical for the reliability of

reported outcomes. However, the authors of AMSTAR 2 tool clarify that the list is not exhaustive nor binding, in that critical and non-critical flaws should be determined on an individual review basis. AMSTAR 2 tool does not give an overall score but rather a rating scheme (high, moderate, low or critically low quality) to avoid disguising underlying review design and conduct weaknesses.

2.2.6 Data Analysis

My primary analysis focused on cohort studies, which represent the gold standard among observational studies. Sensitivity analyses were conducted after including case-control studies. I performed further sensitivity analyses and applied the credibility ceilings threshold to account that a single observational study cannot give more than a maximum certainty, c% (credibility ceiling), that the true effect size is in a different direction from the one suggested by the point estimate.

The evidence from interventional meta-analyses of randomised controlled trials was analysed separately and included only statistically significant studies (random p value <0.05), for which the quality of evidence, as assessed by GRADE, was extracted and presented from original meta-analyses. The main and sensitivity analyses conducted for observational and interventional meta-analyses are illustrated in Figure 2.1. All statistical analyses were performed using Stata version 15 (College Station, TX) (StataCorp 2015), and all P values were two tailed. Statistical analysis of extracted data with Stata software was conducted by Ilkka Kalliala.

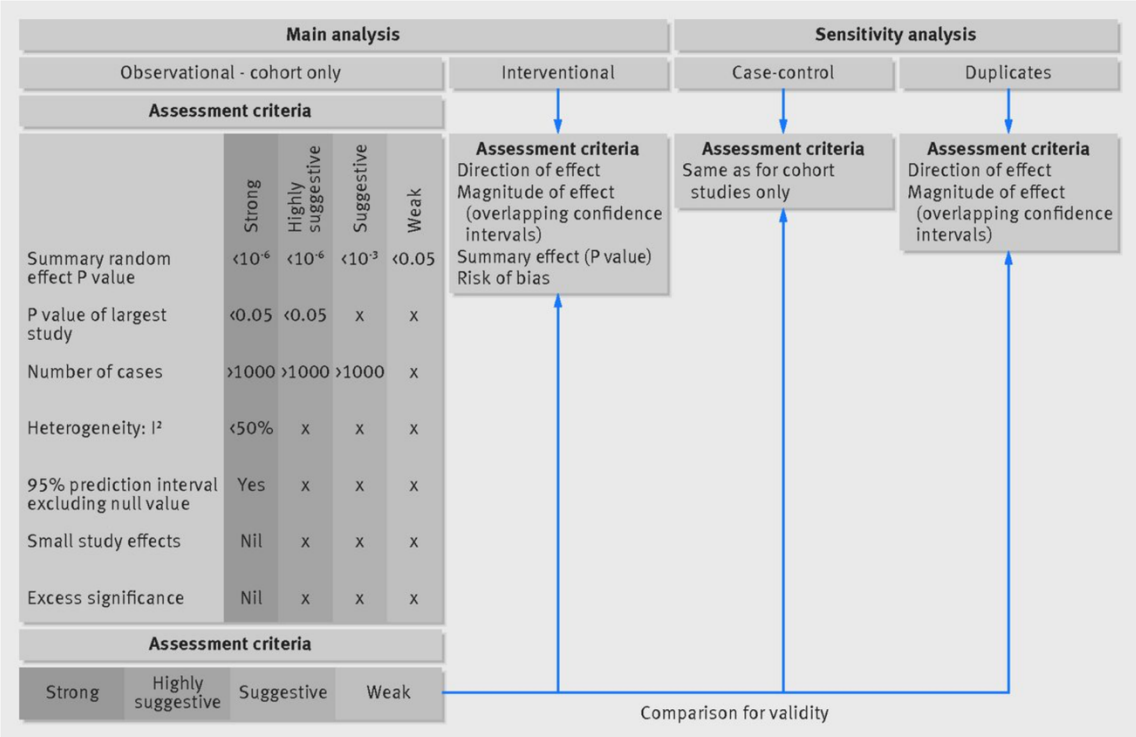


Figure 2.1. Graphical presentation of main and sensitivity analyses of meta-analyses investigating the association of diabetes and anti-diabetic interventions with gynaecological/obstetric conditions.

2.2.6.1 Assessment of summary effect, heterogeneity and prediction intervals

I calculated the summary effect and 95% confidence interval using both fixed and inverse variance weighted random effects methods for each exposure [326]. Inter-study heterogeneity was assessed using the Cochran Q test and the I^2 metric of inconsistency [327] including its 95% confidence intervals [328]. The I^2 statistic describes the percentage of variation across studies that is due to heterogeneity rather than chance [327]. To further account for inter-study heterogeneity, I calculated the 95% prediction intervals for the summary random effect estimates, providing a range in which effect estimates found in a future study will fall [329 330].

2.2.6.2 Assessment of small study effects

Smaller studies in a meta-analysis can generate exaggerated risk estimates when compared to larger studies, for reasons such as publication bias, true heterogeneity or chance [331]. Small study effects were determined by Egger's test at p<0.10 and where more conservative effects in the largest study of a meta-analysis compared to the summary random effects estimate were recorded.

2.2.6.3 Evaluation of excess statistical significance bias

An excess significance test, used to determine whether there is an excess of studies per meta-analysis that have statistically significant results, compares whether the observed number of studies (O) with significant results (so called 'positive' studies, p<0.05) was different from the expected number of significant results (E) [328] under different assumptions about the magnitude of the effect size. The number of significant studies that were expected in each meta-analysis was calculated using the sum of statistical power estimates for each individual study and completed using an algorithm from a non-central *t*-distribution, as used in previous umbrella reviews [320 332-335]. The power estimates of each study depend on the plausible effect size for the tested association, and this was assumed to be the effect of the largest study (i.e., smallest standard error) in each meta-analysis. Sensitivity analyses were performed using the summary fixed and random effects estimates as alternative plausible effect sizes. Excess significance for each individual meta-analysis was defined as two-sided P<0.10.

2.2.6.4 Sensitivity analysis and credibility ceilings

Salanti et al. proposed a sensitivity analysis tool based on the assumption that a single observational study cannot give more than a maximum certainty, c% (credibility ceiling), that the true effect size is in a different direction from the one suggested by the point estimate [336]. I have re-examined the pooled effect size as well as the between-study heterogeneity using a range of credibility ceiling values. This tool accounts for possible methodological limitations of the observational studies, which

can cause overestimation of the combined effect estimates [336 337]. For each meta-analysis and given a ceiling c%, the likelihood ratio of the real effect size being in the direction indicated by the summary estimate for the corresponding unit increase (or level) of exposure was computed [326].

2.3 Analysis of the female genital tract and rectal microbiome

2.3.1 Study population

Women planned for hysterectomy for endometrial cancer or benign conditions (most commonly dysfunctional uterine bleeding, fibroids) were selected. Women were recruited irrespective of the surgical approach (transabdominal open or laparoscopic) or endometrial cancer histological type. Women undergoing hysterectomy for other gynaecological malignancies or pelvic inflammatory disease and endometrial cancer patients that had previous anti-cancer treatment (radio-/chemotherapy) were excluded. Women who reported vaginal douching, antibiotic use within the last two weeks and/or sexual intercourse in the last 48 hours prior to sampling were also excluded. All participants were recruited within Imperial College Healthcare NHS Trust. On the day of surgery, patients gave informed consent and completed a questionnaire, including demographic data, reproductive and menstrual history, oral contraceptive and hormone replacement therapy use, social history, medical and medication history and family history of cancer (see appendix).

2.3.2 Sample collection and storage

Microbiome swabs (VWR Swab Liquid Plastic Amies) were collected from throughout the FGT and rectum by rotating the swab five times against the sites of interest. The lower FGT locations included the lower two thirds of vagina, higher one third of vagina and cervical os. The higher FGT included the lower half of endometrium, fundal endometrium, fallopian tubes and ovaries (Table 2.3). Fallopian tube and ovarian microbiome swabs were not collected in patients with endometrial malignancy as I

thought these are unlikely to contribute to endometrial carcinogenesis. In theatre, prior to cleaning, draping and examination, vaginal and cervical swabs were collected using an unlubricated, disposable, sterile plastic speculum (Medscope Intraspec) before antibiotic administration. Rectal swabs were also collected. In addition to this, a sterile cotton vaginal swab was collected and stored at -80°C in 8% DMSO for bacterial cryopreservation.

The surgical specimen was placed in a sterile bag inside a histology pot and stored at 4°C before being transferred to the histopathology lab on the same day. Under aseptic conditions, the fimbrial end of the fallopian tube and external surface of ovary were swabbed, while endometrial samples were collected after longitudinal dissection of uterus. Tissue samples from the cervix, endometrium and fallopian tube were also collected. All samples were stored at -80°C within 30 minutes (Table 2.3). For the majority of cases, matched samples from the same patient along the genital tract were obtained. Two sets of technical controls were used in the pathology lab. A lysogeny broth (LB) agar plate was left open during sample collection and swabbed afterwards for airborne contaminants. The agar plate was created by mixing 6mg of Agar with 10mg of LB in 500ml of distilled water. The solution was placed in a bottle with a shaker rod and autoclaved for 2 hours. The plates were poured under a fume hood and allowed to set for 45 minutes. The packaged, non-sterile knife used for uterus dissection was also sampled.

Table 2.3. Microbiome swab/tissue collection and storage.

Sample type	Sampling site	Post-sampling processing
Low/High vaginal and cervical swab	Lower 2/3 of vagina, posterior fornix and cervical os	Swab inserted directly into supplied transport vessel and frozen at -80°C within 30 minutes of collection.
Cervical tissue	Ectocervix	Tissue collected in cryovials and stored at -80°C within 30 minutes of collection.
High vaginal swab	Posterior fornix	Bacterial cryopreservation: Cotton swab tip broken off in 1.5mL Eppendorf tubes with 8% DMSO

		and stored at -80°C within 30 minutes of collection.
Low/High endometrial, fallopian tube and ovarian swab	Lower and fundal endometrium, fimbrial end of fallopian tube, external surface of ovary	Swab inserted directly into supplied transport vessel and frozen at -80°C within 30 minutes of collection.
High endometrial, fallopian tube tissue	Fundal endometrium, fimbrial end of fallopian tube	Tissue collected in cryovials and stored at -80°C within 30 minutes of collection.
Rectal swab	Distal 2cm of rectum	Swab inserted directly into supplied transport vessel and frozen at -80°C within 30 minutes of collection.
Controls in path lab Air contamination swab Knife swab	 LB agar plate Knife for uterus dissection	Swab inserted directly into supplied transport vessel and frozen at -80°C within 30 minutes of collection.

2.3.3 Swab bacterial DNA extraction

Bacterial DNA was extracted from microbiome swabs using QiAmp Mini DNA kit (Qiagen, Venlo, Netherlands). Swabs were removed from the -80°C freezer and thawed slowly on ice. Amies transport solution (approx. 500µl/swab) was squeezed from the transport sponge into a 2ml centrifuge tube using a 5ml syringe. Samples were centrifuged at 5000 x *g* for 10 minutes. The Amies supernatant was removed and stored at -20°C for further cytokine studies. Cells in pellet were lysed in a lysis buffer cocktail containing 50µl lysozyme (10mg/ml) (Sigma, Dorset, UK), 6µl mutanolysin (25,000U/ml) (Sigma), 3µl lysostaphin (4,000 U/ml) (Sigma), 41µl TE (10mM Tris, 1mM EDTA at pH 7.4) per sample, at 37 °C for 1 hour. Mechanical lysis was carried out by bead beating by adding 100mg bleached and rinsed 0.1mm zircona/silica beads to each sample and oscillating for 1 minute at 25Hz in a Micro Dismembrator (Sartorius, Goettingen, Germany). After brief centrifugation to bring down the beads, the supernatant lysate was transferred to fresh 2ml centrifuge tubes and a QiAmp Mini DNA kit (Qiagen) used according to the manufacturers protocol. Finally, 50µl elution buffer (supplied with kit) was added to give 50µl template DNA per swab. Polymerase chain reaction (PCR) was carried out using 16S rRNA gene barcoded primer (Invitrogen, Carlsbad, CA, USA) to confirm presence of bacterial DNA as described in section 2.3.5. Twenty-five microliters of bacterial DNA per patient

were sent for sequencing using a MiSeq platform (Illumina, San Diego, CA, USA) to the Digestion, Metabolism & Reproduction Department (St Mary's Hospital, London, UK).

2.3.4 Tissue bacterial DNA extraction

The extraction of bacterial DNA from tissue followed a similar protocol to the extraction from microbiome swabs with chemical and mechanical lysis. However, the initial steps prior to chemical lysis differed as the tissue was dissected using a sterile knife and petri dish. Tissue was suspended in 500 µl PBS (phosphate-buffered saline), vortexed for 30 seconds and placed in the Tissue Lyser at 50 Hz for 2 minutes. The supernatant (250 µl) was extracted and stored at -80°C. The following steps were identical to the above description as per microbiome swab extraction.

2.3.5 16S rRNA polymerase chain reaction (PCR)

The V1-V2 hypervariable regions of 16S rRNA genes were amplified by PCR using a forward and reverse fusion primer. PCR was performed to confirm presence of bacterial DNA prior to MiSeq sequencing. This was done as a quality control step following bacterial DNA extraction. PCR master mix was made as per Table 2.4, containing 2µl template DNA, to give a total of 50µl per reaction. PCR-grade sterile water was used as a negative control, purified bacterial DNA was used as a positive control, and a blank extracted swab was used as a blank. PCR was run on a BioRad Tetra 2 machine (BioRad) using the conditions detailed in Table 2.5. Eight microliters of 6x Blue/Orange Loading Dye was added to each PCR tube and the PCR products were run on a 2% agarose gel, containing SYBR® Safe DNA gel stain with 1x Tris/Borate/EDTA (TBE) used as running buffer. The gel was viewed under ultraviolet (UV) light.

Table 2.4. Bacterial PCR Mastermix (Total 50µl per reaction)

Item	Volume per reaction
10 x AmpliTaq Gold Buffer (ThermoFisher Scientific)	5µl
MgCl2 25µM (ThermoFisher Scientific)	5µl
AmpliTaq Gold DNA Polymerase (ThermoFisher Scientific)	0.25µl
16S rRNA forward primer (Invitrogen) Sequence: 3' GCC TTG CCA GCC CGC TCA GTC AGA GTT TGA TCC TGG CTC AC	1µl
16S rRNA reverse primer (Invitrogen) Sequence: 5' GCC TCC CTC GCG CCA TCA GCA CTG CAT GCT GCC TCC CGT AGG AGT	1µl
Deoxynucleotide mix (dNTP) 10mM (ThermoFisher Scientific)	1µl
PCR-grade sterile water (Sigma)	34.75µl
Template DNA (from bacterial DNA extraction)	2µl

Table 2.5. PCR conditions for 16S rRNA gene primers

Step 1. Incubate at 95^{0}C for 5 minutes.
Step 2. Incubate at 95^{0}C for 30 seconds.
Step 3. Incubate at 55^{0}C for 30 seconds.
Step 4. Incubate at 72^{0}C for 1 minute 30 seconds.
Step 5. Cycle to step 2 for 30 more times.
Step 6. Incubate at 72^{0}C for 7 minutes.
Step 7. Incubate at 4^{0}C till removed.

2.3.6 Quantitative PCR (qPCR) of the 16S rRNA gene

Quantitative PCR (qPCR) was carried out for the quantification of 16S rRNA gene copy number in order to determine the bacterial load at multiple anatomical sites of benign and endometrial cancer patients. Real-time qPCR was performed with universal BactQUANT 16S rRNA primers (Forward primer: 5'-CCT ACG GGA GGC AGC A, Reverse primer: 5'-GGA CTA CCG GGT ATC TAA TC) (Sigma) with the FAM labeled BactQUANT probe ((6FAM) 5′- CAGCAGCCGCGGTA-3′ (MGBNFQ)) [338] on the Applied Biosciences StepOne machine (Thermo Fisher Scientific, Ashford, UK)

with StepOne software (Version 2.3, Life Technologies). Each 20 µl reaction included a standard curve. Lyophilized Genomic DNA from *Escherichia coli (E.coli)* Strain B (Sigma, Dorset, UK) was serially diluted in diethylpyrocarbonate (DEPC) water to make a ten-fold standard curve to obtain concentrations from $3x10^5$ to $3x10^0$ copies of 16S rRNA gene to establish a standard curve from 300,000 to 3 copies (cps) of 16S rRNA gene according to Table 2.6.

Fifteen microliters of Mastermix (Table 2.7) were added into each well of the optical 96-well reaction plate, followed by 5µl of standards or sample. The plate was sealed and spun for 20 seconds in a plate centrifuge immediately prior to StepOnePlus real-time PCR system using the PCR cycle conditions as detailed in Table 2.8. Sterile water was used as negative control. Samples were run in duplicates. Total DNA amount for each sample was calculated by multiplying 16S rDNA quantity in each loaded sample (5µL) by the total volume of extracted DNA (50µL). All apparatus and plasticware were exposed to UV light for 15 minutes prior to use to destroy any DNA contaminants.

Table 2.6. Dilutions for *E. coli* standard curve.

Standards (S)	**S5** **300,000 cps**	**S4** **30,000 cps**	**S3** **3,000 cps**	**S2** **300 cps**	**S1** **30 cps**	**S0** **3 cps**
Recipe	85.71µl (Stock *E. coli*) + 14.29µl H_2O	10µl S5 + 90µl H_2O	10µl S4 + 90µl H_2O	10µl S3 + 90µl H_2O	10µl S2 + 90µl H_2O	10µl S1 + 90µl H_2O

Table 2.7. 16S rRNA qPCR Mastermix (Total 20µl per reaction)

Item	**Volume per reaction**
Diethylpyrocarbonate (DEPC) water (ThermoFisher Scientific)	4.195µl
Platinum Supermix UDG (including ROX) (ThermoFisher Scientific)	0.04µl
BactQuant forward primer 100 µM (0.025 µmol synthesis scale, desalted) (Sigma) Sequence: 5'- CCT ACG GGA GGC AGC A	0.36µl

BactQuant reverse primer 100 µM (0.025 µmol synthesis scale, desalted) (Sigma) Sequence: 5'-GGA CTA CCG GGT ATC TAA TC	0.36µl
BactQuant probe (LifeTech, 6000 pmol scale) Sequence: (6FAM) 5′-CAGCAGCCGCGGTA-3′ (MGBNFQ)	0.045µl

Table 2.8. qPCR conditions for 16S rRNA gene primers

Step 1. Incubate at 50^{0}C for 2 minutes.
Step 2. Incubate at 95^{0}C for 10 seconds.
Step 3. Incubate at 95^{0}C for 15 seconds.
Step 4. Incubate at 60^{0}C for 60 seconds.
Step 5. Cycle to step 3 for 40 more times.

2.3.7 16S rRNA gene sequencing

The V1–V2 hypervariable regions of 16S rRNA genes were amplified for sequencing using 28F primer (5′-GAGTTTGATCNTGGCTCAG-3′) and 388R primer (5′-TGCTGCCTCCCGTAGGAGT-3′). PCR amplification was performed in 25 µl reaction volumes with Qiagen HotStar *Taq* master mix (Qiagen Inc, Valencia, California), 1µl of each 5uM primer, and 1µl of template on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) using the following thermal profile: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 40 sec, 72°C for 1 min, followed by one cycle of 72°C for 10 min and a 4°C hold. Amplicon libraries were cleaned and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana) following the manufacturer's protocol. Pooled sequence libraries were quality checked and quantified using gel electrophoresis, Qubit 2.0 Fluorometer (Life Technologies) and quantitative PCR, and loaded at 10pM on an Illumina MiSeq (Illumina, San Diego, California) using 2x300bp chemistry. Sequencing was performed in the Digestion, Metabolism & Reproduction Department (St Mary's Hospital, London, UK) using an Illumina MiSeq platform (Illumina Inc).

2.3.8 16S rRNA gene sequencing data analysis

Sequence data was processed in Mothur using the MiSeq SOP Pipeline [339]. OTUs were defined using a cut-off value of 97% and resulting data analysed using the Vegan package within the R statistical package for assessment of microbial composition and diversity (R Development Core Team 2008). OTU taxonomies (from Phylum to Genus) were determined using the ribosomal database project (RDP) MultiClassifier script to generate the RDP taxonomy [340], whereas species level taxonomies of the OTUs were determined using the USEARCH algorithm (v.11) combined with the cultured representatives from the RDP [341] and STIRRUPS [342] databases. Alpha and beta indices were calculated from these datasets within Mothur and the Vegan package with the R environment (R Development Core Team 2008). Processing of 16S rRNA gene sequencing data in Mothur using the MiqSeq SOP pipeline and assignment of OTU taxonomies were performed by Dr Ann Smith.

2.3.9 Statistical Analysis of 16S rRNA gene sequencing data

2.3.9.1 Removal of contaminating sequence reads

In the presence of low bacterial biomass samples prone to environmental and kit contamination, I integrated four different sets of technical controls for air, equipment (knife) and kit (DNA extraction and 16S rDNA sequencing) to detect and remove taxonomic units that were likely contaminants using the R package Decontam (v1.6.0) with a prevalence-based threshold of 0.5 (p-value calculated using Chi-square/Fisher's Test that is below 0.5 was identified as a contaminant) [343]. Processing of sequencing data with Decontam package for identification of contaminant sequences was performed by Dr Sherrianne Ng.

2.3.9.2 Determining genuine signatures and microbiota continuum

To assess genuine microbial presence in low-biomass samples (endometrium, fallopian tubes and ovaries) above background contamination following removal of likely contaminants by the Decontam R package, I further filtered my data by including OTUs with at least 5 counts in 10% of samples and low variance (IQR) 5%. The

remaining OTUs in both patient samples and controls were compared through hierarchical clustering analysis (HCA) at genera level and univariate analysis (Mann-Whitney/Kruskal-Wallis, *p* value< 0.05) at species level to determine taxa enriched in low biomass samples versus contaminant controls.

To explore the presence of a microbial continuum along the length of the female genital tract and between the vagina and rectum, I defined as a continuum the presence of bacterial species at a relative abundance of at least 0.5% in all sites of the lower and upper genital tract (vagina, cervix, endometrium, fallopian tube, ovary) in benign patients; in the vagina, cervix and endometrium in endometrial cancer patients and in the vagina and rectum of all patients regardless of malignancy and used Venn diagrams to depict patterns of overlapping colonisation among sites. High vaginal and fundal endometrial samples were used for comparisons.

2.3.9.3 Compositional comparison of microbiota in endometrial cancer versus benign controls

Analysis of statistical differences between microbiota of women with and without endometrial malignancy was performed using the Statistical Analysis of Metagenomic Profiles (STAMP) package (v.2.1.3) [344], ClustVis [345] and Marker-gene Data Profiling (MDP) module of MicrobiomeAnalyst [346]. Data were subjected to multivariate analysis using hierarchical clustering analysis (HCA) by Ward clustering with a density threshold of 0.75 and were rarefied to minimum library size prior to analysis. The most commonly identified genera were included, while remaining OTUs were classified as 'Others'. For α-diversity, which reflects richness and evenness within bacterial populations of each sample, I calculated the Shannon index applying the Mann-Whitney/Kruskal-Wallis statistical test, while β-diversity, which mirrors shared diversity between bacterial populations of different samples, was calculated using the Bray-Curtis index and compared using PERMANOVA statistical test.

Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify taxa significantly overrepresented in endometrial cancer patients when compared to

benign controls, through all taxonomic levels [347]. This analysis was performed using taxonomic relative abundance, with per-sample normalisation and default settings for alpha values (0.05) for the factorial Kruskal–Wallis test among classes and pairwise Wilcoxon test between subclasses. A logarithmic LDA score >2 was used to determine discriminative features.

2.4 Methods for endometrial organoid cultures and comparison with primary tissue

2.4.1 Study population

Women undergoing hysterectomy for endometrial cancer or benign conditions were recruited for endometrial organoid generation. All participants were recruited within Imperial College Healthcare NHS Trust and gave informed consent prior to surgery.

2.4.2 Establishment of endometrial organoid cultures from pipelle biopsies

Pipelle endometrial biopsies were collected from hysterectomy specimens in theatre and transferred to the lab in RPMI-1640 medium within 30 minutes. Biopsies were collected either immediately after uterus extraction or occasionally hysterectomy specimens were stored at 4^0C in theatre fridges for no more than one hour. Biopsies were transferred onto a petri dish and minced with scalpels to 0.5-1mm^3 pieces. Minced tissue was transferred to a disposable Falcon tube and 10mL of collagenase V/DNAse solution was added. The container was placed in the incubator at 37^o C for 1 hour with gentle shaking and/or vortexing every 20 minutes. Further disaggregation was halted with RPMI-1640 medium, the cell suspension was passed through 40µM cell sieves and washed with 5 mL RPMI-1640 medium to remove stromal cells and blood/clots. Sieves were inverted and glandular elements were backwashed with 10 mL RPMI-1640 into a universal tube, which was subsequently centrifuged at 500 x *g* for 5 min. The supernatant was aspirated and the pellet was resuspended with 1 mL of Advanced DMEM/F12 medium followed by gentle pipetting to partially dissociate cells. Glandular elements were transferred to 1.5ml microcentrifuge tubes and

centrifuged at 500g for 5 min. The supernatant was removed and ice-cold Matrigel was added at a quantity 20 times the tissue volume. The cell pellet was resuspended and placed on ice. A total of 20 µL of Matrigel/cell suspension was dropped into the centre of pre-warmed 48-well tissue culture plates using pre-cooled pipette tips and plates were placed in the incubator at 37^{o} C for 15 minutes. Lastly, 250 µL of pre-warmed expansion medium, made up of Advanced DMEM, supplemented with EGF, FGF10, HGF, Rspondin 1, nicotinamide, Noggin and other factors (see Table 2.9), was added in each well and plates were stored in the incubator at 37^{o}C and 5% CO2. Expansion medium was replaced every 2-3 days, cells were passaged every 2-3 weeks as dictated by cell density, the Matrigel becoming unstable, or the cells starting to attach to the bottom of the well, at 1:2-1:3 ratio depending on cell confluence.

Table 2.9. Recipe of 20 mL expansion medium.

Material	Final concentration	Stock concentration
Advanced DMEM/F12	1*	1*
N2 supplement 100x	1*	1*
B27 supplement 50x	1*	1*
N-acetyl-L-cysteine	1.25mM	1*
L-glutamine	2mM	1*
Antibiotic/antimycotic	1*	1*
A83-01	500nM	5.9309mM
Nicotinamide	10mM	1mM
Noggin	100ng/ml	100ug/ml
R-spondin-1	500ng/ml	100ug/ml
EGF	50ng/ml	100ug/ml
FGF10	100ng/ml	100ug/ml
HGF	50ng/ml	100ug/ml
Y-27632	10uM	10mM

2.4.3 Passaging of endometrial organoids

Without removing the culture medium, each well surface was scraped to detach Matrigel and contents were transferred to 1.5 mL tubes at a ratio of 2-4 wells per tube.

Tubes were centrifuged at 600 x *g* for 6 min to pellet. The supernatant was removed, 150 µL of cool Advanced DMEM F12 was added to each tube and pipetted 300 times to break up the organoids and Matrigel. 1ml cool Advanced DMEM F12 was added and centrifuged at 600g for 6 min. The supernatant was again removed, 150 µL cool Advanced DMEM F12 was added and pipetted 80 times using moderate force. 1ml cool Advanced DMEM F12 was added and centrifuged at 600g for 6 min. Supernatant was aspirated and 20 µL of Matrigel per well to be plated added. The cell pellet at this stage contained very little Matrigel. Finally, 20 µL drops of cells/Matrigel was dispensed on centre of well of pre-warmed 48-well culture plates and incubated at 37^{o}C for 15 minutes. 250 µL of warm expansion medium were added in each well and plates were stored in the incubator.

2.4.4 Freezing and thawing of endometrial organoids

Both gland digests, following separation from stromal cells, and established glandular endometrial organoids in culture were frozen using 1 mL Dextran-coated charcoal (DCC)-treated FBS and 1 mL 20% DMSO as cryoprotectant, transferred to labelled cryovials on ice and stored at -80^{0}C. DCC-FBS was made up of 1.25 g of charcoal and 125 mg of dextran 70 added to 500 mL of FBS, mixed thoroughly and incubated at 56 °C in the water bath for 2 h with shaking every 30 min. The mixture was centrifuged at 1,800 *x g*, for 30 min, the supernatant was sterile-filtered using the vacuum-driven filtration system, aliquoted and stored at -20 °C. The benign endometrial gland digests offered by Prof Jan Brosen's lab were frozen following the same protocol except digests were placed in Mr Frosty at -80C overnight and transferred to liquid nitrogen the next day for long-term storage.

For organoid cryopreservation, culture medium was removed from the wells and replaced with 250 µL Cell Recovery Solution (Corning). Organoids and Matrigel were scraped from the well, collected in 1.5 ml low-binding Eppendorf tubes and placed on ice for 60 minutes to dissolve Matrigel. Tubes were centrifuged at 600g for 6 min to pellet organoids. Supernatant was removed, 200 µL Advanced DMEM F12 was added to each tube and suspension was manually pipetted gently up and down 80 times with

moderate force to partially disrupt organoids. 1ml Advanced DMEM F12 was added and centrifuged again. Supernatant was removed and pellets were re-suspended in 1 mL DCC-treated FBS and 1 mL 20% DMSO for each tube with gentle mixing.

For thawing, cryovials were removed from freezer/liquid nitrogen, transferred in dry ice to the lab and placed in water bath for 10' until liquid. 5 mL of RPMI-1640 medium was added and centrifuged at 500g for 5 minutes. Supernatant was aspirated, another 5 mL of RPMI-1640 medium added and pellet was resuspended. The protocol for setting up organoid cultures was then followed as described above.

2.4.5 IncuCyte S3 Live-cell imaging

To visualise endometrial organoid growth in real time, the IncuCyte® S3 Multi-Spheroid Assay was used. 96-well flat bottom plates were coated with 100% Matrigel diluted in RPMI-1640 medium (1:1) (40 µl/well) and placed in a 37^0C incubator for 30 minutes for Matrigel to polymerize. Digested endometrial glands, suspended in expansion medium (150 µl/well), were seeded on top of polymerized Matrigel, the plate was placed inside IncuCyte and scanned every 3h with a 10x objective.

2.4.6 Transmission electron microscopy (TEM)

TEM analysis was performed as previously described in detail [279]. In brief, organoids were removed from Matrigel and sequentially fixed in glutaraldehyde and osmium tetroxide/potassium ferrocyanide. Organoids were incubated with tannic acid for 20 min and uranyl acetate overnight, followed by aspartate for 30 min. Samples were dehydrated and embedded in epoxy resin and 70 nm sections were analysed using a JEM1400 transmission electron microscope (JEOL) equipped with an Olympus SIS Quemesa 11- megapixel camera.

2.4.7 Confocal microscopy

For immunofluorescence, endometrial organoids were fixed in 10% formaldehyde (250 µL/well) for 20-30 minutes at room temperature, washed twice with PBS and

permeabilized in blocking buffer plus 0.3% Triton-X for 10 minutes at 4^0C. Blocking buffer was made up by supplementing 1% w/v BSA in PBS. Organoids were incubated with primary antibodies at 4 °C overnight, followed by incubation with fluorescent conjugated Alexa Fluor secondary antibodies for 2 h at room temperature. Both primary and secondary antibodies were diluted (1:100 primary, 1:200 secondary) in blocking buffer. Samples were washed with PBS twice before visualisation on confocal microscope. For antibodies used, see Table 2.10. Negative controls were prepared by omitting primary antibody. For nucleus and cell membrane staining, DAPI (5µg/ml) and CellMask were used respectively for 5 minutes after fixation in formaldehyde and washed twice with PBS before imaging. Organoids were imaged using the inverted Leica SP5 confocal system and software.

Table 2.10. Primary/Secondary antibodies and stains.

Antibody/Stain	Host	Dilution	Manufacturer	Catalogue number
DAPI stain	-	5µg/ml	Sigma	D9542
CellMask stain	-	1x	Thermo Fisher Scientific	C10046
MUC1	Hamster	1:100	Thermo Fisher Scientific	MA511202
Cytokeratin 7	Rabbit	1:100	Thermo Fisher Scientific	PA529033
E-cadherin	Mouse	1:100	Thermo Fisher Scientific	131700
Ezrin	Mouse	1:100	Thermo Fisher Scientific	MA513862
cd49f	Rabbit	1:100	Thermo Fisher Scientific	710209
TLR-4	Rabbit	1:100	Thermo Fisher Scientific	PA523124
Alexa Fluor® 488	anti-hamster	1:200	Thermo Fisher Scientific	A21110
Alexa Fluor® 555	anti-mouse	1:200	Thermo Fisher Scientific	A21127
Alexa Fluor® 633	anti-rabbit	1:200	Thermo Fisher Scientific	A21071

For time-lapse imaging, organoids were plated in 35mm ibidi µ-dishes and mounted on an inverted confocal laser scanning microscope (Leica SP5), which was continuously held at 37 °C and equipped with a culture chamber for overflow of 5% CO_2. Over 48h, organoids were imaged in XYZT-mode using a ×40 objective. Images were taken at 4 min intervals.

2.4.8 DNA/RNA/miRNA extraction from organoid-tissue pairs

Genomic DNA and total RNA, including miRNA, were extracted simultaneously from the same sample using the Qiagen AllPrep DNA/RNA/miRNA kit. Endometrial

organoid and matched tissue samples were removed from -80^{0}C and tissue was weighed. Between 10-30mg of tissue was used to which 600 µL of buffer RLT Plus was used to disrupt the plasma membranes of samples and release all nucleic acids. Tissue samples required an extra homogenization step using the QIAshredder homogenizer, where lysate was homogenized as centrifuged through a spin column for 2 minutes at maximum speed. Organoid samples were homogenized by vortexing for one minute. The samples were processed as per manufacturer's instructions. In brief, the lysates were passed through an AllPrep DNA Mini spin column, which in combination with a high-salt buffer, allowed binding of genomic DNA. On-column Proteinase K digestion in optimized buffer conditions allowed genomic DNA purification from samples. The column was washed and 100 µL DNA was eluted. Flow-through from the AllPrep DNA Mini spin column was digested by Proteinase K in the presence of ethanol. Subsequent addition of further ethanol, allowed appropriate binding of total RNA, including miRNA, to the RNeasy Mini spin column. DNase I digestion ensured yield of DNA-free RNA. Following DNase I digestion, contaminants were efficiently washed away and 50 µL RNA was eluted. DNA and RNA were quantified using both the Nanodrop spectrophotometer and Qubit™ dsDNA BR Assay Kit, and quality was assessed by 260/280 and 260/230 absorbance ratios.

2.4.9 Targeted gene sequencing of organoid-tissue pairs

NEBNext Direct® Custom Ready Panels (New England BioLabs) for the coding regions of *PTEN, ARID1A, PIK3CA, POLE, CTNNB1, KRAS, TP53* genes were used as per manufacturer's instructions. The NEBNext Direct workflow is graphically illustrated in Figure 2.2. Briefly, DNA extracted from endometrial cancer organoids and matched tissue was removed from -20^{0}C and 200ng of input DNA was used. DNA was enzymatically fragmented. 72 µL of hybridization master mix was used to hybridize DNA to biotinylated oligonucleotide baits for 90 minutes, that captured both strands of the target DNA and defined the 3´ ends of the regions of interest. After hybridization, the bait-target hybrids were bound to 75 µL of streptavidin beads per reaction, and any 3´ off-target sequences were removed enzymatically by adding 100 µL of 3´ Blunting master mix per sample and incubating at 37^{0}C for 10 minutes. dA-Tailing was

performed by adding 100 µL of dA-Tailing master mix to each sample and incubating at 37^0C for 10 minutes. An adaptor was ligated to the 3' end by adding 100 µL of 3´ Adaptor Ligation master mix to each sample followed by incubation at 20^0C for 15 min. 5' Blunting of DNA was next performed by using 100 µL of master mix succeeded by incubation at 20^0C for 10 minutes. The trimmed targets were converted into Illumina-compatible, sequence-ready libraries that included a 12 bp unique molecular identifier (UMI) in the Illumina i5 index location and an 8 bp sample barcode in the Illumina i7 index location. The unique molecular identifier tagged each individual molecule prior to the final PCR amplification to enable identification of PCR duplicates, and barcodes were added to each library during PCR. The NEBNext Direct Index Primer Mix Plate was used for library amplification. Each PCR reaction (100 µL) contained 50 µL Master mix, 5 µL index primer mix and 45 µL resuspended beads. The sequencing was performed on the MiSeq on a PE150 mode. PCR conditions can be found in Table 2.11. NEBNext targeted gene sequencing took place at Imperial BRC Genomics Facility.

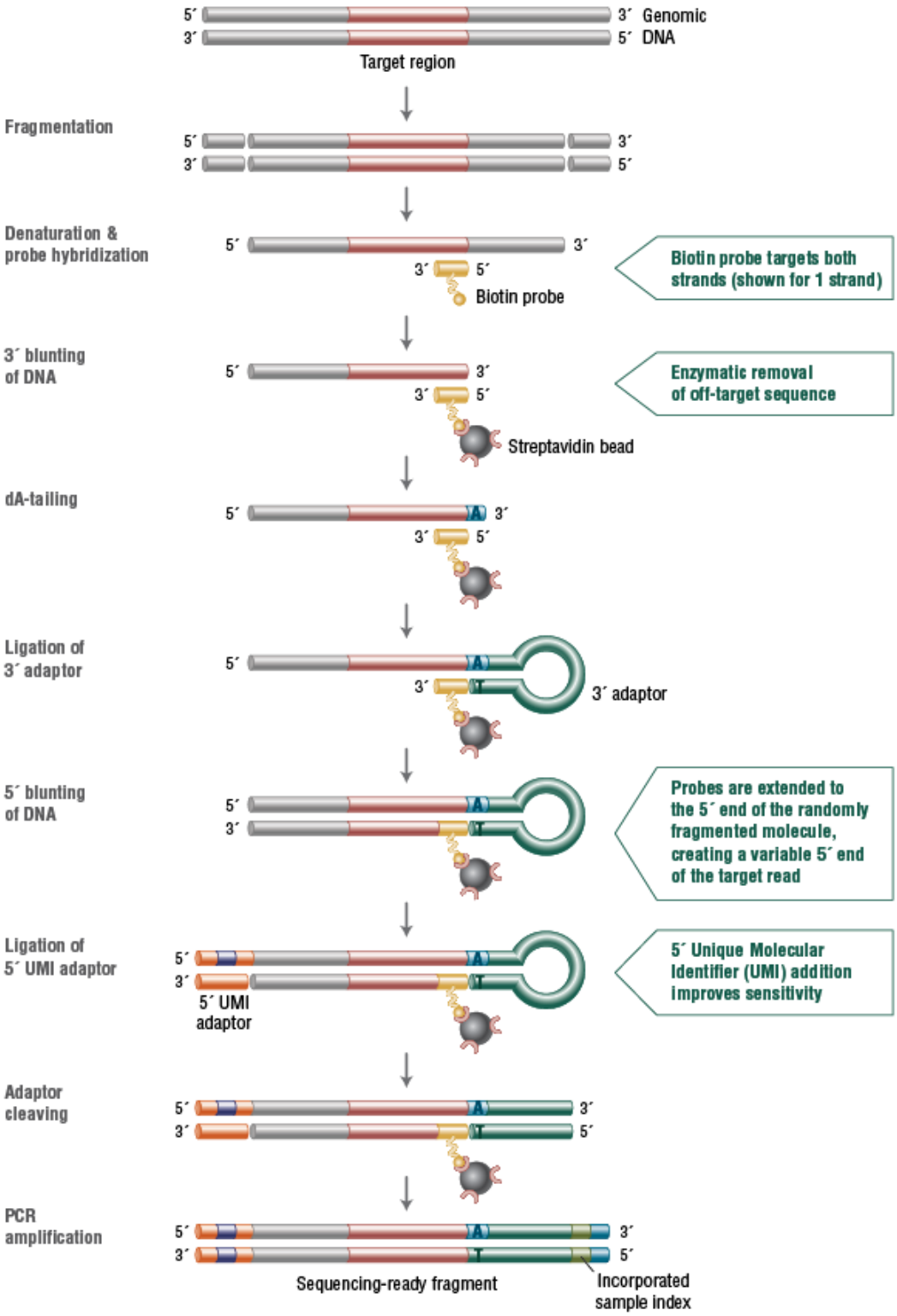


Figure 2.2. NEBNext Direct workflow (adapted from New England BioLabs, permission to reproduce not required).

Table 2.11. PCR conditions for amplification of selected genes.

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing Extension	98°C 62°C 72°C	10 seconds 15 seconds 20 seconds	25
Final Extension	72°C	5 minutes	1
Hold	4°C	Till removed	

2.4.10 Gene panel data analysis

All samples were sequenced on an Illumina MiSeq, generating 3 fastq files: a fastq containing forward reads, a fastq containing reverse reads and a fastq file with the index read containing the UMIs. The sequencing files along with the target BED file, provided by NEB when ordering the NEBNext Direct Custom Gene Panel, were parsed through their Demo Pipeline (https://github.com/DirectedGenomics/DemoPipeline). The pipeline was executed as a simple bash script (pipeline.sh), where an unmapped BAM was generated from the input files, the raw reads were then mapped onto the GRCh38 human genome (GenBank assembly accession number: GCA_000001405.15) and duplicates were marked, consensus reads were generated from the raw reads and these were re-mapped to the reference genome. Finally, both somatic and germline variants were called and filtered. Burrows-Wheeler Aligner was used to align the reads to the genome, Picard was used for various conversions and sorting of file and file content, Fgbio was used to generate consensus reads and filtering of somatic variants, VarDictJava was used to call somatic variants while GATK4 used to call germline variants. Variants were annotated with ANNOVAR (https://annovar.openbioinformatics.org). Among the large number of outputs the pipeline generates, the BAM files with aligned reads and a list of germline variants and somatic variants in vcf format were selected for further analysis. Bioinformatics analysis of targeted gene sequencing was conducted by bioinformaticians, Dr Richard Williams and Dr Nadia Fernandes.

2.4.11 Genome-wide DNA methylation profiling of organoid-tissue pairs

Genome-wide DNA methylation profiling was performed on high quality DNA, which was quantified and normalised to 12 ng/μL, using the Illumina Infinium MethylationEPIC BeadChip array (Illumina, Inc., San Diego, CA, USA), which interrogates over 850,000 methylation sites, including CpG islands. Two hundred fifty nanograms of genomic DNA was bisulphite converted using the EZ-96 DNA Bisulfite Zymo Research conversion protocol (Zymo Research, Irine, CA, USA). Bisulphite-treated samples were amplified, fragmented, purified and hybridized onto the EPIC Beadchip according to the manufacturer's standard protocol. In more detail, bisulfite-converted DNA was added to a 96-well storage plate (Thermo-Fisher Scientific), denatured in 0.014N sodium hydroxide, neutralized and amplified with kit-provided reagents and buffer at 37°C for 20–24 hours. Samples were fragmented using kit-provided reagents and buffer at 37°C for one hour and precipitated by adding 2-propanol. Re-suspended samples were denatured in a 96-well plate heat block at 95°C for 20 minutes. 25 μl of each sample was loaded onto an 8-sample chip and the chips were assembled into hybridization chamber as instructed in the manual. After incubation at 48°C for 16–20 hours, chips were briefly washed and then assembled and placed in a fluid flow-through station for primer-extension and staining procedures. Polymer-coated chips were image-processed in Illumina's HiScan System. The Illumina Infinium MethylationEPIC BeadChip array took place at the Oxford Genomic Centre at the Wellcome Centre for Human Genetics.

2.4.12 Methylation analysis

Raw IDAT files were processed with the R/Bioconductor packages minfi and missMethyl. For clustering purposes, Subset-quantile Within Array Normalization (SWAN) was applied. The methylation level for each probe was expressed as a β-value, ranging between 0 (no methylation) and 1 (complete methylation), or as a logit-transformed M value. Probes were retained for further analysis where the detection p-value was < 0.01 for all samples. The relationship between methylation patterns was assessed by unsupervised hierarchical clustering of the top 1% or 0.1% most variable

probes (β-values, Pearson correlation, average linkage clustering) using the MeV TM4 package, and by multidimensional scaling (MDS) of the M values in R. For differential methylation analysis, a mean detection p-value filter of 0.05 and preprocess Quantile normalisation were applied, M values were calculated, probes with SNPs at CpG sites and known non-specific cross-reactive probes were excluded, and differentially methylated sites were identified using the limma Bioconductor package, with a design matrix paired by patient and an adjusted p-value cut-off of 0.05. Bioinformatics analysis of genome-wide DNA methylation profiling data was conducted by bioinformaticians, Dr Richard Williams and Dr Nadia Fernandes.

2.5 Methods for investigating microbiome function in endometrial cancer

2.5.1 Study population

Women planned to have hysterectomy for endometrial cancer were recruited at Imperial College Healthcare NHS Trust prior to surgery for endometrial cancer organoid generation. Benign organoids were kindly provided by Prof Jan Brosen's laboratory in the University of Warwick and were derived from women that presented in clinic for recurrent miscarriage or recurrent IVF failure.

2.5.2 *L. crispatus* culture supernatant

To investigate the effect of *L. crispatus* on endometrial cell functions, *L. crispatus*, either isolated from the high vaginal swab of a benign patient, previously stored in 8% DMSO at -80^{o}C, or commercially available *L. crispatus* strain ATCC 33820 (DSMZ, Germany), was cultured in MRS broth (Sigma-Aldrich) overnight at 37°C to a density of ~10^{8} CFU/ml. The culture supernatant was collected after 24 hours and NaOH pellets, dissolved in distilled water, were added to achieve a neutral pH. *L. crispatus* isolated from the vaginal swab was confirmed by 16S rRNA gene sequencing as described in section 2.3.7 and the strain (*L. crispatus* ATCC 33820) was determined using the USEARCH and BLASTn software.

2.5.3 Endometrial organoid cultures in *L. crispatus*-conditioned media and LPS treatment

To explore the effect of *L. crispatus*-secreted metabolites on endometrial organoid proliferation and inflammation, benign and malignant endometrial organoids were cultured in *L. crispatus*-conditioned media at increasing concentrations (10%, 20%, 30%) with (benign organoids only) or without 1µg/ml of LPS (*E.coli* O111:B4). Controls grown in plain MRS broth-supplemented medium were also included. TGF-β inhibitor, A83-01 was omitted from organoid culture medium to avoid any interference with inflammatory pathways.

2.5.4 Organoid Viability Assay

To assess the effect of *L. crispatus* metabolites on endometrial organoid proliferation, the CellTiter-GLo® 3D cell viability assay, which quantifies intracellular ATP, was performed according to manufacturer's protocol (Promega). Briefly, medium was removed following treatments and cells were lysed in 100 µl pre-warmed CellTiter-GLo 3D reagent. Then, samples were incubated for 30 min at room temperature and luminescence was recorded using a plate reader (PheraStar).

2.5.5 Magnetic Luminex Cytokine assay

To explore the effect of *L. crispatus* metabolites on endometrial cell inflammatory pathways, cytokine and chemokine secretion by endometrial organoids in response to *L. crispatus*-conditioned medium was interrogated. Supernatants of benign and malignant endometrial glandular organoids stimulated or not with LPS (*E.coli* O111:B4) and grown in *L. crispatus*-conditioned media at increasing concentrations (10%, 20%, 30%) were collected and stored at -80^{o} C. 50 µL of undiluted supernatants were analyzed by a Magnetic Multiplex Cytokine Array (R&D systems**,** Minneapolis, MN, USA), following the manufacturer's instructions. The kit uses colour-coded magnetic microparticles, which are pre-coated with the analyte of interest (e.g., interleukin-8, IL-8). On the day of analysis, the supernatant was thawed slowly on ice

and vortexed. Fifty microliters of diluted microparticle cocktail was added to each well of the supplied 96 well plate, followed by 50µl standard or sample. A standard curve was constructed using the supplied Human Standard Cocktail, serially diluted in the supplied Calibrator Diluent RD6-52. The plate was covered with a foil plate sealer to prevent photobleaching and incubated at room temperature for two hours on a horizontal orbital microplate shaker set to 800rpm, to allow analyte binding by the antibody on the microparticles. Using a magnetic plate holding device (Bio-Rad), the plate was washed 3 times using 100µl wash buffer per well and 50µl biotinylated antibody cocktail was added to each well. The plate was incubated for 1 hour at room temperature on the microplate shaker, washed 3 times using 100µl wash buffer to wash away unbound antibody, and 50µl streptavidin-phycoerthyrin (-PE) conjugate added to each well to bind the biotinylated antibody for detection. After 30 mins incubation at room temperature on the microplate shaker, the plate was washed three times using 100µl wash buffer per well to remove unbound streptavidin-PE and 100µl wash buffer was added to each well to resuspend the microparticles for analysis. Levels of 12 different cytokines/chemokines, including CCL4/MIP1beta, CCL5/Rantes, G-CSF, GM-CSF, IL-1rα, IL-1β/IL-1F2, IL-2, IL-6, IL-8/CXCL8, IFNγ, TNFα and VEGF were measured in endometrial cancer organoid supernatants, while in benign organoid culture supernatants 6 different cytokines, including IL-1β/IL-1F2, IL-6, IL-8/CXCL8, IL-10, IFNγ and TNFα, were determined on a MAGPIX Analyzer (Luminex® Corporation, s-Hertogenbosch, Netherlands), as per manufacturer's instructions. This machine contains a magnet which captures the microparticles in a monolayer and two light emitting diodes (LEDs) to illuminate the beads; one of which identifies the analyte of interest and the second determines the magnitude of PE-derived signal. The Luminex® detection system was able to determine the concentration of selected cytokines/chemokines. All samples were assayed in duplicate. Total protein concentration of supernatant in each well was determined using a Bradford protein assay (Quick Start™ Bradford Protein Assay kit 2, Bio-rad). To correct for different cell number making up the organoids in each well, a correction factor was calculated by dividing the total protein concentration of each well (µg/mL) by the total protein concentration of one of the samples (separately for benign and

malignant organoids), which was multiplied by the cytokine/chemokine concentration (pg/mL) in each well.

2.6 Statistical Analyses

The remaining statistical analyses described in this thesis were performed using the statistical package GraphPad Prism v.8.0.1 (GraphPad Software Inc., California, USA). The appropriate statistical test was selected depending on normality and the number of groups analysed (Figure 2.3). A p-value less than 0.05 was considered statistically significant.

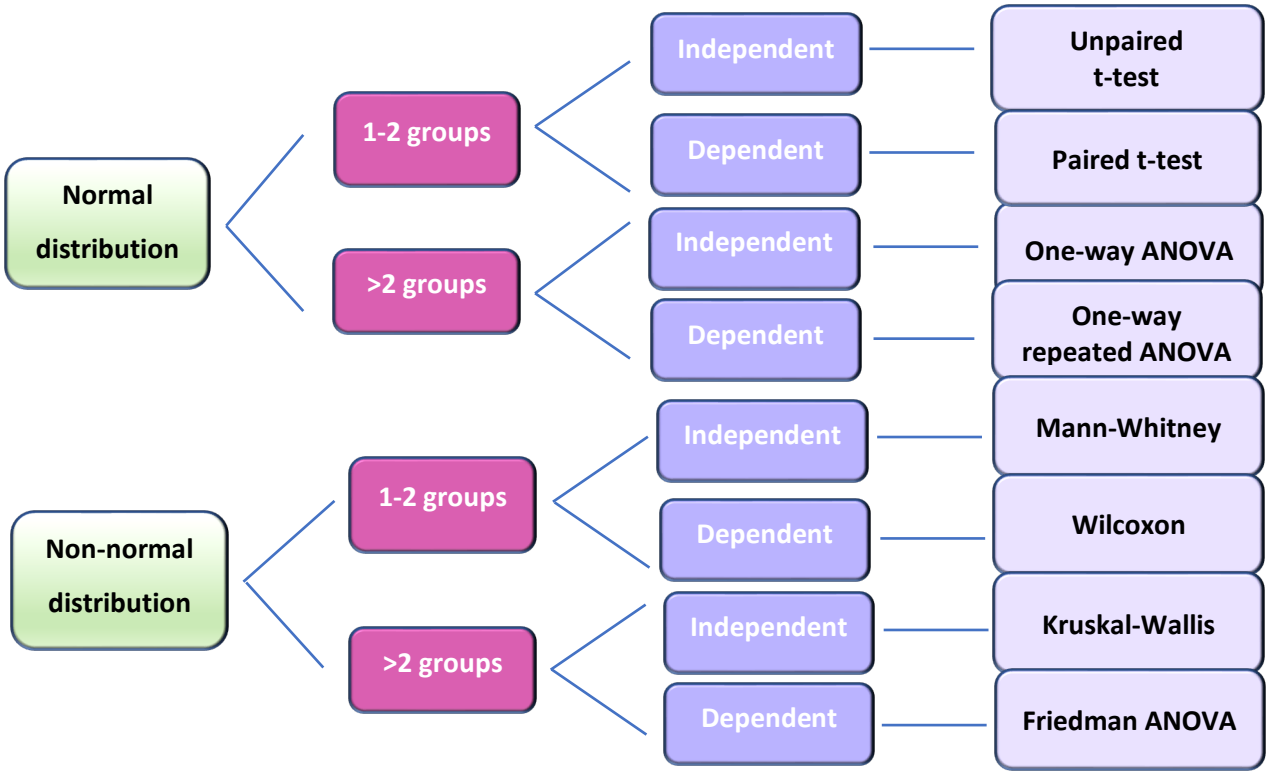


Figure 2.3. Description of selection procedure of statistical analyses used in this thesis.

CHAPTER 3.

Diabetes and anti-diabetic interventions and the risk of gynaecological and obstetric morbidity: an umbrella review of the literature

---

Content from this chapter is currently under preparation as:

Semertzidou A, Grout-Smith H, Kalliala I, , Garg A, Terzidou V, Marchesi J, MacIntyre D, Bennett P, Tsilidis K, Kyrgiou M. Diabetes and anti-diabetic interventions and the risk of gynaecological and obstetric morbidity: an umbrella review of the literature. *(Under preparation)*

3.1 Abstract

Objective

To study the strength and validity of associations between diabetes and anti-diabetic interventions and the risk of any type of gynaecological or obstetric conditions.

Methods

Design: Umbrella review of systematic reviews and meta-analyses.

Data sources: PubMed, Medline, Embase, Cochrane Database of Systematic Reviews, manual screening of references.

Eligibility criteria: Systematic reviews and meta- analyses of observational and interventional studies investigating the relationship between diabetes and gynaecological/obstetric outcomes. In the presence of more than one meta-analysis per outcome, the one with the greatest number of cohort studies was selected for inclusion. Meta-analyses that did not include complete data from individual studies, such as relative risk, 95% confidence intervals, number of cases/controls, or total population were excluded.

Data analysis: The evidence from meta-analyses was graded as strong, highly suggestive, suggestive or weak according to statistical criteria comprising the random effects estimate of meta-analyses and their largest study, the number of cases, 95% prediction intervals, I^2 heterogeneity index between studies, excess significance bias, small studies effect and sensitivity analysis using credibility ceilings. Interventional meta-analyses were assessed separately based on the statistical significance of reported associations, the risk of bias and quality of evidence (GRADE) of included meta-analyses.

Results

A total of 117 meta-analyses of observational studies and 200 meta-analyses of randomised clinical trials were included that evaluated 317 outcomes. Only 2 meta-analyses were supported by strong evidence, i.e., had strongly statistically significant results and no evidence of bias, and demonstrated an increased risk of caesarean

section (RR 1.37, CI 1.24-1.51) and large for gestational age babies (RR 1.53, CI 1.39-1.69) in women with gestational diabetes (WHO criteria), which was reduced by metformin use (RR 0.73, CI 0.61-0.88 and RR 0.8, CI 0.64-0.99 respectively). Highly suggestive evidence supported a relationship between pre-gestational diabetes and the risk of major congenital malformations (RR 2.44, CI 1.92-3.10) and heart defects (OR 3.18, CI 2.77-3.65). Suggestive evidence supported a positive association between diabetes and endometrial cancer (RR 1.56, CI 1.21-2.01) and a better survival in metformin-users (HR 0.47, CI 0.33-0.67), while increased endometrial cancer mortality (RR 1.32, CI 1.10-1.60) among diabetic patients was only supported by weak evidence.

Conclusions

Gestational diabetes appears to be strongly associated with a high risk of caesarean section and large for gestational age babies. Weaker associations were demonstrated between diabetes and anti-diabetic interventions and other obstetric and gynaecological outcomes.

3.2 Introduction

Diabetes affects 223 million women (20-79 years) worldwide according to 2019 estimates, a number which is expected to rise to 343 million by 2045 [348]. According to the International Diabetes Federation (IDF), one in 6 pregnancies is affected by diabetes; 13.6% of cases represent pregestational diabetes mellitus (PGDM), while 86.4% gestational diabetes mellitus (GDM) [348]. In the UK, approximately 16 out of every 100 women will develop gestational diabetes [349]. Several risk factors, such as ethnicity, age, family history, smoking and high blood pressure have been acknowledged as contributing to disease development, but the epidemic proportions noted over the last few decades are attributable to obesity and physical inactivity [349].

The epidemiological burden of diabetes in women is accompanied by a wide spectrum of adverse gynaecological and obstetric outcomes. Reports have associated diabetes

with endometrial and ovarian carcinogenesis affecting both incidence and mortality [350-354], and an even bigger body of evidence has linked diabetes, either pre-existing or gestational, to obstetric morbidity [355-365]. Diabetes-related obstetric complications involve maternal, such as risk of caesarean section, preeclampsia, postnatal depression and foetal, like large for gestational age, macrosomia, preterm delivery, major congenital malformations, respiratory distress syndrome and perinatal mortality.

While systematic reviews and meta-analyses aim to consolidate associations on a broad topic area by incorporating individual study data available in literature, underpowering and bias in the analysis and reporting of findings can lead to erroneous results that overestimate the observed magnitudes of effect [366-368]. Umbrella reviews aim to critically assess the totality of evidence provided by systematic reviews and meta-analyses and test the validity of reported estimates.

I performed an umbrella review of systematic reviews and meta-analyses on the association between diabetes and anti-diabetic interventions and the risk of any type of gynaecological or obstetric morbidity aiming to evaluate the strength of available evidence and guide future healthcare policies.

3.3 Aims

The aim of this umbrella review is:

- To summarise the available evidence of meta-analyses and systematic reviews on diabetes and anti-diabetic interventions and the risk of endometrial cancer and any other type of gynaecological or obstetric morbidity.
- To investigate the strength and validity of existing epidemiological evidence for associations between diabetes and anti-diabetic interventions and the risk of gynaecological and obstetric outcomes and identify the overall most robust associations.

3.4 Results

3.4.1. Meta-analyses of observational studies

3.4.1.1 Characteristics of meta-analyses

Data from 21 eligible publications was extracted, consisting of 63 meta-analyses with 478 study estimates, which investigated the effect of diabetes on gynaecological and obstetric morbidity [20 350 351 355-363 369-375]. Out of the 478 individual studies, 345 (72%) were cohort studies, 66 (14%) case-control studies, 2 (0.4%) were nested case-control studies, 61 (13%) cross-sectional, 2 (0.4%) descriptive cohort studies and 2 (0.4%) case series. There were 2 to 31 individual studies combined per meta-analysis with a median of 13. The median number of cases and total population in each meta-analysis was 1,301 and 33,788 respectively. The lowest number of cases in a meta-analysis was 58 and the highest was 251,558, whereas the smallest total population or controls was 272 and the highest was 15,009,001.

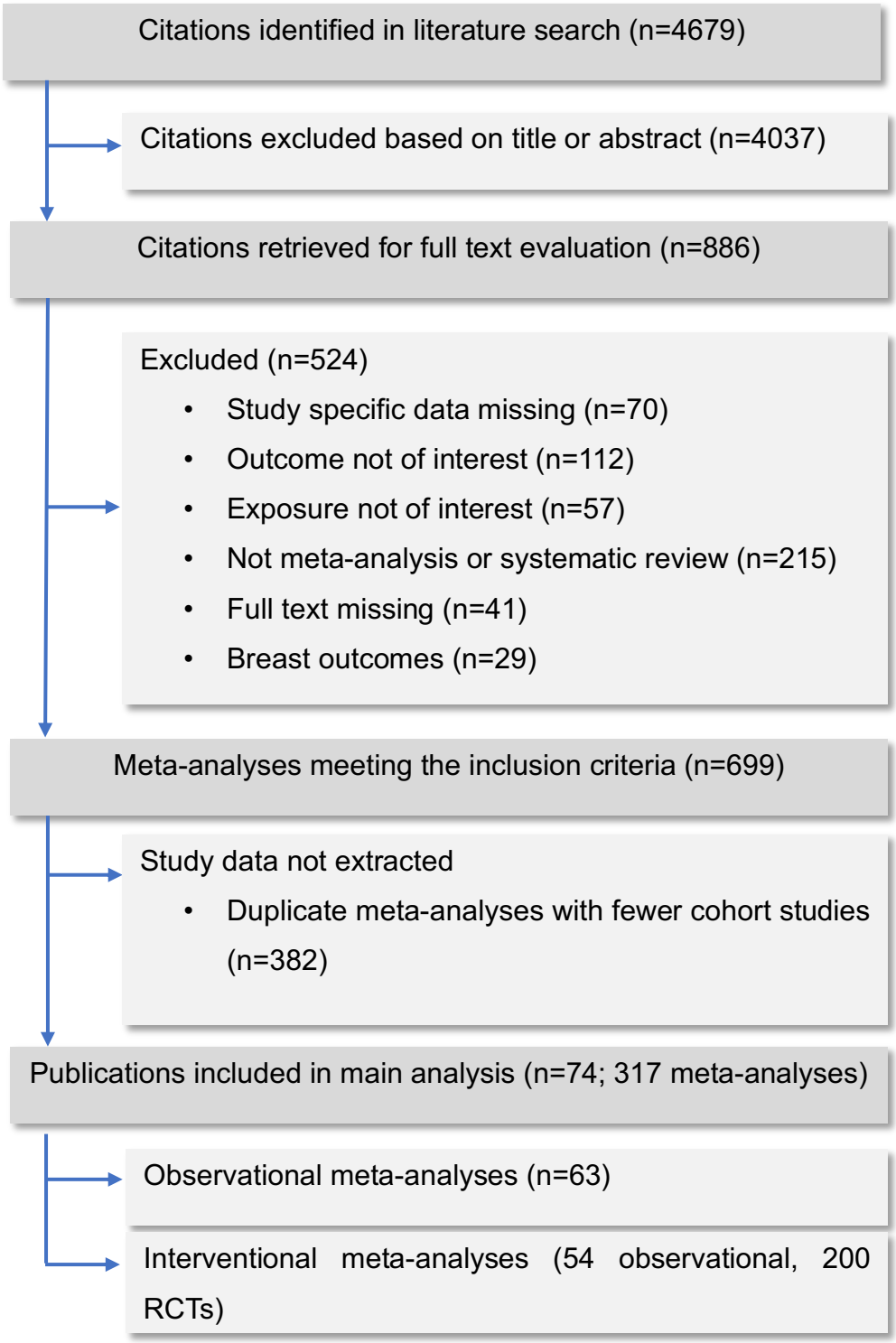


Figure 3.1. Flow diagram of the selection process of meta-analyses included in the review.

3.4.1.2 Summary effect size

Using P<0.05 as a statistical significance threshold, the summary fixed effects estimate reached significance in 35/47 (74%) meta-analyses of cohort studies, while the summary random effects was significant in 33/47 (70%). At a significance cut-off of P<0.001, 27/47 (57%) and 19/47 (40%) meta-analyses yielded significant results using the fixed and random effects model respectively, whereas at a more stringent threshold of $P<10^{-6}$, 22/47 (47%) and 7/47 (15%) of meta-analyses produced significant results. The 7 meta-analyses with strongly statistically significant summary random effect estimates ($P<10^{-6}$) identified a relationship between gestational diabetes and increased risk for caesarean section, large for gestational age babies, macrosomia and brachial plexus palsy as well as a relationship between pregestational diabetes and increased risk for major congenital malformations, and congenital heart defects (Table 3.1).

The summary random effect estimates ranged from 0.03 (95% confidence interval -1.15 to 1.21) for an association between GDM and breastmilk energy up to 4.53 (1.61 to 12.77) for risk of major congenital malformations in women with diabetes type 1,2 (poor vs optimal glycaemic control). 49% (23/47) of the estimates fell between 1.2 and 2. Table 3.1 also shows the effect of the largest study included in each meta-analysis. 55% (n=26/47) of these effects were nominally significant at P<0.05 and showed an increased risk.

3.4.1.3 Heterogeneity between studies

The Cochran's Q test and I^2 index were used as measures of between-study heterogeneity to account for variation in study outcomes between studies that cannot be attributed to chance. The Q test was significant ($P \leq 0.10$) for 8.5% (4/47) of meta-analyses. Moderate to high heterogeneity (I^2 = 50-75%) was noted in 8/47 (17%) of meta-analyses, while substantial heterogeneity (I^2 >75%) was observed in 10/47 (21%) meta-analyses for the following outcomes, congenital heart defects, duration of breastfeeding, major congenital malformations, postnatal depressive symptoms, respiratory distress syndrome of the newborn, LGA, CS, breastmilk energy, ovarian

cancer incidence and disease-specific mortality. I further calculated 95% prediction intervals and found 10 associations in which the null value was excluded (Table 3.2).

3.4.1.4 Small study effects

Small study effects (Egger's test *p* value <0.10 and where more conservative effects in the largest study of a meta-analysis compared to the summary random effects estimate were recorded) were found to be present in five meta-analyses for associations between diabetes and postnatal depressive symptoms (GDM vs non-GDM), congenital malformations (poor vs optimal glycaemic control), brachial plexus palsy (GDM vs non-GDM), cervical cancer screening (diabetes vs non- diabetes) and neonatal mortality (DM2 vs DM1, not significant) (Table 3.2). Only two studies included an adequate number of studies (10 or more) for Egger's test to have adequate statistical power to identify small study effects.

3.4.1.5 Excess significance bias

Two (4%) meta-analyses demonstrated evidence of excess significance bias using the largest study estimate as the plausible effect size (P<0.10). These included the associations between gestational diabetes and postnatal depressive symptoms and pre- or gestational diabetes with respiratory distress syndrome of the newborn. Using the random effects estimate, the association of gestational diabetes with breastmilk energy was additionally highlighted with excess significance bias, while using the summary fixed effects estimate no further meta-analysis was highlighted (Table 3.2).

3.4.1.6 Credibility ceilings

From all 47 meta-analyses, 29 (62%) met nominal significance (P<0.05) with a credibility ceiling of 5%. With ceilings of 10%, 15%, and 20%, 24 (51%), 9 (19%), and 4 (8.5%) meta-analyses remained significant, respectively (Table 3.2).

3.4.1.7 Quality assessment

The methodological quality of the 21 publications included in the main analysis, encompassing 47 meta-analyses of observational studies, was assessed using the AMSTAR 2 tool. Three (3/21) of all the included papers were graded as low and eighteen (18/21) as critically low quality (Table 3.3). Papers assessed with 'low' or 'critically low' quality failed to meet one or more than one 'critical' criteria respectively, i.e. lack of protocol, description of excluded studies and risk of bias assessment. The majority of publications (19/21) did not meet the critical requirement of explicitly stating that the review methods were established prior to the conduct of the review and failed to justify any significant deviations from the protocol, while 18/21 did not provide a list of excluded studies accompanied by justification. With regards to other critical criteria, all included papers provided a comprehensive literature search strategy and used appropriate statistical analyses for combination of results. 10/21 assessed the risk of bias, whereas 17/21 accounted for publication bias (small study bias).

Table 3.1. Description of 47 meta- analyses results investigating the association of diabetes with gynaecological and obstetric morbidity– cohort studies only

Author, year	Exposure	Exposure contrast	N[α]	Sample size cases/ cohort	Summary relative risk (95% CI)			Fixed P-value[ε]	Random P-value[ϕ]	95% Prediction interval[γ]
					Fixed Effects[β]	Random Effects[χ]	Largest Study[δ]			
Gynaecological										
Saed 2019	DM	Diabetes vs non- diabetes	9	3564/429206	1.42 (1.25-1.62)	1.56 (1.21-2.01)	1.16 (0.90-1.48)	5.93E-08	5.04E-04	0.72-3.37
Liao 2014	DM 1,2	DM vs non- diabetes	6	2075/1,268,756	1.32 (1.11-1.57)	1.32 (1.11-1.57)	1.33 (1.07-1.65)	1.6E-3	1.6E-3	1.03-1.69
Pergaliotis 2016	DM (unspecified)	DM vs non- diabetes	8	154/5212	2.85 (1.72-4.72)	2.85 (1.72-4.72)	3.04 (1.02-9.05)	4.53E-05	4.53E-05	1.52-5.35
Wang 2017	DM (unspecified)	DM vs non- diabetes	14	5534/3708313	1.15 (1.07-1.24)	1.19 (1.06-1.34)	1.05 (0.93-1.2)	8.00E-05	4.36E-03	0.87-1.62
Zhang 2017	DM 1,2	DM vs non- diabetes	5	15312/610592	1.31 (1.21-1.42)	1.44 (1.08-1.93)	1.32 (1.16-1.5)	2.09E-10	1.35E-02	0.49-4.25
Bhatia 2020	Diabetes	Diabetes vs non- diabetes	3	2463/4268	0.71 (0.52-0.96)	0.71 (0.52-0.96)	0.74 (0.50-1.10)	0.029	0.029	0.09-5.31
Zhang 2017	DM 1	DM vs non- diabetes	4	868/495012	1.65 (1.30-2.10)	1.83 (1.21-2.78)	1.38 (1.01-1.89)	4.62E-05	0.0045	0.36-9.32
Zhang 2017	DM 2	DM vs non- diabetes	13	4168/2373203	1.20 (1.15-1.26)	1.24 (1.06-1.44)	1.23 (1.15-1.32)	2.37E-14	6.60E-03	0.76-2.02
Wang 2020	GDM	GDM vs non- GDM	3	63694/1163875	1.02 (0.81-1.29)	1.02 (0.81-1.29)	0.90 (0.65-1.26)	0.84	0.84	0.23-4.49
Wang 2020	GDM	GDM vs non- GDM	1	34294/68588	0.31 (0.07-1.42)	0.31 (0.07-1.42)	0.31 (0.07-1.46)	0.13	0.13	N/A
Wang 2020	GDM	GDM vs non- GDM	4	70263/1266775	1.12 (0.93-1.34)	1.14 (0.90-1.44)	0.96 (0.72-1.28)	0.24	0.28	0.53-2.45
Obstetric, maternal										
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	4	5800/30045	1.38 (1.29-1.47)	1.37 (1.25-1.50)	1.42 (1.31-1.53)	1.80E-22	1.08E-11	1.03-1.81
Wendland 2012	GDM (IADPSG-criteria)	GDM vs non- GDM	3	1909/35052	1.80 (1.64-1.99)	1.71 (1.37-2.13)	2.02 (1.78-2.29)	3.07E-33	2.45E-06	0.14-21.04
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	3	1301/26677	1.61 (1.40-1.85)	1.70 (1.31-2.20)	1.55 (1.33-1.81)	3.61E-11	6.27E-05	0.13-22.01
Wilson 2019	GDM	GDM vs non- GDM	12	2083/1064553	1.12 (1.07-1.18)	1.54 (1.22-1.95)	1.06 (1.00-1.11)	4.45E-06	3.03E-04	0.74-3.22
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	3	85/544	2.14 (1.13-4.04)	2.14 (1.13-4.04)	1.77 (0.88-3.72)	1.93E-02	1.93E-02	0.03-131.76
Wilson 2019	GDM	GDM vs non- GDM	2	208/2152	1.87 (1.03-3.39)	1.87 (1.03-3.39)	1.69 (0.88-3.23)	0.039	0.039	N/A

Manerkar 2020	GDM	GDM vs non- GDM	2	58/272	(-)0.36 (-0.67--0.04)	(-)0.36 (-0.67--0.04)	(-)0.45 (-0.86--0.05)	0.025	0.025	N/A
Manerkar 2020	GDM	GDM vs non- GDM	5	901/9716	(-)0.19 (-0.26--0.12)	(-)0.24 (-0.42--0.07)	(-)0.07 (-0.17-0.03)	2.31E-07	0.0073	(-)0.84-0.35
Wendland 2012	GDM (IADPSG-criteria)	GDM vs non- GDM	3	6828/33788	1.28 (1.22-1.35)	1.23 (0.99-1.53)	1.45 (1.36-1.55)	4.25E-24	6.58E-02	0.08-19.86
Wilson 2019	GDM	GDM vs non- GDM	4	47/639	1.16 (0.74-1.83)	1.31 (0.70-2.46)	0.86 (0.48-1.56)	0.52	0.4	0.15-11.12
Manerkar 2020	GDM	GDM vs non- GDM	2	58/272	0.16 (-0.16-0.47)	0.03 (-1.15-1.21)	0.62 (0.22-1.03)	0.34	0.96	N/A
Manerkar 2020	GDM	GDM vs non- GDM	2	58/272	(-)0.13 (-0.45-0.18)	(-)0.13 (-0.45-0.18)	(-)0.17 (-0.57-0.24)	0.42	0.42	N/A
Manerkar 2020	GDM	GDM vs non- GDM	2	58/272	(-)0.10 (-0.42-0.21)	(-)0.13 (-0.61-0.35)	0.09 (-0.32-0.49)	0.53	0.59	N/A
Obstetric, foetal										
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	4	2755/28755	1.53 (1.39-1.68)	1.53 (1.39-1.68)	1.51 (1.36-1.68)	1.10E-17	1.10E-17	1.24-1.89
Zhao 2015	PGDM	PGDM vs non- diabetes	13	33400/1533451	2.48 (2.26-2.71)	2.43 (1.92-3.09)	3.57 (3.00-4.25)	<1.00E-100	1.99E-13	1.13-5.23
Chen 2019	PGDM	PGDM vs non- diabetes	13	66967/6974271	3.93 (3.71-4.17)	3.41 (2.89-4.03)	4.36 (4.02-4.73)	<1.0E-100	<1.0E-100	2.02-5.77
Flenady 2011	PGDM	PGDM vs non- diabetes	3	3614/564896	2.36 (1.71-3.25)	2.52 (1.52-4.17)	1.82 (1.16-2.84)	1.97E-07	3.40E-04	0.01-530.07
Zhao 2015	GDM	GDM vs non- GDM	17	41668/1816289	1.19 (1.11-1.27)	1.18 (1.08-1.28)	1.18 (1.07-1.30)	4.39E-07	1.39E-04	1.02-1.36
Wendland 2012	GDM (IADPSG criteria)	GDM vs non- GDM	3	3392/35902	1.90 (1.77-2.03)	1.75 (1.39-2.20)	1.95 (1.79-2.13)	<1.0E-100	1.51E-06	0.11-28.60
Li 2019	Diabetes (PGDM and GDM)	Diabetes vs non- diabetes	20	81395/2028020	1.24 (1.17-1.32)	1.39 (1.17-1.65)	1.01 (0.91-1.11)	1.70E-13	1.73E-04	0.73-2.66
Manerkar 2020	GDM	GDM vs non- GDM	5	19594/29089	1.36 (1.22-1.52)	1.49 (1.18-1.88)	1.33 (1.17-1.52)	2.24E-08	0.00081	0.75-2.96
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	5	804/11588	1.81 (1.47-2.22)	1.81 (1.47-2.22)	1.66 (1.29-2.13)	2.12E-08	2.12E-08	1.29-2.53
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	8	238/3684	2.33 (1.60-3.40)	2.33 (1.60-3.40)	1.90 (1.19-3.11)	1.05E-05	1.05E-05	1.46-3.73
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	4	95/797	4.53 (1.61-12.77)	4.53 (1.61-12.77)	4.06 (1.12-22.12)	4.22E-03	4.22E-03	0.47-44.01
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	3	72/2701	2.75 (1.46-5.17)	2.75 (1.46-5.17)	2.73 (1.46-5.49)	1.66E-03	1.66E-03	0.05-164.28
Balsells 2009	DM 1,2	DM2 vs DM1	22	279/8797	1.51 (1.16-1.97)	1.51 (1.16-1.97)	1.10 (0.68-1.77)	2.41E-03	2.41E-03	1.14-2.00

Simeone 2015	PGDM	PGDM vs non- diabetes	8	173/1281197	3.59 (3.07-4.20)	3.64 (2.73-4.85)	3.4 (2.6-4.5)	<1.00E-100	1.07E-18	1.61-8.22
Van der Looven 2019	GDM	GDM vs non- GDM	3	244/54484	3.94 (2.70-5.76)	3.94 (2.70-5.76)	3.28 (2.00-5.39)	1.23E-12	1.23E-12	0.34-45.86
Tabrizi 2019	GDM in Iranian women	GDM vs non- GDM	3	103/2491	3.79 (2.34-6.13)	3.77 (1.97-7.21)	2.50 (1.16-5.39)	5.53E-08	5.99E-05	0.01-2276
Li 2019	GDM	GDM vs non -GDM	9	12659/1609618	1.41 (1.28-1.54)	1.44 (1.16-1.78)	1.50 (1.30-1.70)	2.23E-13	0.0011	0.76-2.71
Zhang 2015	GDM	GDM vs non- GDM	6	8212/180374	1.02 (0.90-1.15)	1.04 (0.86-1.27)	1.07 (0.90-1.27)	0.77	0.66	0.68-1.60
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	2	127/9072	1.55 (0.88-2.73)	1.55 (0.88-2.73)	1.48 (0.82-2.66)	0.13	0.13	N/A
Balsells 2009	DM 1,2	DM2 vs DM1	16	74/6531	1.59 (0.96-2.64)	1.59 (0.96-2.64)	0.99 (0.38-2.54)	7.23E-02	7.23E-02	0.91-2.80
Balsells 2009	DM 1,2	DM2 vs DM1	19	168/7407	1.21 (0.85-1.72)	1.23 (0.82-1.85)	1.14 (0.66-1.96)	0.29	0.31	0.62-2.46
Balsells 2009	DM 1,2	DM2 vs DM1	24	514/9693	1.16 (0.94-1.42)	1.19 (0.91-1.56)	0.90 (0.58-1.40)	0.18	0.20	0.59-2.41
Shu 2019	PGDM and GDM	Diabetes vs non- diabetes	5	4470/1937847	1.40 (1.27-1.55)	1.44 (0.95-2.18)	1.40 (1.26-1.55)	6.00E-11	0.086	0.45-4.66

Abbreviations: GDM- Gestational diabetes mellitus; PGDM- Pregestational diabetes mellitus; DM 1,2- Diabetes mellitus type 1,2; WHO- World Health Organisation; IADPSG- International Association of the Diabetes and Pregnancy Study Groups

Key:

$^{\alpha}$ Number of studies

$^{\beta}$ Fixed effects refers to summary relative risk 95% CI) using the meta-analysis fixed- effects model

$^{\chi}$ Random effects refers to summary relative risk (95% CI) using the meta-analysis random -effects model

$^{\delta}$ Relative risk and 95% confidence interval of largest study (smallest SE) in each meta- analysis

$^{\varepsilon}$ P value of summary fixed effects estimate

$^{\phi}$ P value of summary random effects estimate

$^{\gamma}$ Prediction intervals are reported only for meta-analyses including at least 3 studies

All statistical tests were two-sided

Table 3.2. Evaluation of heterogeneity, small study effects, excess significance bias and credibility ceilings in the 47 meta-analyses investigating the association of diabetes with gynaecological and obstetric morbidity– cohort studies only

Author, year	Exposure	Exposure contrast	Egger's P[α]	I^2 (95% CI) P[β]	Studies	Observed[χ]	Expected[δ], P-value[ε]						Credibility ceiling (%) p<0.05
							Fixed effects		Random effects		Largest study		
Gynaecological													
Saed 2019	DM	Diabetes vs non-diabetes	0.18	69 (23-83) 0.0011	9	4	6.39	NP	7.21	NP	2.9	0.43	15
Liao 2014	DM 1,2	DM vs non- diabetes	0.61	0 (0-61) 0.91	6	1	2.24	NP	2.24	NP	2.26	NP	11
Pergaliotis 2016	DM (unspecified)	DM vs non- diabetes	0.49	0 (0-56) 0.97	8	2	4.95	NP	4.95	NP	5.25	NP	22
Wang 2017	DM (unspecified)	DM vs non- diabetes	0.38	44 (0-69) 0.04	14	2	4.30	NP	5.36	NP	1.18	0.43	12
Zhang 2017	DM 1,2	DM vs non- diabetes	0.47	90 (79-94) <1.0E-100	5	4	3.71	0.77	4.15	NP	3.75	0.79	2
Bhatia 2020	Diabetes	DM vs non- diabetes	0.029	0 (0-73) 0.87	3	0	2.86	NP	2.86	NP	2.73	NP	11
Zhang 2017	DM 1	DM vs non- diabetes	0.32	56 (0-83) 0.078	4	3	1.5	0.12	1.66	0.17	1.29	0.068	10
Zhang 2017	DM 2	DM vs non- diabetes	0.73	82 (69-88) 1.78E-09	13	3	4.92	NP	5.73	NP	5.59	NP	3
Wang 2020	GDM	GDM vs non- GDM	0.04	0 (0-73) 0.55	3	0	1.14	NP	1.14	NP	3	NP	0
Wang 2020	GDM	GDM vs non- GDM	N/A	100 (.-.) <1E-100	1	0	1	NP	1	NP	1	NP	0
Wang 2020	GDM	GDM vs non- GDM	0.29	32 (0-77) 0.22	4	1	4	NP	4	NP	2.8	NP	0
Obstetric, maternal													
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	0.92	22 (0-75) 0.28	4	3	3.11	NP	3.09	NP	3.18	NP	13
Wendland 2012	GDM (IADPSG criteria)	GDM vs non- GDM	0.55	74 (0-90) 0.022	3	2	2.94	NP	2.89	NP	2.99	NP	11
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	0.58	40 (0-82) 0.19	3	2	2.30	NP	2.46	NP	2.18	NP	11

Wilson 2019	GDM	GDM vs non- GDM	0.006	79 (61-86) 3.22E-07	12	7	2.41	9.35E-04	5.35	0.34	1.8	2.70E-05	15
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	0.50	0 (0-73) 0.54	3	0	1.47	NP	1.47	NP	0.98	NP	7
Wilson 2019	GDM	GDM vs non- GDM	N/A	0 (.-.) 0.44	2	0	1.11	NP	1.11	NP	1.07	NP	6
Manerkar 2020	GDM	GDM vs non- GDM	N/A	0 (.-.) 0.48	2	1	1.79	NP	1.79	NP	1.93	NP	3
Manerkar 2020	GDM	GDM vs non- GDM	0.46	77 (17-89) 0.0019	5	3	4.37	NP	4.58	NP	2.82	0.87	12
Wendland 2012	GDM (IADPSG-criteria)	GDM vs non- GDM	0.51	95 (88-97) <1.0E-100	3	2	3.00	NP	2.96	NP	3.00	NP	0
Wilson 2019	GDM	GDM vs non- GDM	0.25	34 (0-78) 0.21	4	1	0.23	0.1	0.31	0.2	0.23	0.1	0
Manerkar 2020	GDM	GDM vs non- GDM	N/A	92 (.-.) 0.0003	2	2	0.83	0.09	0.13	<1E-100	2	NP	0
Manerkar 2020	GDM	GDM vs non- GDM	N/A	0 (.-.) 0.76	2	0	0.64	NP	0.64	NP	0.95	NP	0
Manerkar 2020	GDM	GDM vs non- GDM	N/A	55 (.-.) 0.14	2	0	0.43	NP	0.64	NP	0.36	NP	0
Obstetric, foetal													
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	0.478	0 (0-68) 0.94	4	2	2.92	NP	2.92	NP	2.88	NP	15
Zhao 2015	PGDM	PGDM vs non-diabetes	0.94	78 (60-86) <1.0E-100	13	9	10.6	NP	10.6	NP	11.2	NP	27
Chen 2019	PGDM	PGDM vs non-diabetes	0.058	76 (56-85) 9.64E-07	13	10	12.9	NP	12.9	NP	13	NP	22
Flenady 2011	PGDM	PGDM vs non-diabetes	0.36	56 (0-86) 0.11	3	3	3.00	NP	3.00	NP	2.95	0.83	11
Zhao 2015	GDM	GDM vs non- GDM	0.97	10 (0-52) 0.34	17	3	5.72	NP	5.58	NP	5.61	NP	12
Wendland 2012	GDM (IADPSG criteria)	GDM vs non- GDM	0.465	87 (48-94) 0.003	3	2	3.00	NP	3.00	NP	3.00	NP	9
Li 2019	Diabetes (PGDM and GDM)	Diabetes vs non-diabetes	0.20	81 (72-87) 2.78E-13	20	8	11.9	NP	14	NP	1.19	<1E-100	8

Manerkar 2020	GDM	GDM vs non- GDM	0.14	56 (0-82) 0.057	5	3	3.67	NP	3.97	NP	3.58	NP	14
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	0.504	0 (0-64) 0.60	5	3	3.39	NP	3.39	NP	3.07	NP	14
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	0.01	0 (0-56) 0.82	8	2	4.06	NP	4.06	NP	3.01	NP	22
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	0.22	0 (0-68) 0.96	4	0	3.3	NP	3.3	NP	3.16	NP	15
Inkster 2006	DM 1,2	Poor vs optimal glycaemic control	0.73	0 (0-73) 0.96	3	1	1.28	NP	1.28	NP	1.27	NP	4
Balsells 2009	DM 1,2	DM2 vs DM1	0.43	0 (0-41) 0.52	22	2	2.67	NP	2.67	NP	1.14	0.40	10
Simeone 2015	PGDM	PGDM vs non- diabetes	0.97	61 (0-80) 0.013	8	6	5.77	0.857	5.81	0.879	5.60	0.749	16
Van der Looven 2019	GDM	GDM vs non- GDM	0.067	0 (0-73) 0.47	3	3	3	NP	3	NP	3	NP	12
Tabrizi 2019	GDM in Iranian women	GDM vs non GDM	0.91	44 (0-83) 0.17	3	3	2.95	0.82	2.95	0.82	2.44	0.41	11
Li 2019	GDM	GDM vs non- GDM	0.69	71 (31-84) 0.00052	9	5	6.66	NP	6.77	NP	6.96	NP	9
Zhang 2015	GDM	GDM vs non- GDM	0.12	28 (0-71) 0.22	6	0	0.39	NP	0.81	NP	1.45	NP	0
Wendland 2012	GDM (WHO criteria)	GDM vs non- GDM	N/A	0 (.-.) 0.56	2	0	0.87	NP	0.87	NP	0.75	NP	0
Balsells 2009	DM 1,2	DM2 vs DM1	0.08	0 (0-47) 0.80	16	1	1.26	NP	1.26	NP	0.70	0.71	0
Balsells 2009	DM 1,2	DM2 vs DM1	0.64	8 (0-48) 0.36	19	1	1.11	NP	1.16	NP	1.00	NP	0
Balsells 2009	DM 1,2	DM2 vs DM1	0.45	25 (0-54) 0.13	24	1	1.56	NP	1.73	NP	1.39	NP	0
Shu 2019	PGDM and GDM	Diabetes vs non-diabetes	0.65	49 (0-80) 0.098	5	2	2.97	NP	3.02	NP	2.97	NP	0

Abbreviations: GDM- Gestational diabetes mellitus; PGDM- Pregestational diabetes mellitus; DM 1,2- Diabetes mellitus type 1,2; WHO- World Health Organisation; IADPSG- International Association of the Diabetes and Pregnancy Study Groups; NP- not pertinent (because the estimated is larger than the observed, and there is no evidence of excess statistical significance based on the assumption made for the plausible effect size)

Key:

$^{\alpha}$ P-value from the Egger's regression asymmetry test (P<0.10)

$^{\beta}$ I^2 metric of inconsistency (95% confidence interval) and the P-value of the Q test

$^{\chi}$ Observed number of statistically significant studies in each meta-analysis

$^{\delta}$ Expected number of statistically significant studies using the point estimate of each meta-analysis (from fixed effect, random effect of largest study accordingly) as the plausible effect size

$^{\varepsilon}$P value of the excess statistical significance test

All statistical tests were two-sided

Table 3.3. AMSTAR 2 methodological quality assessment for observational systematic reviews investigating the association of diabetes with gynaecological and obstetric morbidity- cohorts only

AMSTAR 2 Questions **Study Author, year**	PICO	'A priori ' design and deviations justified	Study design	Literature search	Duplicate study selection review	Duplicate data extraction	Excluded studies	Description of included studies	Assess risk of bias	Fund-ing	Statistical methods for meta-analysis	Impact of RoB from meta-analysis	RoB in individual studies in results	Heterogeneity	Small study bias	Conflict of interest	**Score**
Balsells 2009	●	○	○	◎	●	○	○	◎	●	○	●	○	○	○	●	●	Critically low
Bhatia 2020	●	●	○	◎	●	○	○	●	●	○	●	○	●	●	●	●	Low
Chen 2019	●	○	○	◎	○	○	○	◎	●	○	●	○	●	●	●	●	Critically low
Flenady 2011	●	○	○	◎	●	●	○	●	●	○	●	●	●	○	○	●	Critically low
Inkster 2006	●	○	○	◎	○	○	○	◎	●	○	●	●	○	○	●	●	Critically low
Li 2019	●	○	○	◎	●	●	○	◎	●	○	●	●	●	●	●	●	Critically low
Liao 2014	●	○	○	◎	●	●	○	◎	●	○	●	●	●	●	●	●	Critically low
Manerkar 2020	●	●	○	●	●	●	●	●	●	○	●	●	●	●	○	●	Low
Pergaliotis 2016	●	○	○	◎	●	○	●	◎	●	○	●	○	○	○	○	○	Critically low
Saed 2019	●	○	○	◎	●	●	○	◎	○	○	●	○	○	●	●	●	Critically low
Shu 2019	●	○	○	◎	●	●	○	●	●	○	●	○	●	●	●	●	Critically low
Simeone 2015	●	○	○	◎	●	●	○	●	○	○	●	○	●	○	○	●	Critically low
Tabrizi 2019	●	○	○	◎	○	○	○	◎	●	○	●	○	○	●	●	●	Critically low
Van der Looven 2019	●	○	○	◎	●	●	○	●	●	○	●	●	●	●	●	●	Critically low
Wang 2017	●	○	○	◎	●	●	○	●	●	○	●	●	○	○	●	●	Critically low
Wang 2020	●	○	○	◎	●	●	○	●	●	○	●	○	○	○	●	●	Critically low
Wendland 2012	●	○	○	◎	●	●	◎	◎	●	○	●	●	●	●	●	●	Low
Wilson 2019	●	○	○	◎	●	●	○	●	●	○	●	●	○	●	●	○	Critically low
Zhang 2015	●	○	○	◎	○	●	○	◎	○	○	●	○	○	●	●	●	Critically low
Zhang 2017	●	○	○	◎	○	○	○	◎	○	○	●	○	○	●	●	●	Critically low
Zhao 2015	●	○	○	◎	●	●	○	◎	●	○	●	●	○	●	●	○	Critically low

Abbreviations: PICO: Patient/Population- Intervention- Comparison- Outcomes, RoB: Risk of Bias

Key: ● Yes

◎ Partial yes

○ No

Critical flaw

3.4.1.8 Grading of evidence

Each of the outcomes identified as being associated with diabetes was graded into four groups according to the strength of reported evidence in cohort studies: strong, highly suggestive, suggestive or weak evidence (Table 3.4). Detailed explanation of the assessment criteria is presented in Table 3.5 (for cohort studies only), while the results for both cohort and case–control studies are shown in Table 3.6.

Only two out of 47 (4%) meta-analyses fulfilled the criteria of strong evidence of an association with diabetes; they examined the association between GDM and increased risk of caesarean section (GDM vs non- GDM, RR 1.37, 95% CI 1.24-1.51) and large for gestational age babies (GDM vs non- GDM, RR 1.53, 95% CI 1.39-1.69). Highly suggestive evidence was presented by two meta-analyses (4%), which reported associations between pre-gestational diabetes and increased risk of major congenital malformations (PGDM vs non- diabetes, RR 2.44, 95% CI 1.92-3.10) and congenital heart defects (PGDM vs non- diabetes, OR 3.18, 95% CI 2.77–3.65). Nine meta-analyses (19%) described suggestive evidence for associations of diabetes with several outcomes, including pre-eclampsia, stillbirth (>20 weeks or >400g), postnatal depression, respiratory distress syndrome, introduction of breastmilk substitute before hospital discharge and endometrial cancer. 20 meta-analyses (43%) described weak evidence and 14 meta-analyses (30%) showed no association (P>0.05).

Table 3.4. Summary of evidence grading for meta-analyses associating diabetes and anti-diabetic interventions with risk of obstetric and gynaecological morbidity– cohort studies only*

Evidence	Criteria used	Decreased risk	Increased risk
Strong	$P<10^{-6}$‖; >1,000 cases; $I^2<50\%$; no small study effects¶; prediction interval excludes the null value; no excess significance bias† survives 10% credibility ceiling *n*=2		**Maternal outcomes** Caesarean section (GDM vs non- GDM)
			Foetal outcomes Large for gestational age (GDM vs non- GDM)
Highly Suggestive	$P<10^{-6}$‖; >1,000 cases; P<0.05 of the largest study in a meta-analysis *n*=3	**Gynaecological outcomes** Ovarian cancer occurrence (Metformin vs non-metformin, T2DM)	**Foetal outcomes** Major congenital malformations (unspecified) (PGDM vs non-diabetes) Congenital heart defects (PGDM vs non- diabetes)
Suggestive	$P<10^{-3}$‖; >1000 cases *n*=10		**Maternal outcomes** Preeclampsia (GDM vs non- GDM, IADPSG criteria) Preeclampsia (GDM vs non- GDM, WHO criteria) Postnatal depression (GDM vs non- GDM)
			Foetal outcomes Stillbirth (>20 weeks or >400g) (PGDM vs non- diabetes)

			Major congenital malformations (unspecified) (GDM vs non-GDM) Large for gestational age (GDM vs non- GDM, IADPSG criteria) Respiratory distress syndrome (Diabetes vs non- diabetes) Introduction of formula milk/breastmilk substitute before hospital discharge (GDM vs non- GDM)
			Gynaecological outcomes Endometrial cancer incidence (Diabetes vs non- diabetes) Improved endometrial cancer survival (Metformin vs other anti-diabetics)
Weak	*P*<0.05 *n*=29		**Maternal outcomes** Miscarriage (Poor vs optimal glycaemic control of T1/2DM) Antenatal depressive symptoms (GDM vs non- GDM) Decreased duration of breastfeeding (GDM vs non- GDM) Low breastmilk protein content (GDM vs non- GDM) Miscarriage (Continuous sc Ins infusion vs Multiple daily inj, T1DM)
		Foetal outcomes Congenital malformations (Preconception vs no preconception care, PGDM) Perinatal mortality (Preconception vs no preconception care, PGDM)	**Foetal outcomes** Macrosomia (GDM vs non- GDM, WHO criteria) Macrosomia in Iranian women (GDM vs non- GDM) Congenital malformations (unspecified) (Poor vs optimal glycaemic control of T1/2DM)

		Preterm delivery (Preconception vs no preconception care, PGDM)	Major congenital malformations (Poor vs optimal glycaemic control of T1/2DM) Perinatal mortality (Poor vs optimal glycaemic control of T1/2DM) Perinatal mortality (T2DM vs T1DM) Congenital heart defects (PGDM vs non- diabetes) Brachial plexus palsy (GDM vs non- GDM) Respiratory distress syndrome (GDM vs non- GDM) LGA (Lispro vs Regular Ins or NPH, GDM, T1/2DM) LGA (Lispro vs Regular Ins, T1DM) Macrosomia >4.5kg (Continuous sc Ins infusion vs Multiple daily inj, T1DM) Higher birth weight (Lispro vs Regular Ins or NPH, GDM/ T1,2DM)
		Gynaecological outcomes Cervical cancer occurrence (Metformin vs non- metformin, T2DM)	**Gynaecological outcomes** Endometrial cancer mortality (disease-specific) (T1/2DM vs non-diabetes) Premalignant/Malignant endometrial polyps (Diabetes vs non-diabetes) Ovarian cancer incidence (Diabetes vs non- diabetes) Ovarian cancer mortality (disease-specific) (T1/2DM vs non-diabetes) Infrequent cervical cancer screening (Diabetes vs non- diabetes) Ovarian cancer incidence (T1DM vs non- diabetes) Ovarian cancer incidence (T2DM vs non- diabetes)

Abbreviations: GDM- Gestational diabetes mellitus; PGDM- Pregestational diabetes mellitus; T1/2DM- Type 1/2 diabetes mellitus; LGA- Large for gestational age; CS- Caesarean section;WHO- World Health Organisation; IADSPG- International Association of Diabetes and Pregnancy Groups; Ins- Insulin; sc- subcutaneous; inj- injections.

Key:

*only meta-analyses meeting at least weak grade of evidence listed

‖*P* indicates the *p*-values of the meta-analysis random effects model

¶Small study effect is based on the *P*-value from the Egger's regression asymmetry test (*P*>0.1) where the random effects summary estimate was larger compared to the point estimate of the largest study in a meta-analysis

†Based on the *p*-value (*P*>0.1) of the excess significance test using the largest study (smallest standard error) in a meta-analysis as the plausible effect size.

Table 3.5. Details of evidence grading for meta-analyses associating diabetes and anti-diabetic interventions with risk of obstetric and gynaecological morbidity– cohort studies only*

Exposure	Exposure contrast	*N*'	Sample size Cases/cohort	Largest study#	Random effects summary RR (95% CI)¥	Random *P*-value‖	95% Prediction interval	Egger's *P*∞	I^2 (%)	Excess significance§	
										O/E ¤	*P*-value♀
Strong evidence											
Obstetric, maternal											
Caesarean section	GDM vs non- GDM	4	5800/30045	1.42 (1.31-1.53)	1.37 (1.25-1.50)	1.08E-11	1.03-1.81	0.92	22	3/3.18	NP
Obstetric, foetal											
Large for gestational age	GDM vs non- GDM	4	2755/28755	1.51 (1.36-1.68)	1.53 (1.39-1.68)	1.10E-17	1.24-1.89	0.478	0	2/2.88	NP
Highly suggestive evidence											
Obstetric, foetal											
Major congenital malformations (unspecified)	PGDM vs non-diabetes	13	33400/1533451	3.57 (3.00-4.25)	2.43 (1.92-3.09)	1.99E-13	1.13-5.23	0.94	78	9/11.2	NP
Congenital heart defects	PGDM vs non-diabetes	13	66967/6974271	4.36 (4.02-4.73)	3.41 (2.89-4.03)	<1.0E-100	2.02-5.77	0.058	76	10/13	NP
Gynaecological											
Ovarian cancer occurrence	Metformin vs non-metformin, T2DM	3	3288/513702	0.16 (0.14-0.17)	0.18 (0.12-0.25)	2.52E-23	0.01-4.31	0.38	14	2/2.42	NP
Suggestive evidence											
Obstetric, maternal											
Preeclampsia	GDM vs non- GDM, IADPSG criteria	3	1909/35052	2.02 (1.78-2.29)	1.71 (1.37-2.13)	2.45E-06	0.14-21.04	0.55	74	2/2.99	NP
Preeclampsia	GDM vs non- GDM, WHO criteria	3	1301/26677	1.55 (1.33-1.81)	1.70 (1.31-2.20)	6.27E-05	0.13-22.01	0.58	40	2/2.18	NP

Postnatal depression	GDM vs non- GDM	12	2083/1064553	1.06 (1.00-1.11)	1.54 (1.22-1.95)	3.03E-04	0.74-3.22	0.006	79	7/1.8	2.70E-05
Obstetric, foetal											
Stillbirth (>20 weeks or >400g)	PGDM vs non-diabetes	3	3614/564896	1.82 (1.16-2.84)	2.52 (1.52-4.17)	3.40E-04	0.01-530.07	0.36	56	3/2.95	0.83
Major congenital malformations (unspecified)	GDM vs non- GDM	17	41668/1816289	1.18 (1.07-1.30)	1.18 (1.08-1.28)	1.39E-04	1.02-1.36	0.97	10	3/5.61	NP
Large for gestational age	GDM vs non- GDM, IADPSG criteria	3	3392/35902	1.95 (1.79-2.13)	1.75 (1.39-2.20)	1.51E-06	0.11-8.60	0.465	87	2/3.0	NP
Respiratory distress syndrome	Diabetes vs non-diabetes	20	81395/2028020	1.01 (0.91-1.11)	1.39 (1.17-1.65)	1.73E-04	0.73-2.66	0.20	81	8/1.19	<1E-100
Introduction of formula milk/breastmilk substitute before hospital discharge	GDM vs non- GDM	5	19594/29089	1.33 (1.17-1.52)	1.49 (1.18-1.88)	0.00081	0.75-2.96	0.14	56	3/3.58	NP
Gynaecological											
Endometrial cancer incidence	Diabetes vs non-diabetes	9	3564/429206	1.16 (0.90-1.48)	1.56 (1.21-2.01)	5.04E-04	0.72-3.37	0.18	69	4/2.9	0.43
Endometrial cancer survival	Metformin vs other anti-diabetics	3	1368/2015	0.43 (0.24-0.77)	0.47 (0.33-0.67)	3.84E-05	0.04-4.88	0.19	0	2/2.99	NP
Weak evidence											
Obstetric, maternal											
Miscarriage	Poor vs optimal glycaemic control (T1/2DM)	3	85/544	1.77 (0.88-3.72)	2.14 (1.13-4.04)	1.93E-02	0.03-131.76	0.50	0	0/0.98	NP
Antenatal depression	GDM vs non- GDM	2	208/2152	1.69 (0.88-3.23)	1.87 (1.03-3.39)	0.039	N/A	N/A	0	0/1.07	NP
Duration of breastfeeding	GDM vs non- GDM	5	901/9716	(-)0.07 (-0.17-0.03)	(-)0.24 (-0.42--0.07)	0.0073	(-)0.84-0.35	0.46	77	3/2.82	0.87

Breastmilk protein content	GDM vs non- GDM	2	58/272	(-)0.45 (-0.86--0.05)	(-)0.36 (-0.67--0.04)	0.025	N/A	N/A	44	1/1.93	NP
Miscarriage	Continuous sc Ins infusion vs Multiple daily inj (T1DM)	10	425/3732	2.15 (1.59-2.910	1.76 (1.39-2.23)	2.23E-06	1.11-2.80	0.11	20	3/5.19	NP
Obstetric, foetal											
Macrosomia	GDM vs non- GDM, WHO criteria	5	804/11588	1.66 (1.29-2.13)	1.81 (1.47-2.22)	2.12E-08	1.29-2.53	0.504	0	3/3.07	NP
Macrosomia in Iranian women	GDM vs non- GDM	3	103/2491	2.50 (1.16-5.39)	77 (1.97-7.21)	5.99E-05	0.01-2276	0.91	44	3/2.44	0.41
Congenital malformations (unspecified)	Poor vs optimal glycaemic control (T1/2DM)	8	238/3684	1.9 (1.19-3.11)	2.33 (1.60-3.40)	1.05E-05	1.46-3.73	0.01	0	2/3.01	NP
Major congenital malformations	Poor vs optimal glycaemic control (T1/2DM)	4	95/797	4.06 (1.12-22.12)	4.53 (1.61-12.77)	4.22E-03	0.47-44.01	0.22	0	0/3.16	NP
Perinatal mortality	Poor vs optimal glycaemic control (T1/2DM)	3	72/2701	2.73 (1.46-5.49)	2.75 (1.46-5.17)	1.66E-03	0.05-164.28	0.73	0	1/1.27	NP
Perinatal mortality	T2DM vs T1DM	22	279/8797	1.10 (0.68-1.77)	1.51 (1.16-1.97)	2.41E-03	1.14-2.00	0.43	0	2/1.14	0.40
Congenital heart defects	PGDM vs non-diabetes	8	173/1281197	3.4 (2.6-4.5)	3.64 (2.73-4.85)	1.07E-18	1.61-8.22	0.97	61	6/5.60	0.749
Brachial plexus palsy	GDM vs non- GDM	3	244/54484	3.28 (2.00-5.39)	3.94 (2.70-5.76)	1.23E-12	0.34-45.86	0.067	0	3/3	NP
Respiratory distress syndrome	GDM vs non- GDM	9	12659/1609618	1.50 (1.30-1.70)	1.44 (1.16-1.78)	0.0011	0.76-2.71	0.69	71	5/6.96	NP
LGA	Lispro vs Regular Ins or NPH (GDM, T1/2DM)	4	329/875	1.50 (1.15-1.96)	1.42 (1.19-1.70)	8.45E-05	0.97-2.09	0.65	0	2/1.54	0.64

LGA	Lispro vs Regular Ins (T1DM)	2	164/355	1.41 (1.06-1.86)	1.41 (1.09-1.83)	0.0084	N/A	N/A	0	1/0.57	0.51
Macrosomia >4.5kg	Continuous sc Ins infusion vs Multiple daily inj (T1DM)	2	27/482	2.37 (1.06-5.31)	2.50 (1.20-5.20)	0.014	N/A	N/A	0	1/0.76	0.73
Birth weight	Lispro vs Regular Ins or NPH (GDM, T1/2DM)	6	318/1018	128.00 (-23.76-279.76)	116.44 (28.78-204.11)	0.0092	(-)7.74-240.63	0.84	0	1/3.66	NP
Congenital malformations	Preconception vs no preconception care (PGDM)	11	124/2361	1.60 (0.52-4.93)	0.29 (0.15-0.56)	2.31E-04	0.06-1.34	0.3	31	4/1.44	0.021
Perinatal mortality	Preconception vs no preconception care (PGDM)	5	33/1015	0.28 (0.08-0.96)	0.36 (0.15-0.87)	0.023	0.08-1.51	0.27	0	1/1.84	NP
Preterm delivery	Preconception vs no preconception care (PGDM)	4	216/583	0.64 (0.47-0.88)	0.70 (0.55-0.89)	0.0038	0.42-1.19	0.49	0	1/1.28	NP
Gynaecological											
Endometrial cancer mortality (disease-specific)	T1/2DM vs non-diabetes	6	2075/1,268,756	1.33 (1.07-1.65	1.32 (1.11-1.57)	1.6E-3	1.03-1.69	0.61	0	1/2.26	NP
Premalignant/ Malignant endometrial polyps	Diabetes vs non-diabetes	8	154/5212	3.04 (1.02-9.05)	2.85 (1.72-4.72)	4.53E-05	1.52-5.35	0.49	0	2/5.25	NP
Ovarian cancer incidence	Diabetes vs non-diabetes	14	5534/3708313	1.05 (0.93-1.2)	1.19 (1.06-1.34)	4.36E-03	0.87-1.62	0.38	44	2/1.18	0.43
Ovarian cancer mortality (disease-specific)	T1/2DM vs non-diabetes	5	15312/610592	1.32 (1.16-1.5)	1.44 (1.08-1.93)	1.35E-02	0.49-4.25	0.47	90	4/3.75	0.79
Cervical cancer screening	Diabetes vs non-diabetes	3	2463/4268	0.74 (0.50-1.10)	0.71 (0.52-0.96)	0.029	0.09-5.31	0.029	0	0/2.73	NP

Ovarian cancer incidence	T1DM vs non-diabetes	4	868/495012	1.38 (1.01-1.89)	1.83 (1.21-2.78)	0.0045	0.36-9.32	0.32	56	3/1.29	0.068
Ovarian cancer incidence	T2DM vs non-diabetes	13	4168/2373203	1.23 (1.15-1.32)	1.24 (1.06-1.44)	6.60E-03	0.76-2.02	0.73	82	3/5.59	NP
Cervical cancer occurrence	Metformin vs non-metformin (T2DM)	2	481/144262	0.60 (0.43-0.84)	0.60 (0.43-0.83)	0.0023	N/A	N/A	0	1/1.1	NP

Abbreviations: GDM- Gestational diabetes mellitus; PGDM- Pregestational diabetes mellitus; T1/2DM- Type 1/2 diabetes mellitus**;** WHO- World Health Organisation; IADPSG- International Association of the Diabetes and Pregnancy Study Groups; NP- not pertinent (because the estimated is larger than the observed, and there is no evidence of excess statistical significance based on the assumption made for the plausible effect size)

Summary of evidence grading criteria

Weak	$P<0.05$[ll]
Suggestive	$P<10^{-3}$[ll]; >1,000 cases
Highly suggestive	$P<10^{-6}$[ll]; >1,000 cases; $P<0.05$ of the largest study in a meta-analysis
Strong	$P<10^{-6}$[ll]; >1,000 cases; $P<0.05$ of the largest study in a meta-analysis; $I^2<50\%$; no small study effect[¶]; prediction interval excludes the null value; no excess significance bias[†]

Key:

*only meta-analyses meeting at least weak grade of evidence listed

* Number of studies

Relative risk and 95% confidence interval of largest study (smallest standard error) in each meta-analysis

¥ Random effects refer to summary risk ratio (95% confidence interval) using the random-effects model

ǁ *P* value of summary random effects estimate

ºº *P*-value from the Egger's regression asymmetry test

§ Expected number of statistically significant studies using the point estimate of the largest study (smallest standard error) as the plausible effect size

ᵅ Observed/Expected number of statistically significant studies (largest study)

φ*P* value of the excess statistical significance test (largest study)

¶Small study effect is based on the *P*-value from the Egger's regression asymmetry test (*P*>0.1) where the random effects summary estimate was larger compared to the point estimate of the largest study in a meta-analysis

†Based on the *p*-value (*P*>0.1) of the excess significance test using the largest study (smallest standard error) in a meta-analysis as the plausible effect size.

All statistical tests were two-sided

3.4.1.9 Sensitivity analysis

When both cohort and case-control studies were included in the analysis, two further meta-analyses met the criteria for strong evidence; these reported the association of PGDM and stillbirth (>20 weeks or >400g), which was upgraded from suggestive and the association of diabetes and cervical cancer screening, previously classified as weak evidence. The association of GDM and brachial plexus palsy was upgraded from weak to highly suggestive, while the correlation of GDM with respiratory distress syndrome was upgraded from weak to suggestive (Table 3.6).

24 duplicate meta-analyses were identified investigating the same exposure and outcome association that were not selected for further analysis either because of missing study specific data (number of cases/controls, relative risk, 95% confidence interval) or because another study included a bigger number of cohort studies (Table 3.7). For the majority of these duplicate meta-analyses, there was agreement in principle on the direction, magnitude, and significance of the summary associations with the meta-analyses included in the analysis instead.

Table 3.6. Details of evidence grading for meta-analyses associating diabetes and anti-diabetic interventions with risk of obstetric and gynaecological morbidity– all study types included

Exposure	Exposure contrast	*N*[*]	Sample size Cases/cohort	Largest study[#]	Random effects summary RR (95% CI)[¥]	Random *P*-value[ll]	95% Predictio n interval	Egger's *P*[∞]	I^2 (%)	Excess significance[§]	
										O/E [α]	*P*-value[φ]
Strong evidence											
Obstetric, maternal											
Caesarean section	GDM vs non- GDM	4	5800/30045	1.42 (1.31-1.53)	1.37 (1.25-1.50)	1.08E-11	1.03-1.81	0.92	22	3/3.18	NP
Obstetric, foetal											
Large for gestational age	GDM vs non- GDM	4	2755/28755	1.51 (1.36-1.68)	1.53 (1.39-1.68)	1.10E-17	1.24-1.89	0.478	0	2/2.88	NP
Stillbirth (>20 weeks or >400 g)	PGDM vs non- DM	5	7680/1892890	3.34 (2.46-4.55)	2.90 (2.05-4.09)	1.43E-09	1.05-7.99	0.83	49	5/5	NP
Gynaecological											
Cervical ca screening	Diabetes vs non- diabetes	14	251558/311368	0.73 (0.66-0.81)	0.76 (0.71-0.81)	2.55E-16	0.70-0.81	0.94	0	4/13.6	NP
Highly suggestive evidence											
Obstetric, foetal											
Major congenital malformations (unspecified)	PGDM vs non- diabetes	13	33400/1533451	3.57 (3.00-4.25)	2.43 (1.92-3.09)	1.99E-13	1.13-5.23	0.94	78	9/11.2	NP
Brachial plexus palsy	GDM vs non GDM	10	3209/1651281	4.58 (3.34-6.30)	5.33 (3.77-7.55)	3.83E-21	1.99-14.26	0.88	59	8/9.69	NP
Congenital heart defects	PGDM vs non- diabetes	31	236491/15009001	4.36 (4.02-4.73)	3.18 (2.78-3.65)	<1E-100	1.73-5.86	0.06	79	26/30.8	NP
Gynaecological											
Ovarian ca occurrence	Metformin vs non-metformin, T2DM	3	3288/513702	0.16 (0.14-0.17)	0.18 (0.12-0.25)	2.52E-23	0.01-4.31	0.38	14	2/2.42	NP
Suggestive evidence											

Obstetric, maternal											
Preeclampsia	GDM vs non- GDM, IADPSG criteria	3	1909/35052	2.02 (1.78-2.29)	1.71 (1.37-2.13)	2.45E-06	0.14-21.04	0.55	74	2/2.99	NP
Preeclampsia	GDM vs non- GDM, WHO criteria	3	1301/26677	1.55 (1.33-1.81)	1.70 (1.31-2.20)	6.27E-05	0.13-22.01	0.58	40	2/2.18	NP
Postnatal depression	GDM vs non- GDM	15	2164/1066123	1.06 (1.00-1.11)	1.59 (1.26-2.00)	7.53E-05	0.74-3.41	0.003	79	8/1.96	3.80E-06
Obstetric, foetal											
Major congenital malformations (unspecified)	GDM vs non- GDM	17	41668/1816289	1.18 (1.07-1.30)	1.18 (1.08-1.28)	1.39E-04	1.02-1.36	0.97	10	3/5.61	NP
Large for gestational age	GDM vs non- GDM, IADPSG criteria	3	3392/35902	1.95 (1.79-2.13)	1.75 (1.39-2.20)	1.51E-06	0.11-8.60	0.465	87	2/3.0	NP
Respiratory distress syndrome	Diabetes vs non- diabetes	24	81768/2029896	1.01 (0.91-1.11)	1.47 (1.24-1.74)	6.34E-06	0.75-2.89	0.09	81	11/1.4	<1E-100
Respiratory distress syndrome	GDM vs non- GDM	13	13032/1611494	1.50 (1.30-1.70)	1.57 (1.28-1.93)	2.00E-05	0.82-3.03	0.36	71	8/8.68	NP
Introduction of formula milk/breastmilk substitute before hospital discharge	GDM vs non- GDM	5	19594/29089	1.33 (1.17-1.52)	1.49 (1.18-1.88)	0.00081	0.75-2.96	0.14	56	3/3.58	NP
Gynaecological											
Endometrial cancer incidence	Diabetes vs non- diabetes	9	3564/429206	1.16 (0.90-1.48)	1.56 (1.21-2.01)	5.04E-04	0.72-3.37	0.18	69	4/2.9	0.43
Endometrial cancer survival	Metformin vs other anti-diabetics	3	1368/2015	0.43 (0.24-0.77)	0.47 (0.33-0.67)	3.84E-05	0.04-4.88	0.19	0	2/2.99	NP
Weak evidence											
Obstetric, maternal											
Miscarriage	Poor vs optimal glycaemic control (T1/2DM)	4	126/1117	1.77 (0.88-3.72)	3.15 (1.61-6.14)	7.72E-04	0.37-26.86	0.71	28	1/1.47	NP

Antenatal depression	GDM vs non- GDM	6	358/4387	1.52 (1.09-2.12)	2.08 (1.42-3.05)	0.00018	0.77-5.61	0.41	46	2/2	NP
Duration of breastfeeding	GDM vs non- GDM	5	901/9716	(-)0.07 (-0.17-0.03)	(-)0.24 (-0.42--0.07)	0.0073	(-)0.84-0.35	0.46	77	3/2.82	0.87
Breastmilk protein content	GDM vs non- GDM	2	58/272	(-)0.45 (-0.86--0.05)	(-)0.36 (-0.67--0.04)	0.025	N/A	N/A	44	1/1.93	NP
Miscarriage	Continuous sc Ins infusion vs Multiple daily inj (T1DM)	14	476/4257	2.15 (1.59-2.91)	1.74 (1.37-2.20)	4.91E-06	1.04-2.91	0.17	24	4/6.35	NP
Obstetric, foetal											
Macrosomia	GDM vs non- GDM, WHO criteria	5	804/11588	1.66 (1.29-2.13)	1.81 (1.47-2.22)	2.12E-08	1.29-2.53	0.504	0	3/3.07	NP
Congenital malformations (unspecified)	Poor vs optimal glycaemic control (T1/2DM)	12	306/4943	1.90 (1.19-3.11)	2.96 (2.07-4.22)	2.05E-09	1.77-4.96	0.067	5	4/4.17	NP
Major congenital malformations	Poor vs optimal glycaemic control (T1/2DM)	6	134/1785	3.49 (1.20-10.42)	5.05 (2.58-9.87)	2.23E-06	1.95-13.05	0.520	0	2/4.62	NP
Perinatal mortality	Poor vs optimal glycaemic control (T1/2DM)	4	91/3136	2.73 (1.46-5.49)	3.01 (1.75-5.17)	6.71E-05	0.92-9.89	0.69	0	2/1.93	0.94
Perinatal mortality	T2DM vs T1DM	22	279/8797	1.10 (0.68-1.77)	1.51 (1.16-1.97)	2.41E-03	1.14-2.00	0.43	0	2/1.14	0.40
Anorectal malformations	PGDM vs non-diabetes	4	828/53789	8.18 (3.86-17.34)	4.51 (2.55-7.96)	2.10E-07	0.74-27.53	0.37	27	3/3.99	NP
Anorectal malformations	GDM vs non GDM	5	1100/93772	1.18 (0.71-1.98)	1.81 (1.23-2.65)	2.54E-03	0.68-4.84	0.460	30	2/1.34	0.50
Persistent pulmonary hypertension of the newborn	Diabetes vs non diabetes	7	4950/1939461	1.40 (1.26-1.55)	1.66 (1.18-2.36)	0.0041	0.67-4.16	0.27	56	3/4.19	NP
LGA	Lispro vs Regular Ins or NPH (GDM, T1/2DM)	5	372/1071	1.50 (1.15-1.96)	1.42 (1.20-1.68)	3.47E-05	1.08-1.86	0.6	0	2/1.8	0.85

LGA	Lispro vs Regular Ins (T1DM)	3	299/816	1.41 (1.06-1.86)	1.39 (1.14-1.68)	8.99E-04	0.40-4.83	0.69	0	2/1.03	0.24
Macrosomia >4.5kg	Continuous sc Ins infusion vs Multiple daily inj (T1DM)	2	27/482	2.37 (1.06-5.31)	2.50 (1.20-5.20)	0.014	N/A	N/A	0	1/0.76	0.73
Birth weight	Lispro vs Regular Ins or NPH (GDM, T1/2DM)	6	318/1018	128.00 (-23.76-279.76)	116.44 (28.78-204.11)	0.0092	(-)7.74-240.63	0.84	0	1/3.66	NP
Birth weight	Myonisitol vs placebo (GDM)	3	180/353	(-)162.00 (-295.08--28.92)	(-)114.52 (-213.67--15.37)	0.024	(-)871.93-642.89	0.31	13	1/ 2.85	NP
Congenital malformations	Preconception vs no preconception care (PGDM)	11	124/2361	1.60 (0.52-4.93)	0.29 (0.15-0.56)	2.31E-04	0.06-1.34	0.3	31	4/1.44	0.021
Perinatal mortality	Preconception vs no preconception care (PGDM)	5	33/1015	0.28 (0.08-0.96)	0.36 (0.15-0.87)	0.023	0.08-1.51	0.27	0	1/1.84	NP
Preterm delivery	Preconception vs no preconception care (PGDM)	4	216/583	0.64 (0.47-0.88)	0.70 (0.55-0.89)	0.0038	0.42-1.19	0.49	0	1/1.28	NP
Gynaecological											
Endometrial cancer mortality (disease-specific)	T1/2DM vs non- diabetes	6	2075/1,268,756	1.33 (1.07-1.65	1.32 (1.11-1.57)	1.6E-3	1.03-1.69	0.61	0	1/2.26	NP
Premalignant/Malignant endometrial polyps	Diabetes vs non- diabetes	8	154/5212	3.04 (1.02-9.05)	2.85 (1.72-4.72)	4.53E-05	1.52-5.35	0.49	0	2/5.25	NP
Ovarian cancer incidence	Diabetes vs non- diabetes	14	5534/3708313	1.05 (0.93-1.2)	1.19 (1.06-1.34)	4.36E-03	0.87-1.62	0.38	44	2/1.18	0.43
Ovarian cancer mortality (disease-specific)	T1/2DM vs non- diabetes	5	15312/610592	1.32 (1.16-1.5)	1.44 (1.08-1.93)	1.35E-02	0.49-4.25	0.47	90	4/3.75	0.79
Ovarian cancer incidence	T1DM vs non- diabetes	4	868/495012	1.38 (1.01-1.89)	1.83 (1.21-2.78)	0.0045	0.36-9.32	0.32	56	3/1.29	0.068
Ovarian cancer incidence	T2DM vs non- diabetes	13	4168/2373203	1.23 (1.15-1.32)	1.24 (1.06-1.44)	6.60E-03	0.76-2.02	0.73	82	3/5.59	NP
Hirsutism prevalence	T1DM vs non-diabetes	8	120/471	0.38 (0.27-0.5)	0.29 (0.21-0.40)	6.10E-14	0.11-0.76	0.14	71	7/3.24	0.0068

Hyperandrogenemia prevalence	T1DM vs non-diabetes	6	88/383	0.45 (0.31-0.59)	0.27 (0.19-0.37)	1.20E-14	0.09-0.76	0.36	67	6/1.86	2.63E-04
Menstrual dysfunction prevalence	T1DM vs non-diabetes	8	117/473	0.28 (0.20-0.37)	0.25 (0.19-0.34)	9.28E-21	0.11-0.56	0.11	56	8/4.48	0.012
PCOM prevalence	T1DM vs non-diabetes	7	105/343	0.55 (0.39-0.70)	0.35 (0.26-0.47)	1.09E-12	0.15-0.83	0.29	65	7/1.32	4.00E-08
PCOS prevalence	T1DM vs non-diabetes	8	99/413	0.37 (0.28-0.46)	0.25 (0.17-0.34)	3.13E-16	0.09-0.64	0.02	63	8/2.85	1.41E-04
Cervical cancer occurrence	Metformin vs non-metformin (T2DM)	2	481/144262	0.60 (0.43-0.84)	0.60 (0.43-0.83)	0.0023	N/A	N/A	0	1/1.1	NP

Abbreviations: GDM- Gestational diabetes mellitus; PGDM- Pregestational diabetes mellitus; T1/2DM- Type 1/2 diabetes mellitus; ca- cancer; PCOM- Polycystic ovary morphology; PCOS- Polycystic ovary syndrome; WHO- World Health Organisation; IADPSG- International Association of the Diabetes and Pregnancy Study Groups; NP- not pertinent (because the estimated is larger than the observed, and there is no evidence of excess statistical significance based on the assumption made for the plausible effect size)

Summary of evidence grading criteria

Weak	$P<0.05$[II]
Suggestive	$P<10^{-3}$[II]; >1,000 cases
Highly suggestive	$P<10^{-6}$[II]; >1,000 cases; $P<0.05$ of the largest study in a meta-analysis
Strong	$P<10^{-6}$[II]; >1,000 cases; $P<0.05$ of the largest study in a meta-analysis; $I^2<50\%$; no small study effect[¶]; prediction interval excludes the null value; no excess significance bias[†]

Key:

*only meta-analyses meeting at least weak grade of evidence listed

* Number of studies

Relative risk and 95% confidence interval of largest study (smallest standard error) in each meta-analysis

¥ Random effects refer to summary risk ratio (95% confidence interval) using the random-effects model

ll *P* value of summary random effects estimate

°° *P*-value from the Egger's regression asymmetry test

§ Expected number of statistically significant studies using the point estimate of the largest study (smallest standard error) as the plausible effect size

α Observed/Expected number of statistically significant studies

φ*P* value of the excess statistical significance test

¶Small study effect is based on the *P*-value from the Egger's regression asymmetry test (*P*>0.1) where the random effects summary estimate was larger compared to the point estimate of the largest study in a meta-analysis

†Based on the *p*-value (*P*>0.1) of the excess significance test using the largest study (smallest standard error) in a meta-analysis as the plausible effect size.

All statistical tests were two-sided

Table 3.7. Duplicate and excluded meta-analyses and meta-analyses included instead on the effect of diabetes on gynaecological/obstetric outcomes- cohorts only

Exposure	Exposure contrast	Author, year	Outcome	N[a] studies	N cohort studies	Summary point estimate & 95% CI[b]	Included	Evidence grade[c]
Gynaecological outcomes								
DM	DM vs non-diabetes	Saed 2019	Endometrial ca incidence	9	9	1.56 (1.21–2.01)	Yes	Suggestive
GDM	GDM vs non-GDM	Wang 2020	Endometrial ca incidence	2	1	0.77 (0.20–2.98)	Yes	NS
DM 1/2	DM vs non-diabetes	Liao 2014	Endometrial ca incidence	7	7	1.71 (1.48- 1.97)	No	
DM (largely type 2)	DM vs non-diabetes	Zhang 2013	Endometrial ca incidence	8	8	1.92 (1.23- 3.01)	No	
DM (Glycemic index)	Highest vs lowest	Galeone 2013	Endometrial ca incidence	7	5	1.09 (0.92- 1.29)	No	
DM (Glycemic load)	Highest vs lowest	Galeone 2013	Endometrial ca incidence	7	5	1.19 (1.06- 1.34)	No	
DM (Glycemic index)	Highest vs lowest	Choi 2012	Endometrial ca incidence	5	5	1 (0.87- 1.14)	No	
DM (Glycemic load)	Highest vs lowest	Choi 2012	Endometrial ca incidence	5	5	1.21 (1.07- 1.37)	No	
DM	DM vs population	Noto 2010	Endometrial ca incidence	4	1	3.43 (1.53- 7.72)	No	
DM	DM vs population	Friberg 2007	Endometrial ca incidence	16	3	2.1 (1.75- 2.53)	No	
DM 1/2	DM vs non-diabetes	Liao 2014	Endometrial ca mortality (disease-specific)	6	6	1.32 (1.10–1.60)	Yes	Weak
DM (largely type 2)	DM vs non-diabetes	Zhang 2013	Endometrial ca mortality	3	3	1.47 (1.06- 2.04)	No	
DM (unspecified)	DM vs non-diabetes	Wang 2017	Ovarian ca incidence	14	14	1.19 (1.06- 1.34)	Yes	Weak
GDM	GDM vs non GDM	Wang 2020	Ovarian ca incidence	4	4	1.14 (0.90–1.44)	Yes	NS
DM	DM vs controls	Lee 2013	Ovarian ca incidence	19	12	1.17 (1.02- 1.33)	No	
DM 1	DM vs non-diabetes	Zhang 2017	Ovarian ca incidence	4	4	1.83 (1.21- 2.78)	Yes	Weak
DM 2	DM vs non-diabetes	Zhang 2017	Ovarian ca incidence	13	13	1.24 (1.06- 1.44)	Yes	Weak
Maternal obstetric outcomes								
GDM (WHO criteria)	GDM vs non-GDM	Wendland 2012	CS	4	4	1.37 (1.24-1.51)	Yes	Strong
GDM (IADPSG-criteria)	GDM vs non-GDM	Wendland 2012	CS	3	3	1.23 (1.01- 1.51)	Yes	NS
PGDM	PGDM vs non-diabetes	Yu 2017	CS	42	NK	3.52 (2.91- 4.25)	No	

GDM	GDM vs non-GDM	Farrar 2016	CS	4	4	1.66 (1.52- 1.82)	No	
GDM (IADPSG-criteria)	GDM vs non-GDM	Wendland 2012	Pre-eclampsia	3	3	1.71 (1.37- 2.14)	Yes	Suggestive
PGDM	PGDM vs non-diabetes	Yu 2017	Pre-eclampsia	48	NK	3.48 (3.01- 4.02)	No	
GDM	GDM vs non-GDM	Wilson 2019	Postnatal depression	15	12	1.59 (1.26, 2.00)	Yes	Suggestive
GDM	GDM vs non-GDM	Ross 2016	Postnatal depression	6	NK	NK	No	
PGDM	PGDM vs non-diabetes	Ross 2016	Postnatal depression	5	NK	NK	No	
Foetal obstetric outcomes								
GDM (WHO criteria)	GDM vs non-GDM	Wendland 2012	LGA	4	4	1.53 (1.39- 1.69)	Yes	Strong
GDM (IADPSG-criteria)	GDM vs non-GDM	Wendland 2012	LGA	3	3	1.73 (1.28- 2.35)	Yes	Suggestive
GDM	GDM vs non-GDM	Roeckner 2016	LGA	7	7	1.12 (0.66- 1.90)	No	
PGDM	PGDM vs non-diabetes	Zhao 2015	MCM (unspecified)	13	13	2.44 (1.92- 3.10)	Yes	Highly suggestive
PGDM	PGDM vs control	Balsells 2012	MCM	10	9	2.66 (2.04- 3.47)	No	
GDM	GDM vs non-GDM	Zhao 2015	MCM	17	17	1.18 (1.11- 1.26)	Yes	Suggestive
GDM	GDM vs control	Balsells 2012	MCM	17	15	1.16 (1.07- 1.25)	No	
DM 1/2	DM2 vs DM1	Balsells 2009	MCM	24	24	1.19 (0.91- 1.56)	Yes	NS
DM 1/2	Poor vs optimal glycaemic control	Inkster 2006	MCM	6	4	5.14 (2.94- 9.01)	Yes	Weak
PGDM	PGDM vs non- DM	Flenady 2011	Stillbirth (>20 weeks or >400 g)	5	3	2.9 (2.05- 4.09)	Yes	Suggestive
PGDM	PGDM vs non-diabetes	Yu 2017	Stillbirth	39	NK	3.52 (3.19- 3.88)	No	
DM 1/2	DM2 vs DM1	Balsells 2009	Stillbirth	19	19	1.23 (0.82- 1.85)	Yes	NS
PGDM	PGDM vs non-diabetes	Chen 2019	Congenital heart defects	31	13	3.18 (2.77–3.65)	Yes	Highly suggestive
PGDM	PGDM vs non-diabetes	Simeone 2015	Congenital heart defects	12	8	3.8 (3.0- 4.9)	No	
Diabetes (PGDM and GDM)	Diabetes vs non-diabetes	Li 2019	RDS	24	20	1.47 (1.24–1.74)	Yes	Suggestive
PGDM	PGDM vs non-diabetes	Yu 2017	RDS	NK	NK	2.05 (1.55- 2.83)	No	
GDM	GDM vs non-GDM	Li 2019	RDS	13	9	2.66 (2.06–3.44)	Yes	Weak
GDM (WHO criteria)	GDM vs non-GDM	Wendland 2012	Macrosomia	5	5	1.81 (1.47- 2.22)	Yes	Weak
GDM	GDM vs non-GDM	Roeckner 2016	Macrosomia	9	9	0.98 (0.55- 1.76)	No	

GDM	GDM vs non-GDM	He 2015	Macrosomia (over 4000 / 4500g)	14	4	1.71 (1.52- 1.94)	No	
PGDM	PGDM vs non-diabetes	Yu 2017	Macrosomia> 4kg	17	NK	1.91(1.74- 2.10)	No	
DM 1/2	Poor vs optimal glycaemic control	Inkster 2006	Perinatal mortality	4	3	3.03 (1.87- 4.92)	Yes	Weak
DM 1/2	DM2 vs DM1	Balsells 2009	Perinatal mortality	22	22	1.5 (1.15- 1.96)	Yes	Weak
PGDM	PGDM vs non-diabetes	Yu 2017	Perinatal mortality	27	NK	3.39 (3.02- 3.81)	No	
GDM (WHO-criteria)	GDM vs non-GDM	Wendland 2012	Perinatal mortality	2	2	1.55 (0.88- 2.73)	Yes	NS

Abbreviations: DM, diabetes mellitus; GDM, gestational diabetes mellitus; PGDM, pregestational diabetes mellitus; WHO, World Health Organisation; IADPSG, International Association of Diabetes and Pregnancy Study Groups; ca, cancer; CS, caesarean section; LGA, large for gestational age; MCM, major congenital malformations; RDS: respiratory distress syndrome; NK, not known; NS, non- significant.

Summary of evidence grading criteria

Weak	$P<0.05$[d]
Suggestive	$P<10^{-3}$[d]; >1,000 cases
Highly suggestive	$P<10^{-6}$[d]; >1,000 cases; P<0.05 of the largest study in a meta-analysis
Strong	$P<10^{-6}$[d]; >1,000 cases; P<0.05 of the largest study in a meta-analysis; I^2<50%; no small study effect[e]; prediction interval excludes the null value; no excess significance bias[f]

Key:

[a] Number of studies

[b] Summary relative risk of random effects model including all study types

[c] Summary of evidence grading criteria:

[d] P value of summary random effects estimate

[e] Small study effect is based on the P-value from the Egger's regression asymmetry test (P>0.1) where the random effects summary estimate was larger compared to the point estimate of the largest study in a meta-analysis

[f] Based on the p-value (P>0.1) of the excess significance test using the largest study (smallest standard error) in a meta-analysis as the plausible effect size.

3.4.2 Meta-analyses of interventional observational studies

A total of 254 meta-analyses of interventional studies were identified from 53 publications [353 354 376-422], of which 54 were observational studies (49 cohorts) and 200 randomised controlled trials, examining the effect of anti-diabetic interventions on gynaecological and obstetric conditions (Figure 3.1).

3.4.2.1 Characteristics of meta-analyses

Data from 16 eligible publications were extracted, consisting of 54 observational meta-analyses with 266 study estimates, which investigated the effect of anti-diabetic interventions on gynaecological and obstetric morbidity. Out of the 266 individual studies, 200 (75%) were cohort studies, 44 (17%) case-control studies, 5 were nested case-control studies, 13 randomized controlled trials and 4 retrospective analyses of RCT data. There were 2 to 16 individual studies combined per meta-analysis with a median of 6. The median number of cases and total population in each meta-analysis was 121 and 638 respectively. The lowest number of cases in a meta-analysis was 8 and the highest was 8,723, whereas the smallest total population or controls was 103 and the highest was 5,295,969. Only cohort studies were used for main analysis.

3.4.2.2 Summary effect size

Using $P<0.05$ as a statistical significance threshold, the summary fixed effects estimate reached significance in 17/49 (35%) meta-analyses of cohort studies, while the summary random effects was significant in 11/49 (22%). At a significance cut-off of $P<0.001$, 9/49 (18%) and 5/49 (10%) meta-analyses yielded significant results using the fixed and random effects model respectively, whereas at a more stringent threshold of $P<10^{-6}$, 4/49 (8%) and 2/49 (4%) of meta-analyses produced significant results. The two meta-analyses with strongly statistically significant summary random effect estimates ($P<10^{-6}$) yielded a decreased risk of ovarian cancer occurrence in metformin users of type 2 diabetes and a decreased risk of congenital malformations in women with pregestational diabetes who received preconception care (Table 3.8).

The summary random effect estimates ranged from 0.18 (95% confidence interval 0.12 to 0.25) for an association between metformin use and ovarian cancer occurrence up to 92.22 (-73.15 to 257.59) for the association between insulin analog use and birth weight.

3.4.2.3 Heterogeneity between studies

Moderate to high heterogeneity (I^2 = 50-75%) was noted in 4/49 (8%) of meta-analyses, while substantial heterogeneity (I^2 >75%) was observed in 3/49 (6%) meta-analyses for associations of anti-diabetic medication, including metformin, with endometrial cancer incidence (Table 3.9).

3.4.2.4 Small study effects

Small study effects (Egger's test *p* value <0.10 and where more conservative effects in the largest study of a meta-analysis compared to the summary random effects estimate were recorded) was found to be present in one meta-analysis, consisting of only 3 individual studies and describing a reduced risk of preterm delivery in type 1 diabetics treated with multiple daily insulin injections vs continuous subcutaneous infusion (Table 3.9).

3.4.2.5 Excess significance bias

One meta-analysis demonstrated evidence of excess significance bias using the largest study estimate as the plausible effect size (P<0.10), which reported deceased risk of congenital malformations in women receiving preconception care and decreased risk of preeclampsia in type 1 diabetic patients using continuous subcutaneous insulin infusion (vs multiple daily injections). Using the random effects estimate the association of metformin use with increased endometrial cancer occurrence and survival were also highlighted with excess significance bias, while using the summary fixed effects estimate no further meta-analysis was highlighted (Table 3.9).

3.4.2.6 Credibility ceilings

From all 49 meta-analyses, 9 (18%) met nominal significance (P<0.05) with a credibility ceiling of 5%. With a ceiling of 10%, 3 (6%) meta-analyses remained significant, one meta-analysis met significance with a 15% ceiling whereas no meta-analysis survived the 20% credibility ceiling (Table 3.9).

3.4.2.7 Quality assessment

The methodological quality of the 16 publications included in main analysis, encompassing 49 meta-analyses of interventional observational studies, was assessed using the AMSTAR 2 tool. One (1/16) of all the included papers was graded as low and fifteen (15/16) as critically low quality (Table 3.10). Papers assessed with 'low' or 'critically low' quality failed to meet one or more than one 'critical' criteria respectively, i.e. lack of protocol, comprehensive literature search, description of excluded studies and risk of bias assessment. The majority of publications (12/16) did not meet the critical requirement of explicitly stating that the review methods were established prior to the conduct of the review and failed to justify any significant deviations from the protocol, while 13/16 did not provide a list of excluded studies accompanied by justification. With regards to other critical criteria, most included papers (12/16) provided a comprehensive literature search strategy and used appropriate statistical analyses for combination of results (12/14). 5/16 assessed the risk of bias, whereas 8/16 accounted for publication bias (small study bias).

Table 3.8. Description of 49 meta- analyses results investigating the association of anti-diabetic interventions with gynaecological and obstetric morbidity– cohort studies only

Author, year	Exposure	Exposure contrast	N[α]	Sample size cases/ cohort	Summary relative risk (95% CI)			Fixed P-value[ε]	Random P-value[ϕ]	95% Prediction interval[γ]
					Fixed Effects[β]	Random Effects[χ]	Largest Study[δ]			
Gynaecological										
Wen 2019	Metformin (T2DM)	Metformin vs non- metformin	3	3288/513702	0.16 (0.15-0.18)	0.18 (0.12-0.25)	0.16 (0.14-0.17)	<1.0E-100	2.52E-23	0.01-4.31
Chu 2018	Metformin (Diabetes)	Metformin vs other antidiabetic drugs	3	1368/2015	0.47 (0.33-0.67)	0.47 (0.33-0.67)	0.43 (0.24-0.77)	3.84E-05	3.84E-05	0.04-4.88
Wen 2019	Metformin (T2DM)	Metformin vs non- metformin	2	481/144262	0.60 (0.43-0.83)	0.60 (0.43-0.83)	0.60 (0.43-0.84)	0.0023	0.0023	N/A
Chu 2018	Metformin (Diabetes)	Metformin vs other antidiabetic drugs	5	5793/5267300	0.85 (0.79-0.92)	1.02 (0.73-1.42)	0.68 (0.61-0.74)	2.29E-05	0.92	0.29-3.60
Wen 2019	Metformin (T2DM)	Metformin vs non- metformin	4	3822/1052116	0.31 (0.29-0.33)	0.71 (0.29-1.73)	0.23 (0.21-0.25)	<1.0E-100	0.45	0.01-51.90
Tian 2019	Anti-diabetic medication (Diabetes)	Metformin vs non- metformin	3	3391/491029	0.57 (0.52-0.61)	1.04 (0.46-2.39)	0.50 (0.46-0.54)	<1.0E-100	0.92	0-40828
Raffone 2019	Conservative Mx of endometrial hyperplasia and cancer (Diabetes)	Diabetes vs non- diabetes	2	37/404	0.96 (0.30-3.07)	0.96 (0.30-3.07)	0.79 (0.17-3.60)	0.95	0.95	N/A
Raffone 2019	Conservative Mx of endometrial hyperplasia and cancer (Diabetes)	Diabetes vs non- diabetes	5	48/383	1.86 (0.61-5.67)	1.72 (0.43-6.80)	7.40 (1.18-46.39)	0.27	0.44	0.05-56.96
Obstetric, maternal										
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs Multiple daily inj	10	425/3732	1.86 (1.55-2.23)	1.76 (1.39-2.23)	2.15 (1.59-2.910	1.59E-11	2.23E-06	1.11-2.80
Ranasinghe 2015	Ins analogs/Regular Ins in MDI arm(T1DM)	MDI vs CSII	3	191/293	1.08 (0.88-1.33)	1.11 (0.77-1.58)	1.14 (0.88-1.48)	0.45	0.58	0.02-49.28

Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	3	147/220	1.01 (0.90-1.14)	1.01 (0.90-1.14)	1.00 (0.87-1.14)	0.82	0.82	0.47-2.17
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	3	25/330	0.43 (0.16-1.19)	0.52 (0.14-1.97)	0.24 (0.07-0.87)	0.1	0.34	0-90676
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	7	55/672	0.64 (0.34-1.19)	0.47 (0.19-1.20)	2.34 (0.84-6.51)	0.16	0.11	0.04-6.01
Alqudah 2018	Metformin (GDM)	Metformin vs control	4	49/1404	1.17 (0.67-2.04)	1.22 (0.57-2.62)	0.91 (0.45-1.84)	0.58	0.62	0.10-14.89
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	7	148/1125	0.91 (0.65-1.27)	1.13 (0.60-2.15)	0.76 (0.49-1.16)	0.57	0.71	0.20-6.36
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	10	313/2741	0.77 (0.60-0.99)	0.94 (0.55-1.62)	0.92 (0.63-1.34)	0.038	0.84	0.18-5.03
Obstetric, foetal										
Lv 2015	Insulin analogs (GDM,T1/2DM)	Lispro vs Regular Ins or NPH	4	329/875	1.42 (1.19-1.70)	1.42 (1.19-1.70)	1.50 (1.15-1.96)	8.45E-05	8.45E-05	0.97-2.09
Wahabi 2010	Preconception care (PGDM)	Preconception vs no preconception care	11	124/2361	0.32 (0.19-0.54)	0.29 (0.15-0.56)	1.60 (0.52-4.93)	1.92E-05	2.31E-04	0.06-1.34
Wahabi 2010	Preconception care (PGDM)	Preconception vs no preconception care	5	33/1015	0.36 (0.15-0.87)	0.36 (0.15-0.87)	0.28 (0.08-0.96)	0.023	0.023	0.08-1.51
Wahabi 2010	Preconception care (PGDM)	Preconception vs no preconception care	4	216/583	0.70 (0.55-0.89)	0.70 (0.55-0.89)	0.64 (0.47-0.88)	0.0038	0.0038	0.42-1.19
Blanco 2011	Lispro/Regular Ins (T1DM)	Lispro vs Regular Ins	2	164/355	1.41 (1.09-1.83)	1.41 (1.09-1.83)	1.41 (1.06-1.86)	0.0084	0.0084	N/A
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs Multiple daily inj	2	27/482	2.50 (1.20-5.20)	2.50 (1.20-5.20)	2.37 (1.06-5.31)	0.014	0.014	N/A
Lv 2015	Insulin analogs (GDM,T1/2DM)	Lispro vs Regular Ins or NPH	6	318/1018	116.44 (28.78-204.11)	116.44 (28.78-204.11)	128.00 (-23.76-279.76)	0.0092	0.0092	(-)7.74-240.63
Waugh 2010	Mx for GDM	Glibenclamide vs Insulin	3	14/626	3.15 (0.99-9.95)	3.15 (0.99-9.95)	3.03 (0.81-11.28)	0.051	0.051	N/A
Waugh 2010	Mx for GDM	Glibenclamide vs Insulin	3	161/1030	1.21 (0.91-1.59)	1.21 (0.91-1.59)	1.16 (0.84-1.59)	0.18	0.18	0.20-7.28
Waugh 2010	Mx for GDM	Glibenclamide vs Insulin	4	68/778	1.82 (1.08-3.07)	1.71 (0.58-5.10)	1.99 (1.00-3.95)	0.026	0.33	0.02-175.14
Ranasinghe 2015	Ins (T1DM)	MDI vs CSII	3	9/219	2.12 (0.38-11.79)	2.12 (0.38-11.79)	2.20 (0.26-18.28)	0.39	0.39	N/A

Ranasinghe 2015	Ins analogs/Regular Ins in MDI arm (T1DM)	MDI vs CSII	3	39/306	1.18 (0.65-2.17)	1.18 (0.65-2.17)	1.20 (0.56-2.58)	0.58	0.58	0.02-59.61
Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	3	39/220	0.97 (0.51-1.84)	0.97 (0.51-1.84)	1.15 (0.55-2.40)	0.92	0.92	0.02-62.42
Ranasinghe 2015	Ins analogs/Regular Ins in MDI arm (T1DM)	MDI vs CSII	3	50/303	1.21 (0.69-2.13)	1.21 (0.69-2.13)	1.10 (0.49-2.49)	0.51	0.51	0.03-47.48
Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	3	61/210	0.90 (0.59-1.39)	0.90 (0.59-1.39)	0.94 (0.56-1.58)	0.64	0.64	0.06-14.53
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	5	40/508	0.78 (0.38-1.58)	0.78 (0.38-1.58)	1.09 (0.38-3.11)	0.49	0.49	0.25-2.46
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	4	76/355	1.20 (0.71-2.03)	1.20 (0.71-2.03)	1.10 (0.45-2.68)	0.49	0.49	0.38-3.80
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	6	117/620	0.95 (0.61-1.47)	0.95 (0.61-1.47)	1.27 (0.55-2.96)	0.82	0.82	0.51-1.77
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	6	188/581	0.80 (0.48-1.35)	0.79 (0.45-1.38)	1.19 (0.47-2.99)	0.4	0.41	0.28-2.22
Pollex 2011	Ins glargine/NPH (PGDM, GDM)	Glargine vs NPH	5	28/325	0.97 (0.47-2.01)	0.97 (0.47-2.01)	1.08 (0.44-2.63)	0.94	0.94	0.20-4.77
Pollex 2011	Ins glargine/NPH (PGDM, GDM)	Glargine vs NPH	3	51/291	1.28 (0.77-2.12)	1.28 (0.77-2.12)	1.08 (0.52-2.25)	0.35	0.35	0.05-33.99
Pollex 2011	Ins glargine/NPH (PGDM, GDM)	Glargine vs NPH	7	123/650	0.96 (0.69-1.33)	0.94 (0.64-1.38)	0.82 (0.45-1.52)	0.8	0.77	0.44-2.02
Syed 2011	Mx for GDM	Optimal vs suboptimal control	3	58/469	0.39 (0.24-0.64)	0.48 (0.21-1.08)	0.31 (0.17-0.55)	1.50E-04	0.077	0-1920
Syed 2011	Mx for GDM	Optimal vs suboptimal control	2	8/3376	0.52 (0.12-2.26)	0.61 (0.09-4.30)	0.29 (0.05-1.60)	0.39	0.62	N/A
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	9	331/1373	0.95 (0.78-1.15)	0.95 (0.78-1.15)	0.87 (0.61-1.25)	0.61	0.61	0.75-1.20
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	10	622/2236	0.96 (0.82-1.11)	0.96 (0.82-1.11)	1.08 (0.85-1.36)	0.56	0.56	0.80-1.14
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	9	291/2560	0.86 (0.67-1.11)	0.86 (0.67-1.11)	0.83 (0.57-1.22)	0.26	0.26	0.63-1.17
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	6	70/2196	1.21 (0.72-2.02)	1.21 (0.72-2.02)	1.60 (0.82-3.11)	0.47	0.47	0.58-1.51
Gilbert 2006	Metformin (Diabetes)	Metfomin vs non metformin	3	13/200	0.84 (0.14-5.08)	0.84 (0.14-5.08)	0.37 (0.02-7.05)	0.85	0.85	0-98251

Lv 2015	Insulin analogs (PGDM)	Glargine vs NPH	2	64/105	19.59 (-68.06-107.24)	19.59 (-68.06-107.24)	20.00 (-69.25-109.25)	0.66	0.66	N/A
Ranasinghe 2015	Ins analogs/Regular Ins in MDI arm (T1DM)	MDI vs CSII	2	150/244	(-)24.80 (-245.58-195.99)	(-)24.80 (-245.58-195.99)	(-)2.00 (-265.15-261.15)	0.83	0.83	N/A
Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	3	86/220	92.22 (-73.15-257.59)	92.22 (-73.15-257.59)	147.70 (-96.24-391.64)	0.27	0.27	(-)979.87-1164.31
Zheng 2015	Myoinositol (GDM)	Myonisitol vs placebo	2	134/270	(-)106.03 (-216.21-4.16)	(-)88.28 (-260.13-83.57)	(-)162.00 (-295.08--28.92)	0.059	0.31	N/A

Abbreviations: GDM- Gestational diabetes mellitus; PGDM- Pregestational diabetes mellitus; T1/2DM- Type 1/2 diabetes mellitus; sc- subcutaneous; inj- injections; Ins- Insulin; Mx- Management; NPH- Neutral Protamine Hagedorn; CSII- Continuous subcutaneous insulin infusion; MDI- Multiple daily injections

Key:

$^{\alpha}$ Number of studies

$^{\beta}$ Fixed effects refers to summary relative risk 95% CI) using the meta-analysis fixed- effects model

$^{\chi}$ Random effects refers to summary relative risk (95% CI) using the meta-analysis random -effects model.

$^{\delta}$ Relative risk and 95% confidence interval of largest study (smallest SE) in each meta- analysis

$^{\varepsilon}$ P value of summary fixed effects estimate

$^{\phi}$ P value of summary random effects estimate

$^{\gamma}$ Prediction intervals are reported only for meta-analyses including at least 3 studies

All statistical tests were two-sided

Table 3.9. Evaluation of heterogeneity, small study effects, excess significance bias and credibility ceilings in the 49 meta-analyses investigating the association of anti-diabetic interventions with gynaecological and obstetric morbidity– cohort studies only

Author, year	Exposure	Exposure contrast	Egger's P[α]	I^2 (95% CI) P[β]	Studies	Observed χ	Expected[δ], P-value[ε]						Credibility ceiling (%) p<0.05
							Fixed effects		Random effects		Largest study		
Gynaecological													
Wen 2019	Metformin (T2DM)	Metformin vs non-metformin	0.38	14 (0-77) 0.31	3	2	2.41	NP	2.38	NP	2.42	NP	9
Chu 2018	Metformin (Diabetes)	Metformin vs other antidiabetic drugs	0.19	0 (0-73) 0.69	3	2	2.97	NP	2.97	NP	2.99	NP	12
Wen 2019	Metformin (T2DM)	Metformin vs non-metformin	N/A	0 (.-.) 0.87	2	1	1.1	NP	1.1	NP	1.1	NP	3
Chu 2018	Metformin (Diabetes)	Metformin vs other antidiabetic drugs	0.17	93 (87-96) 1.01E-11	5	3	3.5	NP	0.31	6.20E-07	4.98	NP	0
Wen 2019	Metformin (T2DM)	Metformin vs non metformin	0.14	98 (98-99) <1.0E-100	4	2	3.99	NP	2.91	NP	4	NP	0
Tian 2019	Anti-diabetic medication (Diabetes)	Metformin vs non metformin	0.093	97 (96-98) 1.22E-17	3	2	2.94	NP	0.39	0.0056	2.99	NP	0
Raffone 2019	Conservative Mx of endometrial hyperplasia and cancer (Diabetes)	Diabetes vs non diabetes	N/A	0 (.-.) 0.70	2	0	0.1	NP	0.1	NP	0.16	NP	0

Raffone 2019	Conservative Mx of endometrial hyperplasia and cancer (Diabetes)	Diabetes vs non diabetes	0.66	29 (0-74) 0.23	5	1	0.81	0.82	0.67	0.66	3.9	NP	0
Obstetric, maternal													
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs Multiple daily inj	0.11	20 (0-62) 0.26	10	3	4.27	NP	3.87	NP	5.19	NP	7
Ranasinghe 2015	Ins analogs/ Regular Ins in MDI arm (T1DM)	MDI vs CSII	0.77	58 (0-86) 0.094	3	0	0.17	NP	0.19	NP	0.22	NP	0
Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	0.86	0 (0-73) 0.91	3	0	0.15	NP	0.15	NP	0.15	NP	0
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	0.28	30 (0-80) 0.24	3	1	0.72	0.71	0.5	0.44	1.42	NP	0
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	0.023	49.9 (0-77) 0.062	7	1	0.7	0.7	1.33	NP	1.61	NP	0
Alqudah 2018	Metformin (GDM)	Metformin vs control	0.86	30 (0-76) 0.23	4	0	0.24	NP	0.26	NP	0.21	NP	0
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	0.36	54 (.-.) 0.04	7	1	0.39	0.32	0.42	0.35	0.69	0.69	0
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	0.29	71 (33-83) 0.00035	10	2	1.17	0.42	0.53	0.038	0.57	0.0499	0
Obstetric, foetal													
Lv 2015	Insulin analogs (GDM,T1/2DM)	Lispro vs Regular Ins or NPH	0.65	0 (0-68) 0.90	4	2	1.25	0.42	1.25	0.42	1.54	0.64	15

Wahabi 2010	Preconception care (PGDM)	Preconception vs no preconception care	0.3	31 (0-66) 0.16	11	4	5.1	NP	5.58	NP	1.44	0.021	11
Wahabi 2010	Preconception care (PGDM)	Preconception vs no preconception care	0.27	0 (.-.) 0.93	5	1	1.41	NP	1.41	NP	1.84	NP	5
Wahabi 2010	Preconception care (PGDM)	Preconception vs no preconception care	0.49	0 (0-68) 0.48	4	1	0.9	0.9	0.9	0.9	1.28	NP	4
Blanco 2011	Lispro/Regular Ins (T1DM)	Lispro vs Regular Ins	N/A	0 (.-.) 0.95	2	1	0.58	0.52	0.58	0.52	0.57	0.51	5
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs Multiple daily inj	N/A	0 (.-.) 0.75	2	1	0.83	0.81	0.83	0.81	0.76	0.73	6
Lv 2015	Insulin analogs (GDM,T1/2DM)	Lispro vs Regular Ins or NPH	0.84	0 (0-61) 0.47	6	1	3.34	NP	3.34	NP	3.66	NP	7
Waugh 2010	Mx for GDM	Glibenclamide vs Insulin	N/A	0 (.-.) 0.91	3	0	0.73	NP	0.73	NP	0.7	NP	0
Waugh 2010	Mx for GDM	Glibenclamide vs Insulin	0.23	0 (0-73) 0.85	3	0	0.32	NP	0.32	NP	0.26	NP	0
Waugh 2010	Mx for GDM	Glibenclamide vs Insulin	0.87	70 (0-88) 0.017	4	2	0.99	0.24	0.84	0.16	1.23	0.4	0
Ranasinghe 2015	Ins (T1DM)	MDI vs CSII	N/A	0 (.-.) 0.95	3	0	0.27	NP	0.27	NP	0.29	NP	0
Ranasinghe 2015	Ins analogs/Regular Ins in MDI arm (T1DM)	MDI vs CSII	0.6	0 (0-73) 0.99	3	0	0.18	NP	0.18	NP	0.19	NP	0
Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	0.17	0 (0-73) 0.56	3	0	0.15	NP	0.15	NP	0.17	NP	0

Ranasinghe 2015	Ins analogs/ Regular Ins in MDI arm (T1DM)	MDI vs CSII	0.27	0 (0-73) 0.91	3	0	0.2	NP	0.2	NP	0.16	NP	0
Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	0.054	0 (0-73) 0.90	3	0	0.17	NP	0.17	NP	0.16	NP	0
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	0.82	0 (0-64) 0.56	5	0	0.33	NP	0.33	NP	0.26	NP	0
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	0.25	0 (0-68) 0.79	4	0	0.27	NP	0.27	NP	0.22	NP	0
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	0.032	0 (0-61) 0.51	6	0	0.31	NP	0.31	NP	0.49	NP	0
Lepercq 2012	Ins glargine/NPH (DM)	Glargine vs NPH	0.48	11 (0-65) 0.34	6	0	0.51	NP	0.54	NP	0.43	NP	0
Pollex 2011	Ins glargine/NPH (PGDM, GDM)	Glargine vs NPH	0.96	0 (0-68) 0.76	5	0	0.2	NP	0.2	NP	0.21	NP	0
Pollex 2011	Ins glargine/NPH (PGDM, GDM)	Glargine vs NPH	0.19	0 (0-73) 0.76	3	0	0.24	NP	0.24	NP	0.16	NP	0
Pollex 2011	Ins glargine/NPH (PGDM, GDM)	Glargine vs NPH	0.57	19 (0-66) 0.28	7	0	0.36	NP	0.36	NP	0.49	NP	0
Syed 2011	Mx for GDM	Optimal vs suboptimal control	0.042	49.9 (0-84) 0.14	3	1	1.43	NP	1.06	NP	1.8	NP	0
Syed 2011	Mx for GDM	Optimal vs suboptimal control	N/A	36 (.-.) 0.21	2	0	0.22	NP	0.17	NP	0.55	NP	0
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	0.42	0 (0-54) 0.54	9	0	0.47	NP	0.47	NP	0.63	NP	0

Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	0.11	0 (0-53) 0.54	10	0	0.53	NP	0.53	NP	0.6	NP	0
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	0.63	0 (0-54) 0.75	9	0	0.65	NP	0.65	NP	0.77	NP	0
Rys 2018	Insulin (T1DM)	Continuous sc Ins infusion vs multiple daily inj	0.17	0 (0-65) 0.53	6	0	0.39	NP	0.39	NP	0.84	NP	0
Gilbert 2006	Metformin (Diabetes)	Metfomin vs non metformin	0.19	0 (0-73) 0.77	3	0	0.16	NP	0.16	NP	0.57	NP	0
Lv 2015	Insulin analogs (PGDM)	Glargine vs NPH	N/A	0 (.-.) 0.96	2	0	0.19	NP	0.19	NP	0.19	NP	0
Ranasinghe 2015	Ins analogs/ Regular Ins in MDI arm (T1DM)	MDI vs CSII	N/A	0 (.-.) 0.76	2	0	0.12	NP	0.12	NP	0.1	NP	0
Ranasinghe 2015	Only Ins analogs in MDI arm (T1DM)	MDI vs CSII	0.75	0 (0-73) 0.62	3	0	0.76	NP	0.76	NP	1.56	NP	0
Zheng 2015	Myoinositol (GDM)	Myonisitol vs placebo	N/A	54 (.-.) 0.14	2	1	1.43	NP	1.15	NP	1.89	NP	0

Abbreviations: GDM- Gestational diabetes mellitus; PGDM- Pregestational diabetes mellitus; T1/2DM- Type 1/2 diabetes mellitus; sc-subcutaneous; inj-injections; Ins- Insulin; Mx- Management; NPH- Neutral Protamine Hagedorn; CSII- Continuous subcutaneous insulin infusion; MDI- Multiple daily injections; NP- not pertinent (because the estimated is larger than the observed, and there is no evidence of excess statistical significance based on the assumption made for the plausible effect size)

Key:

[α] P-value from the Egger's regression asymmetry test (P<0.10)

[β] I^2 metric of inconsistency (95% confidence interval) and the P-value of the Q test

[χ] Observed number of statistically significant studies in each meta-analysis

[δ] Expected number of statistically significant studies using the point estimate of each meta-analysis (from fixed effect, random effect of largest study accordingly) as the plausible effect size

[ε] P value of the excess statistical significance test

All statistical tests were two-sided

Table 3.10. AMSTAR 2 methodological quality assessment for observational systematic reviews investigating the association of anti-diabetic interventions with gynaecological and obstetric morbidity- cohort studies only

AMSTAR 2 Questions **Study Author, year**	PICO	'A priori ' design and deviations justified	Study design	Literature search	Duplicate study selection review	Duplicate data extraction	Excluded studies	Description of included studies	Assess risk of bias	Fund-ing	Statistical methods for meta-analysis	Impact of RoB from meta-analysis	RoB in individual studies in results	Heterogeneity	Small study bias	Conflict of interest	**Score**
Alqudah 2018	●	○	○	●	●	○	●	●	●	○	●	●	●	●	●	●	Low
Blanco 2011	●	◎	●	◎	●	○	○	◎	○	○	○	○	○	○	○	●	Critically low
Chu 2018	●	○	●	○	●	●	○	●	●	○	●	●	○	●	●	●	Critically low
Gilbert 2006	●	○	●	○	○	○	●	○	○	○	○	○	○	○	●	○	Critically low
Lepercq 2012	●	○	○	◎	●	○	○	●	○	○	●	○	○	●	○	●	Critically low
Lv 2015	●	○	●	○	●	○	○	○	●	○	○	○	○	○	○	●	Critically low
Pollex 2011	●	○	●	◎	●	●	○	◎	○	○	●	○	○	●	○	●	Critically low
Raffone 2019	●	◎	○	●	●	●	○	●	●	○	●	○	○	●	●	●	Critically low
Ranasinghe 2015	●	◎	○	◎	●	○	○	●	●	○	●	○	●	●	○	●	Critically low
Rys 2018	●	○	●	◎	●	○	○	●	●	○	○	●	○	●	●	●	Critically low
Syed 2011	●	○	○	◎	●	●	○	◎	◎	○	●	○	○	○	○	●	Critically low
Tian 2019	●	○	○	◎	○	○	○	◎	●	○	●	●	○	●	●	○	Critically low
Wahabi 2010	●	○	○	◎	●	●	●	●	●	○	●	●	●	●	○	○	Critically low
Waugh 2010	●	○	○	◎	●	●	○	●	●	●	●	●	●	●	●	●	Critically low
Wen 2019	○	◎	○	○	○	●	○	◎	○	○	●	○	●	●	●	●	Critically low
Zheng 2015	●	○	○	◎	●	●	○	◎	●	○	●	○	○	●	○	○	Critically low

Abbreviations: PICO: Patient/Population- Intervention- Comparison- Outcomes, RoB: Risk of Bias

Key: ● Yes

◎ Partial yes

○ No

Critical flaw

3.4.2.8 Grading of evidence

With regards to anti-diabetic interventions, no association was supported by strong evidence, while a single (1/49) meta-analysis presented highly suggestive evidence reporting a decreased risk of ovarian cancer in metformin users of Type 2 diabetes versus non- metformin users (OR 55, 95% CI 0.36-0.84). Suggestive evidence was presented by one meta-analysis demonstrating improved endometrial cancer survival in diabetic patients using metformin versus other anti-diabetics (HR 0.47, 95% CI 0.33-0.67). Nine meta-analyses (18%) described weak evidence for several outcomes, including metformin use and decreased cervical cancer occurrence, preconception care in PGDM and decreased rate of congenital malformations, perinatal mortality and preterm delivery (Table 3.4). Thirty-eight (38/49, 78%) meta-analyses showed no association (P>0.05) between anti-diabetic interventions and gynaecological/obstetric outcomes. Detailed explanation of evidence grading is presented in Table 3.5 (for cohort studies only).

3.4.2.9 Sensitivity analysis

When both cohort and case-control studies were included in the analysis, the association of myoinositol use with reduced birth weight in GDM was upgraded from non-significant to weak.

3.4.3 Meta-analyses of randomised controlled trials (RCTs)

A total of 200 meta-analyses of randomised controlled trials were retrieved from 38 publications describing the effect of lifestyle interventions (n=20), dietary interventions (n=25), exercise (n=4), oral anti-diabetics and/or insulin (n=110) and omega-3 supplements (n=4) on 37 outcomes.

I observed nominally significant associations (p random value < 0.05) in 40/200 (20%) meta-analyses for 16 associations on 16 separate outcomes: between lifestyle interventions and reduced rates of large for gestational age (LGA) babies, macrosomia and shoulder dystocia; between metformin (vs insulin) and reduced rates of LGA,

macrosomic babies, caesarean section, gestational hypertension, pregnancy-induced hypertension, preeclampsia, NICU admission, neonatal hypoglycaemia and hyperbilirubinaemia; between any specific/intensive treatment and reduction of neonatal hypoglycaemia, preeclampsia, shoulder dystocia, hypertensive disorders of pregnancy, LGA, macrosomia and increased rates of induction of labour (Table 3.11).

To assess the quality of evidence and risk of bias in the interventional meta-analyses, I extracted and present here the method of quality assessment performed in each original meta-analysis. Altogether 16 out of 17 publications that yielded statistically significant results assessed the risk of bias of the included studies (Table 3.12). All publications used the Cochrane risk of bias tool or a modification of this (Risk of bias assessment tool recommended by the Cochrane Neonatal Review group) [423-425]. The GRADE score was used to assess the quality of evidence in five meta-analyses [426]. Five publications used more than one tool to assess methodological quality, and one publication used none. For outcomes that were assessed by GRADE method, high quality evidence supported the association between GDM treatment and reduced rate of LGA/macrosomia; moderate quality evidence demonstrated a reduction in LGA when lifestyle interventions were in place and a drop in rates of hypertensive disorders of pregnancy when GDM was treated; finally, low and very low quality of evidence supported the link between GDM treatment and reduced shoulder dystocia, metformin and reduced CS/hypertensive disorders of pregnancy, dietary interventions (DASH, soy protein) and reduced birth weight (Table 3.9).

Interventional meta-analyses (RCTs) reached statistical significance for both outcomes that met the strong criteria in observational studies. Metformin and internet-based self- monitoring were found to reduce the risk of CS among women with GDM versus insulin and usual care respectively. Decreased rates of LGA were also demonstrated when metformin or GDM treatment were used versus insulin and usual antenatal care respectively. No RCTs have been published for highly suggestive outcomes (major congenital malformations and heart defects in pre-gestational diabetes) but one observational interventional study showed weak evidence of

preconception care reducing the risk of congenital abnormalities in patients with PGDM. With regards to suggestive outcomes from observational studies analysis, metformin or any treatment were nominally significant in reducing the risk of preeclampsia when compared to insulin and no treatment respectively. No RCTs were retrieved on diabetes and gynaecological outcomes.

Table 3.11. Meta-analyses including only statistically significant* RCTs over the effect of any anti-diabetic intervention on the incidence of any obstetric or gynaecological disease included in current umbrella review.

Author year	Intervention	Intervention contrast	Population	Outcome	Participants total	Events total	N of studies	Level of evidence (GRADE)	Random summary effect estimate (95% CI)
Brown 2017a	Lifestyle interventions	Lifestyle intervention vs usual care/control	GDM	Shoulder dystocia	2,894	57	5	-	0.37 (0.21-0.65)
Brown 2017c	Ins/Diet	Ins vs diet/standard care	GDM	Macrosomia	717	70	3	-	0.30 (0.18-0.50)
Brown 2017c	Ins/Other Ins	NPH vs other Ins	GDM	Macrosomia	84	9	2	-	0.10 (0.02-0.65)
Brown 2017a	Lifestyle interventions	Lifestyle intervention versus usual care/control	GDM	LGA	2,994	450	6	Moderate quality	0.60 (0.50-0.72)
Brown 2017a	Lifestyle interventions	Lifestyle intervention versus usual care/control	GDM	Macrosomia	3,422	499	7	-	0.64 (0.48-0.86)
Falavigna 2012	GDM Tx/Usual antenatal care	GDM Tx vs usual antenatal care	GDM	Hypertensive disorders in pregnancy	2,084	269	3	Moderate quality	0.64 (0.51-0.81)
Falavigna 2012	GDM Tx/Usual antenatal care	GDM Tx vs usual antenatal care	GDM	LGA >90th centile	2,242	356	4	High quality	0.57 (0.44-0.74)
Falavigna 2012	GDM Tx/Usual antenatal care	GDM Tx vs usual antenatal care	GDM	Macrosomia (>4000g)	3,157	412	6	High quality	0.47 (0.35-0.63)
Falavigna 2012	GDM Tx/Usual antenatal care	GDM Tx vs usual antenatal care	GDM	Shoulder dystocia	1,958	48	2	Low quality	0.41 (0.22-0.75)

Horvath 2010	Tx for GDM	Intensive vs less intensive Tx	GDM	Shoulder dystocia	775	20	6	-	0.31 (0.13-0.71)
Lau 2016	Internet-Based Self-Monitoring	Internet-Based Self-Monitoring vs usual care	GDM, T1/2DM	CS	307	133	2	-	0.73 (0.56-0.97)
Tieu 2017	Metformin/Ins	Metformin vs Ins	T2DM, GDM	CS	241	160	3	Low quality	0.75 (0.57-0.99)
Tieu 2017	Metformin/Ins	Metformin vs Ins	T2DM, GDM	Hyperbilirubinaemia	220	40	2	-	0.45 (0.25-0.83)
Alwan 2009	Treatment	Any specific Tx vs routine antenatal care	GDM	IOL	1,068	374	2	-	1.33 (1.13-1.57)
Poolsup 2014	Metformin/Ins	Metformin vs Ins	GDM	Gestational hypertension	1,110	58	3	-	0.53 (0.31-0.91)
Hartling 2013	Any Tx	Any Tx vs no Tx	GDM	Preeclampsia	2,014	191	3	-	0.62 (0.43-0.89)
Hartling 2013	Any Tx	Any Tx vs no Tx	GDM	Shoulder dystocia	2040	51	3	-	0.42 (0.23-0.76)
Tufnell 2003	Treatment	Any intensive Tx vs any minimal Tx	GDM/IGT	Neonatal hypoglycaemia	194	17	2		0.25 (0.75-0.84)
Butalia 2017	Metformin/Ins	Metformin vs Ins	GDM/T2DM	LGA	1,549	315	7	-	0.80 (0.64-0.99)
Butalia 2017	Metformin/Ins	Metformin vs Ins	GDM/T2DM	Neonatal hypoglycaemia	2,120	296	14	-	0.62 (0.45-0.86)
Butalia 2017	Metformin/Ins	Metformin vs Ins	GDM/T2DM	NICU admission	1,822	360	10	-	0.72 (0.57-0.91)
Butalia 2017	Metformin/Ins	Metformin vs Ins	GDM/T2DM	PIH	1,160	87	4	-	0.56 (0.37-0.85)
Immanuel 2017	Treated GDM in developed countries	Early onset vs late onset GDM (both treated)	GDM	NICU admission	7,872	1,806	4	Low quality	1.13 (1.04-1.22)
Immanuel 2017	Treated GDM in developed countries	Early onset vs late onset GDM (both treated)	GDM	Perinatal mortality	9,010	41	6	Low quality	3.61 (1.90-6.84)

Kalafat 2018	Metformin	Metformin vs Ins	GDM	Hypertensive disorders in pregnancy	1,724	233	8	Low quality	0.71 (0.55-0.91)
Kalafat 2018	Metformin	Metformin vs other drugs/placebo	GDM/ obesity	Hypertensive disorders in pregnancy	3,120	376	15	Very low quality	0.75 (0.60-0.94)
Guo 2019	Ins/Oral Tx	Metformin vs Ins	GDM	Preeclampsia	3,402	289	14	-	0.67 (0.47-0.95)
Guo 2019	Ins/Oral Tx	Metformin vs Ins	GDM	Neonatal hypoglycaemia	2,755	581	15	-	0.57 (0.50-0.66)
Guo 2019	Ins/Oral Tx	Glyburide vs Ins	GDM	Neonatal hypoglycaemia	2,406	174	12	-	1.70 (1.11-2.59)
Guo 2019	Ins/Oral Tx	Metformin vs Ins	GDM	Macrosomia >4kg	2,331	272	13	-	0.70 (0.54-0.90)
Bao 2019	Ins/Oral Tx	Metformin vs Ins	GDM	PIH	1,526	105	5	-	0.64 (0.44-0.95)
Brown 2017c	Ins/Diet	Ins vs diet/standard care	GDM	Birth weight	106	61	2	-	(-)332.5 (-579.9 - -85.1)
Brown 2017a	Lifestyle interventions	Lifestyle intervention vs usual care/control	GDM	Birth weight	3,074	1,521	6	-	(-)109.6 (-149.8 - -69.5)
Giuffrida 2003	Diet/Ins	Diet alone vs diet/Ins combined	GDM	Macrosomia	1,281	644	6	-	(-)0.10 (-0.17 - -0.03)
Yamamoto 2018	Dietary intervention	DASH vs control	GDM	Birth weight	119	60	3	Very low quality	(-)598.2 (-663.1 - -533.3)
Yamamoto 2018	Dietary intervention	Soy protein vs control	GDM	Birth weight	131	67	2	Very low quality	(-)184.7 (-319.4 - -50.0)
Guo 2019	Ins/Oral Tx	Ins vs Metformin	GDM	GA at delivery	2295	1209	12	-	0.23 (0.12-0.34)
Tarry-Adkins 2019	Ins/Oral Tx	Metformin vs Ins	GDM	Neonatal ponderal index	986	488	3	-	(-)0.13 (-0.26 - -0.002)
Tarry-Adkins 2019	Ins/Oral Tx	Metformin vs Ins	GDM	Birth weight	2,816	1,386	17	-	(-)107.7 (-182.7 - -32.7)
Brown 2017c	Ins/Oral Tx	Ins vs Metformin	GDM	NICU admission	2,306	445	10	-	1.45 (1.11-1.89)

Abbreviations: RCT- Randomised controlled trial; Ins- Insulin; GDM- Gestational diabetes mellitus; NPH- Neutral protamine hagedorn; LGA- Large for gestational age; CS- Caesarean section; IOL- Induction of labour; Tx- Treatment; T1/2DM- Type 1/2 diabetes mellitus; NICU- Neonatal intensive care unit; PIH- Pregnancy-induced hypertension; GA- Gestational age

Key:

* random p value< 0.05

Table 3.12. Risk of bias in the included interventional meta-analyses (RCTs).

Author, year	Study type	Tool used	Subscale	Range	Results		
Brown, 2017a					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Handbook for Systematic reviews of Interventions	Random sequence generation	Low/Unclear/High risk	6/10	4/10	0/10
			Allocation concealment		4/10	6/10	0/10
			Blinding of participants/personnel		4/10	1/10	5/10
			Blinding of outcome		5/10	5/10	0/10
			Incomplete outcome data		7/10	1/10	2/10
			Selective reporting		3/10	1/10	6/10
			Other bias		9/10	0/10	1/10
Brown, 2017c					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Handbook for Systematic reviews of Interventions	Random sequence generation	Low/Unclear/High risk	1/5	3/5	1/5
			Allocation concealment		0/5	4/5	1/5
			Blinding of participants/personnel		0/5	1/5	4/5
			Blinding of outcome		0/5	5/5	0/5
			Incomplete outcome data		4/5	0/5	1/5
			Selective reporting		0/5	1/5	4/5
			Other bias		1/5	1/5	3/5
Falavigna, 2012					**Low**	**Uncertain**	**High**
	RCT	Cochrane Handbook for Systematic reviews of Interventions	Random sequence generation	Low/Uncertain/High quality	3/7	1/7	3/7
			Allocation concealment		2/7	2/7	3/7
			Blinding of participants/personnel		0/7	0/7	7/7
			Incomplete outcome data		4/7	2/7	1/7
Horvath, 2010					**Yes**	**Unclear**	**No**
	RCT		Adequate randomisation	Yes/Unclear/No	3/6	3/6	0/6
			Adequate allocation concealment		2/6	4/6	0/6
			Blinding of participants/caregivers		1/6	0/6	5/6
			Blinding of end point assessment		0/6	6/6	0/6

			ITT analyses		3/6	1/6	2/6
Overall High potential for study bias							
Lau, 2016					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Handbook Guidelines (2011)	Random sequence generation	Low/Unclear/High risk	1/2	1/2	0/2
			Allocation concealment		2/2	0/2	0/2
			Blinding of participants/personnel		2/2	0/2	0/2
			Blinding of outcome		0/2	2/2	0/2
			Incomplete outcome data		0/2	0/2	2/2
			Selective reporting		0/2	1/2	1/2
Tieu, 2017							
	RCT	Cochrane Handbook Guidelines (2011)	Random sequence generation	Low/Unclear/High risk	1/3	0/3	2/3
			Allocation concealment		1/3	0/3	2/3
			Blinding of participants/personnel		3/3	0/3	0/3
			Blinding of outcome		0/3	3/3	0/3
			Incomplete outcome data		1/3	1/3	1/3
			Selective reporting		0/3	2/3	1/3
			Other bias		0/3	3/3	0/3
Alwan. 2009					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Handbook Guidelines (2011)	Allocation concealment	Low/Unclear/High risk	2/2	0/2	0/2
Poolsup, 2014					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Handbook Guidelines (2011)	Random sequence generation	Low/Unclear/High risk	0/3	1/3	2/3
			Allocation concealment		0/3	2/3	1/3
			Blinding of participants/personnel		3/3	0/3	0/3
			Blinding of outcome		2/3	0/3	1/3
			Incomplete outcome data		0/3	0/3	3/3
			Selective reporting		0/3	0/3	3/3
			Other bias		0/3	0/3	3/3
Hartling, 2013							

	RCT		Risk of bias Consistency Directness Precision Overall strength of evidence		**Preeclampsia/Shoulder dystocia** Low/Medium Consistent/Consistent Direct/Direct Imprecise/Precise Moderate/Moderate (favours treatment)		
Tufnell, 2003					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Reviewer's Handbook (Clarke 2000)	Allocation concealment		1/2	1/2	0/2
Butalia, 2017					**Yes**	**Unclear**	**No**
	RCT	Risk of bias assessment tool recommended by the Cochrane Neonatal Review Group	Allocation concealment Selection criteria (inclusion/exclusion) Group comparability Assessors blinded ITT Loss to follow-up reported	Yes/Unclear/No	8/15 14/15 10/15 3/15 10/15 14/15	6/15 0/15 1/15 1/15 1/15 0/15	1/15 1/15 14/15 11/15 4/15 1/15
Kalafat, 2018					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane risk of bias tool	Random sequence generation Allocation concealment Blinding of participants/personnel Blinding of outcome Incomplete outcome data Selective reporting Other bias	Low/Unclear/High risk	10/15 7/15 15/15 15/15 13/15 9/15 5/15	4/15 7/15 0/15 0/15 1/15 3/15 10/15	1/15 1/15 0/15 0/15 1/15 3/15 0/15
Guo, 2019					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Handbook for Systematic reviews of Interventions	Random sequence generation Allocation concealment Blinding of participants/personnel	Low/Unclear/High risk	2/32 0/32 0/32	19/32 21/32 23/32	11/32 11/32 9/32

			Blinding of outcome		0/32	22/32	10/32
			Incomplete outcome data		0/32	1/32	31/32
			Selective reporting		0/32	16/32	16/32
			Other bias		0/32	32/32	0/32
Bao, 2019					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Handbook for Systematic reviews of Interventions	Random sequence generation	Low/Unclear/High risk	1/5	0/5	4/5
			Allocation concealment		0/5	0/5	5/5
			Blinding of participants/personnel		0/5	5/5	0/5
			Blinding of outcome		0/5	5/5	0/5
			Incomplete outcome data		0/5	0/5	5/5
			Selective reporting		0/5	0/5	5/5
			Other bias		0/5	0/5	5/5
Yamamoto, 2018					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Collaboration tool	Random sequence generation	Low/Unclear/High risk	0/5	1/5	4/5
			Allocation concealment		0/5	5/5	0/5
			Blinding of participants/personnel		1/5	3/5	1/5
			Blinding of outcome		2/5	2/5	1/5
			Incomplete outcome data		2/5	2/5	1/5
			Selective reporting		2/5	3/5	0/5
			Other bias		3/5	0/5	2/5
Tarry-Adkins, 2019					**Low (-)**	**Unclear (?)**	**High (+)**
	RCT	Cochrane Collaboration tool	Random sequence generation	Low/Unclear/High risk	3/17	2/17	12/17
			Allocation concealment		0/17	12/17	5/17
			Blinding of participants/personnel		17/17	0/17	0/17
			Blinding of outcome		3/17	11/17	3/17
			Incomplete outcome data		5/17	1/17	11/17
			Selective reporting		0/17	1/17	16/17
			Other bias		1/17	1/17	15/17

3.5 Discussion

3.5.1 Diabetes and gynaecological malignancy

This umbrella review investigated the association between diabetes and anti-diabetic interventions and the risk of obstetric and gynaecological outcomes. A total of 117 meta-analyses of observational studies and 200 meta-analyses of clinical trials were included that evaluated 317 outcomes, which were subsequently graded based on the strength of association (observational only).

Suggestive evidence supported increased endometrial cancer incidence among diabetic patients and improved endometrial cancer survival in metformin users, while the evidence showing an association of diabetes with endometrial cancer mortality was weak. The results are consistent with the 2018 report of the World Cancer Research Fund Continuous Update Project (WCRF CUP), which identifies glycaemic load as a probable cause of endometrial cancer [427]. Interestingly, a large prospective cohort study using the Women's Health Initiative (WHI) dataset and encompassing 88107 postmenopausal participants, concluded that the significant higher risk of endometrial cancer in diabetic patients (HR=1.44, 95% CI: 1.13–1.85 for diabetes, HR=1.57, 95% CI: 1.19–2.07 for treated diabetes) was rendered non- significant when adjusting for BMI [428]. This finding suggests that confounders, like body weight, may distort exposure- outcome relationships and should be considered before establishing associations. A Mendelian randomisation study did not demonstrate an association between T2DM variants and endometrial cancer but supported a causal association of hyperinsulinaemia with endometrial cancer risk, irrespectively of BMI [429]. From a molecular perspective, increased expression of the glucose transporters GLUT1, GLUT3 and GLUT8 proportional to grade and stage has been observed in endometrial cancer suggesting that hyperglycaemia may promote tumour growth by supplying malignant cells with a carbon source for biosynthetic pathways necessary for cell proliferation [430 431]. Interestingly, the relationship between diabetes and premalignant or malignant endometrial polyps was weak.

The association of ovarian cancer incidence and mortality with diabetes was supported by weak evidence irrespective of exposure (diabetes type 1, type 2 or both). However, ever use of metformin demonstrated highly suggestive evidence for a lower ovarian cancer risk (RR 0.18, 95% CI 0.12-0.25). The antineoplastic effect of metformin is thought to be mediated by regulation of the mitogenic IGF1/AKT/mTOR pathways, which diminishes cell growth and proliferation [432]. Conversely, decreased risk for cervical cancer development among metformin users was supported by weak evidence. A history of gestational diabetes did not seem to impact on the incidence of endometrial, ovarian or cervical cancer, as demonstrated by Wang's meta-analysis that did not, however, reach statistical significance [374].

3.5.2 Diabetes and obstetric outcomes

Strong evidence was observed for only two obstetric outcomes; women with gestational diabetes seem to be at higher risk of caesarean section and large for gestational age babies. LGA is explained pathophysiologically by the Pedersen hypothesis according to which maternal hyperglycaemia leads to fetal hyperglycaemia and hyperinsulinaemia, which incites fetal overgrowth [433]. Higher risk of LGA in women treated with Lispro insulin versus regular or NPH was supported by weak evidence. When case-control studies were included in the analysis, two further associations were considered strong, pregestational diabetes and stillbirth (>20 weeks or >400g) as well as diabetes and infrequent cervical screening. The positive association of pregestational diabetes with major congenital malformations and congenital heart defects was graded as highly suggestive, while the evidence linking gestational diabetes with brachial plexus palsy was also upgraded to highly suggestive, when case control studies were included. Animal studies have showed that pregestational diabetes induces cellular oxidative stress, impairs endogenous antioxidant capacity and triggers apoptotic pathways in target organs resulting in structural birth defects, including the embryonic heart and neural tube [434].

Eight obstetric outcomes were supported by suggestive evidence including preeclampsia, postnatal depression, and neonatal respiratory distress syndrome.

Increased oxidative stress, placental endothelial dysfunction and dysregulated angiogenesis underpin both preeclampsia and gestational diabetes suggesting a potential link [435]. Surprisingly, the link of macrosomia with gestational diabetes was only supported by weak evidence. This finding partly contradicts a Mendelian randomisation study which revealed genetic evidence for a possible causal association between higher maternal fasting glucose and higher birth weight and also the HAPO study that demonstrated a higher risk of birth weight above the 90th centile (OD 1.38, 95% CI 1.32-1.44) in women with fasting hyperglycaemia [436] [437]. The discrepancy noted could be potentially attributed to the fact that our analysis included diabetes as exposure and not hyperglycaemia, which may be left untreated. Evidence showing that continuous subcutaneous insulin infusion favours macrosomia versus multiple daily injections was graded as weak.

Of the meta-analyses of randomised controlled trials exploring the impact of anti-diabetic interventions on gynaecological or obstetric adverse outcomes, 20% (40/200) reached nominal significance for 18 outcomes. GDM treatment is associated with a reduced risk of preeclampsia, macrosomia, LGA, shoulder dystocia, neonatal hypoglycaemia; insulin is associated with decreased rates of macrosomia versus diet and increased NICU admission versus metformin; metformin is linked with reduced rates of hypertensive disorders in pregnancy, CS, hyperbilirubinaemia, LGA, neonatal hypoglycaemia and NICU admission compared to insulin; lifestyle interventions correlate with reduced birth weight, LGA, macrosomia and shoulder dystocia, while dietary interventions (DASH diet, soy protein) with reduced birth weight. Insights from mouse models corroborate above epidemiological findings. Metformin has been shown to improve preeclamptic symptoms in mice by increasing matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) placental levels [438]**.** Furthermore, intrauterine exposure to metformin leads to lower birth weight in offspring born to high fat diet-fed mice [439].

3.6 Strengths and Limitations

This umbrella review represents the most comprehensive overview of published literature investigating associations between diabetes and anti-diabetic interventions and the risk of any type of obstetric or gynaecological morbidity to date. The strength and validity of meta-analyses of observational studies was assessed against a transparent and replicable set of statistical criteria that categorised observational evidence as strong, highly suggestive, suggestive and weak.

This review has a few inherent limitations that should be considered when interpreting findings. The analysis relied on previously published meta-analyses and literature searches performed by the authors of meta-analyses, therefore some individual studies might have been missed. Nevertheless, this is unlikely to have significantly influenced our findings because the assessment of duplicate meta-analyses on the same associations between exposure- outcome pairs reported similar summary results. In addition to this, reporting bias by the authors of meta-analyses could have led to underreporting of associations that did not reach statistical significance or did not conform with general knowledge and expectations [440] [441]. Furthermore, some associations were derived from a small number of studies included in a meta-analysis making the assessment of small study effects and excess significance bias potentially misleading given the low statistical power. Considering the large observed heterogeneity and some hints of bias in several of these meta-analyses, false-positives and inflated results could not be definitively excluded.

Even though a wide range of statistical tools was used to explore risk of bias in individual primary studies of a meta-analysis, other types of biases may have been overlooked and not detected by the statistical tests used. For example, confounding bias may have undermined the reliability of associations [442] [443]. Absence of patient stratification and statistical adjustment for imbalances in risk factors such as obesity, commonly encountered in diabetic patients, or age, diet, physical activity, alcohol, smoking, co-morbidities and drug use could have impacted on observed associations

and shifted relationships. For example, several observational studies on diabetes and gynae-, obstetric outcomes did not specify whether patients received treatment, diet modifications, left untreated or concurrently used other drugs like aspirin, which is commonly prescribed to diabetic patients with additional cardiovascular risk factors [444]. Finally, lack of clear definition or consistency of the clinical criteria used for diagnosing diabetes and several outcomes e.g., macrosomia, stillbirth, congenital malformations may have led to spurious data collation. In the same context, ambiguity remains on whether epidemiological studies were based on self-report questionnaires or medical records of diabetes given that a study has showed that self-report of diabetes is <66% sensitive compared to medical record data [445].

3.7 Future Work

Future epidemiological studies identifying associations between diabetes and gynaecological or obstetric outcomes should clearly define the diagnostic criteria used for diabetes and outcomes (e.g., definition of macrosomia, prematurity etc.) and also take into consideration confounding factors that may co-occur with diabetes, e.g., obesity, hypertension, dyslipidaemia. In the same context, diabetes as part of the metabolic syndrome should be considered a separate epidemiological entity and clearly stated when under investigation. Given that the diagnosis of diabetes usually prompts some kind of intervention, whether dietary or anti-diabetic medication, observational studies should thoroughly search for information on anti-diabetic treatments the patients might be receiving.

To establish causality between diabetes and reported outcomes, cross-study validation, Mendelian randomisation analysis and rigorous lab experimentation are essential adjuncts. For under-researched outcomes with small sample size and substantial heterogeneity, larger prospective studies should be conducted to reveal links with diabetes.

3.8 Conclusions

This umbrella review provides a comprehensive summary of the published body of evidence on gynaecological and obstetric ramifications of the diabetes epidemic. There is a strong association between GDM and increased risk of caesarean section and LGA, while interventional studies demonstrated that metformin mitigates the risk for both outcomes. Highly suggestive evidence supports the association of PDGM and increased risk of major congenital malformations and heart defects as well as metformin use in type 2 diabetes and decreased risk of ovarian cancer. The association between diabetes and increased incidence of endometrial cancer was supported by suggestive evidence, which was also the case for the association between metformin and better endometrial cancer survival. On the other hand, the evidence on the connection between diabetes and increased endometrial cancer mortality was classified as weak. Weaker associations were demonstrated for remaining outcomes that could still be valid but mandate further investigation.

The identification of robust relationships between diabetes and gynaecological/obstetric outcomes, following stringent scrutiny of studies against clearly defined statistical criteria that account for small sample size and potential bias, is crucial to reveal actionable risk factors and best interventional strategies. This umbrella review aims to guide targeted prevention initiatives, further epidemiological studies with standardised design and reporting of analysis but also biomedical studies that could shed light on the underlying molecular mechanisms of diabetes-induced adverse outcomes.

3.9 Statement of Contribution

Statistical analysis of extracted data with Stata software (version 15) was conducted by Ilkka Kalliala.

CHAPTER 4.

Female genital tract microbiota: genuine signatures in low biomass sites, continuum and correlation with rectal microbiota

---

Content from this chapter is currently under preparation as:

Semertzidou A, Smith A, Brosens J, Marchesi J, Bennett P, MacIntyre D and Kyrgiou M. The role of genital tract microbiota continuum in endometrial cancer. *(Under preparation)*

4.1 Introduction

Microbiome research has gained tremendous popularity over the past 15 years alongside the concept that indigenous microbiota co-exist with the human host in a mutualistic relationship, while opportunistic pathogens can incur infections and life-threatening diseases. Even though the vaginal and cervical microbiota have been described in literature in sufficient detail both in health and disease states, e.g., bacterial vaginosis, STIs and cervical dysplasia, the upper genital tract ecosystems are much less characterised. A reason for this is that several microbes resist cultivation in the laboratory [446].

Hence, the endometrium and other internal female organs have traditionally been considered sterile. It was not until 2007 with the advent of 16S rRNA gene sequencing that the sterile womb dogma was challenged [447]. 16S rRNA sequencing has enabled to comprehensively survey microbial compositions by extracting genetic material from biological samples, thus circumventing the need to cultivate microbes. Since the introduction of high throughput sequencing technologies, accumulating evidence has reported endometrial colonisation in up to 60-97% of non-malignant uteri [142 143 157 448-452], while a smaller body of evidence has focused on fallopian tube and ovarian microbial milieu.

Anatomical sites of low bacterial biomass present the additional challenge of contamination proneness in taxonomic studies. Experimental design pitfalls often fail to account for DNA contamination from patient sources, as in transcervical sample collection, or non-patient sources from airborne and “kitome” contaminants [453-455], leading to bacterial biomass overestimation. Hence, there is currently no consensus on a core benign endometrial bio-signature even though some commonly encountered genera have been consistently reported, including *Lactobacillus, Prevotella*, *Gardnerella*, *Bacteroides, Enterobacter*, *Acinetobacter, Bifidobacterium, Staphylococcus, Streptococcus, Sneathia, Escherichia, Atopobium and Pseudomonas* [141-143 157 449-452 456-459]. Bacteria identified in fallopian tube samples

include *Pseudomonas, Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria* [141 159]. There is a scarcity of data describing the ovarian microbiome and these are mostly in the context of ovarian malignancy [159].

The recent establishment of microbial fingerprints in low bacterial biomass sites has instigated the question of microbial continuum along the female genital tract, which merits investigation since the sampling of the easily accessible vagina could provide useful information of upstream colonisation and be used for determining disease susceptibility in cases of dysbiosis. Intra-individual microbial correlations between the lower and upper genital tract consistent with anatomical contingency have been reported by Chen *et al*. [141], whereas Walsh *et al.* reported that the microbiome of lower genital tract is correlated with uterine microbiome in patients with endometrial cancer [308]. In addition to this, associations of FGT with rectal microbiota have been suggested based on bacterial species overlap between the two sites and the presence of enteric oestrobolome, whereby the gut microbiome metabolises oestrogens and indirectly facilitates vaginal *Lactobacillus* growth [193-195 198 460-462].

Overall, microbiome studies properly designed and conducted to address contamination vulnerability of samples can lend credence to theories of upper genital tract symbiotic or dysbiotic microenvironments. On the other hand, interrogation of microbiota continuum in the female genital tract and correlations with rectal microbiota could unlock colonisation mechanisms and microbial dialogue across sites.

4.2 Aims

- To interrogate the presence of a genuine microbial signature above background noise in low bacterial biomass sites (endometrium, fallopian tubes, ovaries) in both benign and endometrial cancer patients.
- To assess the correlation of lower genital tract (vagina, cervix) microbiota with upper and rectum in both benign and endometrial cancer patients.

4.3 Results

4.3.1 Microbial signatures above background contamination in low bacterial biomass sites

4.3.1.1 Patient demographics and characteristics

Overall, 44 microbiome swabs (n= 23 patients, 20 patients double-swabbed in lower and higher endometrium) were collected from benign endometrium, 22 samples (n= 22 patients) from benign fallopian tubes, 20 samples (n=20 patients) from benign ovaries, 49 samples (n= 26 patients, 23 double-swabbed in lower and higher endometrium) from malignant endometrium and 51 samples from controls. Patient and clinical characteristics are shown in Table 4.1.

Table 4.1. Patient and clinical characteristics of the low biomass samples cohort.

Patient/Clinical characteristics	Benign (n=23)	Endometrial ca (n=26)
Ethnicity, n/N (%)		
White	18/23 (78)	12/26 (46)
Asian	1/23 (4)	9/26 (35)
Black/African/Caribbean	3/23 (13)	4/26 (15)
Other	1/23 (4)	1/26 (4)
Age (years), n/N (%)		
18- 34	1/23 (4)	0/26 (0)
35- 49	4/23 (17)	1/26 (4)
50- 64	14/23 (61)	13/26 (50)
≥ 65	4/23 (17)	12/26 (46)
BMI status, n/N (%)		
Underweight (<18.5)	1/23 (4)	2/24 (8)
Normal (18.5- 24.9)	6/23 (26)	4/24 (16)
Overweight (25- 29.9)	5/23 (22)	8/26 (30)
Obese (≥ 30)	11/23 (48)	12/26 (46)
Parity, n/N (%)		
Nulliparous	4/23 (17)	7/26 (27)

Parous	19/23 (83)	19/26 (73)
Menopausal status, n/N (%)		
Premenopausal	3/23 (13)	2/26 (8)
Postmenopausal	20/23 (87)	24/26 (92)
Surgical approach		
Laparoscopic	15/23 (65)	21/26 (81)
Transabdominal	8/23 (35)	5/26 (19)

4.3.1.2 Residual sequence reads after removal of contaminants

The presence of microbial signature above background contamination was investigated in the endometrium, fallopian tubes and ovaries of benign patients and endometrium of endometrial cancer patients. Sequencing read data was obtained from all samples, both from upper and lower genital tract.

To detect and account for potential sources of sequence contamination, several strategies were employed. An agar plate, which was left open in pathology lab during sample collection, and the knife used for uterus dissection were swabbed and sequenced to account for airborne and equipment contaminants respectively. In addition to this, negative controls were used during DNA extraction and 16S rRNA gene sequencing to assess kit and reagent contamination (Table 4.2). The Decontam R package was subsequently applied to detect and remove likely contaminant sequence reads firstly on the basis of *frequency*, where contaminant DNA concentrations will be present in equal and low amounts across all samples versus true samples where DNA concentration can vary widely, and *prevalence*, where contaminant DNA occurs more in negative controls than true samples [343].

Table 4.2. Potential (de)contamination sources, samples affected and management.

Sources of (de)contamination	Samples affected	Management
Air	All	Controls included
Pre-op enema	Rectal	Unknown effect on rectal microbiome
In theatre		
Intra-op antibiotics	Endometrial, fallopian tube, ovarian	Administered after collection of vaginal, cervical, rectal samples Dead bacteria still picked up by 16S rRNA gene sequencing
Uterine manipulator in laparoscopic procedures	Endometrial	Comparison with transabdominal hysterectomies
Vaginal retrieval of specimen in laparoscopic procedures	Fallopian tube, ovarian	Vaginal disinfection pre-op Comparison with transabdominal hysterectomies
In pathology lab		
Knife for uterus dissection	Endometrial	Controls included
In lab		
DNA extraction kit	All	Negative controls included
16S rRNA gene sequencing	All	Negative controls included
Sample carryover during DNA extraction/sequencing	All	Layout of sample loading taken into account during analysis

A total of 1037/6818 (15%) of OTUs detected in the dataset were identified as likely contaminants and were removed from further analysis (Supplementary material). As expected, the effect of environmental and kit contaminant removal was more pronounced in low-biomass sites (endometrium, fallopian tubes, ovaries) (Figure 4.1).

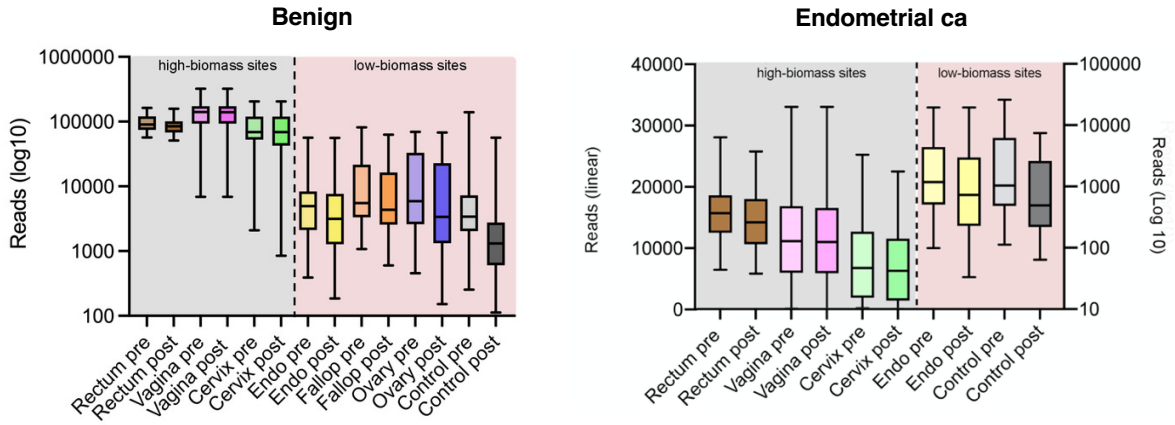


Figure 4.1. 16S rRNA gene sequence read counts pre- and post-removal of contaminant sequence reads.

4.3.1.3 Quantitative comparison of microbial signals in real samples and controls

The bacterial copy numbers at different locations of the female genital tract and rectum were estimated in both benign and endometrial cancer samples by qPCR of the 16S rRNA gene. The presence of a low-abundance microbiome was confirmed in the endometrium, fallopian tubes and ovaries comparable to the bacterial biomass of controls, which was 1- to 4- orders of magnitude lower than the vagina, cervix and rectum (Figure 4.2). Applying a cut-off above control counts at 700 bacterial copies, a prominent microbial signature above background contamination was observed in 62% of benign endometrium, 50% of malignant endometrium, 85% of benign fallopian tube and 95% of benign ovary based on sequencing reads.

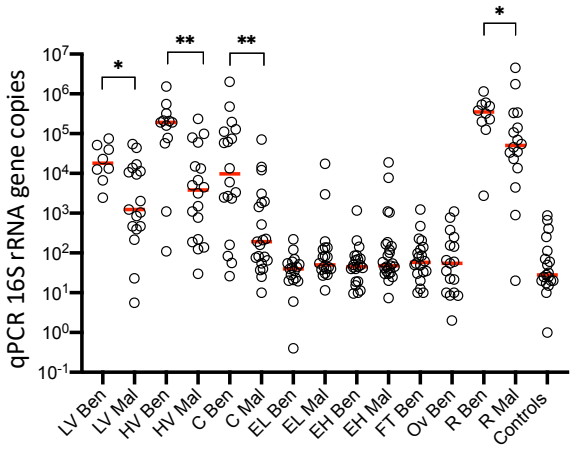


Figure 4.2. qPCR bacterial load at different locations in benign, endometrial cancer patients and controls. Red line represents median. *Ben: Benign, Mal: Malignant, LV: Lower Vagina, HV: Higher Vagina, C: Cervix, EL: Endometrium Lower, EH: Endometrium Higher, FT: Fallopian tube, Ov: Ovary, R: Rectum.*

4.3.1.4 Compositional comparison of microbial signals between patient samples and controls

Compositional differences between low biomass samples and controls in both benign and endometrial cancer patients was next explored. Following the exclusion of likely contaminants, hierarchical clustering analysis (HCA) was performed at genera level**.** In samples from benign gynaecological conditions, several bacterial genera were observed to be over-represented in the endometrium, fallopian tubes and ovaries compared to controls, indicating the presence of a genuine microbial fingerprint in these sites, while the microbiome of malignant endometrial samples and technical controls overlapped (Figure 4.3).

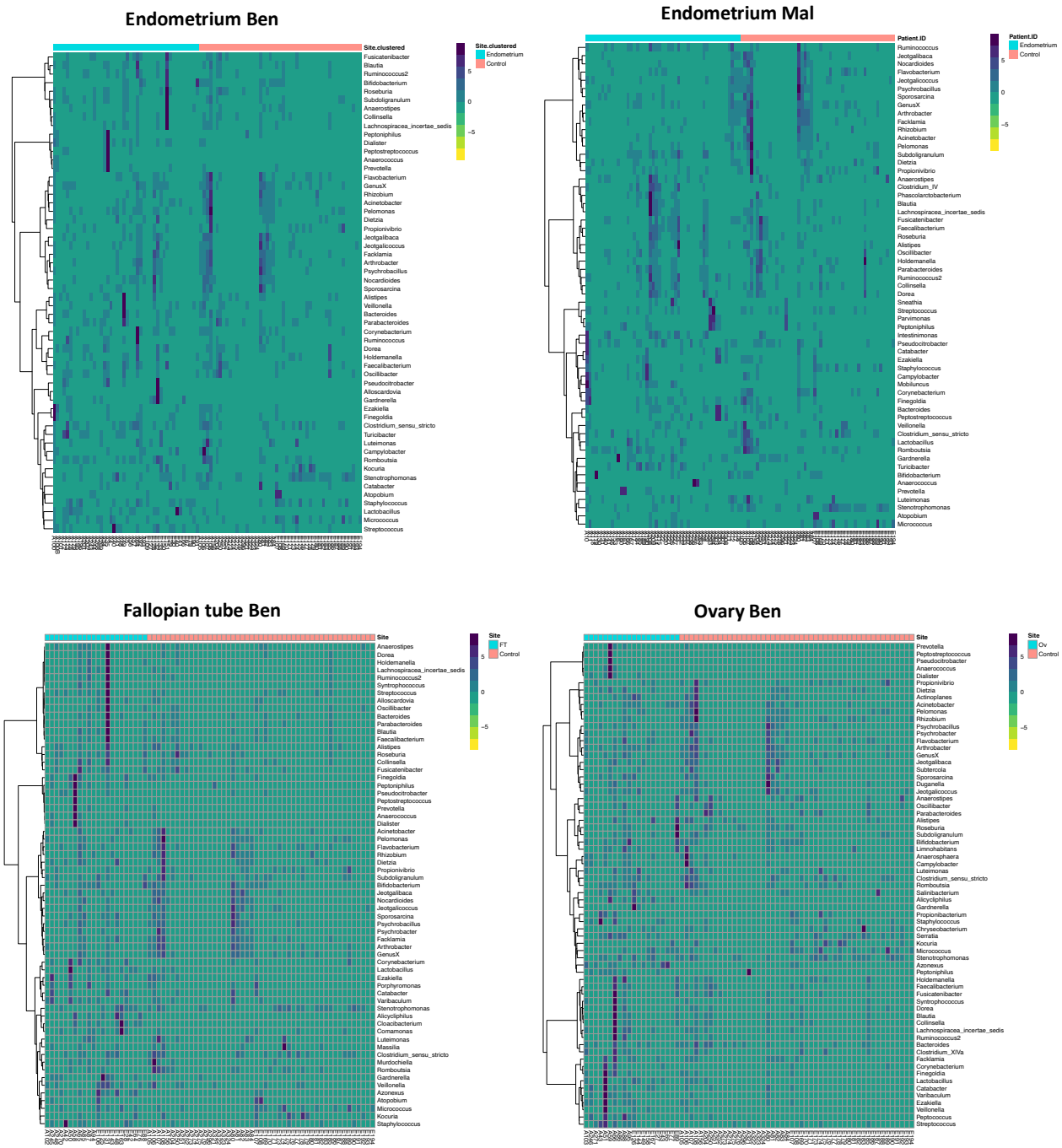
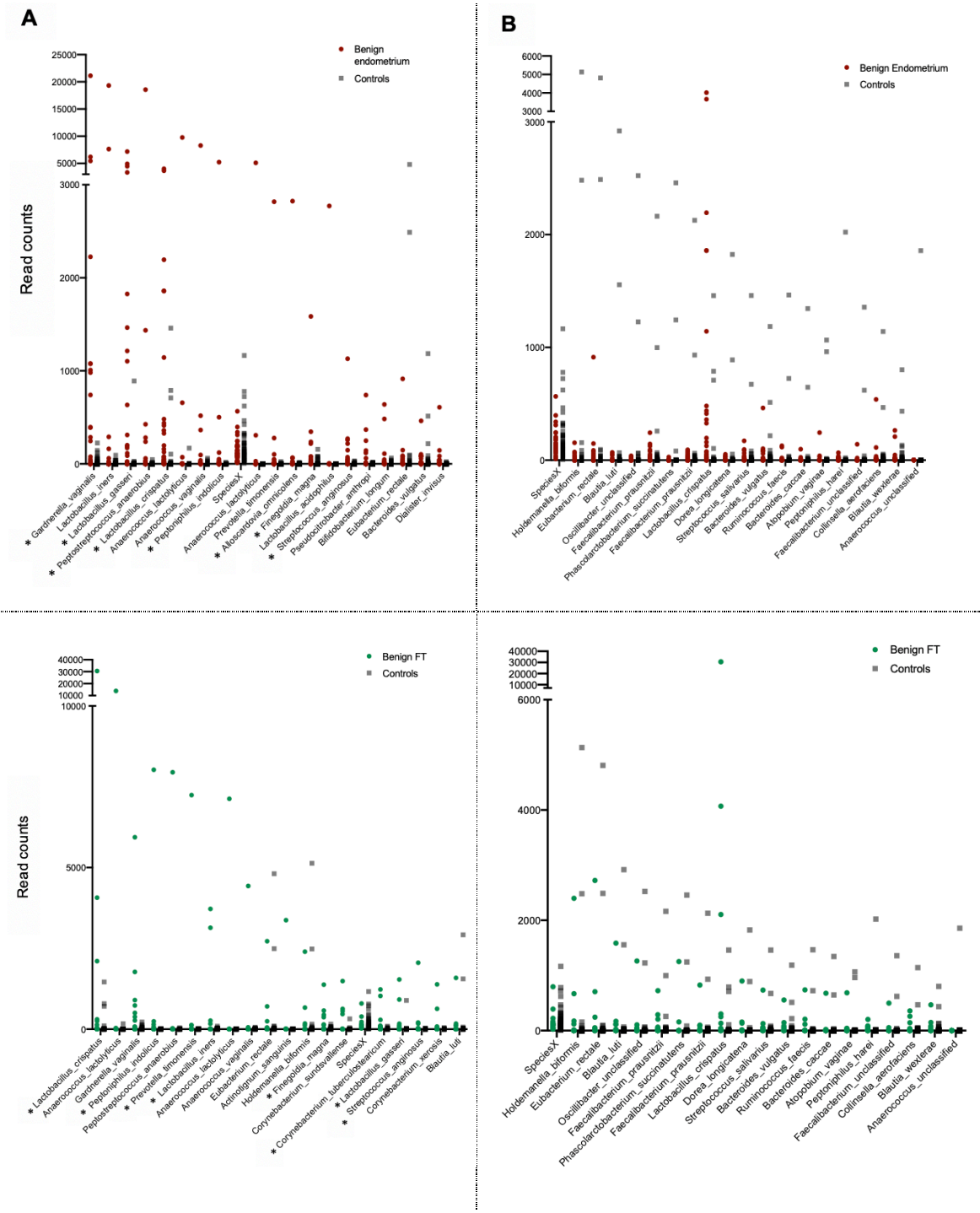


Figure 4.3. Prominent operational taxonomic units (OTUs) among low biomass patient samples and technical controls mapped to genera. *Ben: Benign, Mal: Malignant, FT: Fallopian tube, Ov: Ovary.*

To investigate this further, I next compared the relative abundances of the top 20 most abundant bacterial species encountered in low biomass sites (separately for benign endometrium, fallopian tubes, ovaries and malignant endometrium) with the top 20 species detected in control samples. This was done for each anatomical site of the upper genital tract (Figure 4.4). Several dominant bacterial species were significantly higher in the benign endometrium, fallopian tubes and ovaries compared to controls, providing evidence that a proportion of benign patients have a microbiota signature above background contamination in low bacterial biomass sites. A list of species significantly enriched in the benign upper genital tract in relation to controls is presented in Table 4.3. In contrast, substantial overlap was noted between the top 20 most abundant taxa in controls and malignant endometrium samples, implying that endometrial cancer patients may not harbour a true microbiome in their uterus.


A
Benign endometrium
Controls
Read counts
25000
20000
15000
10000
5000
3000
2000
1000
0
* Gardnerella_vaginalis
Lactobacillus_iners
* Lactobacillus_gasseri
* Peptostreptococcus_anaerobius
* Lactobacillus_crispatus
Anaerococcus_lactolyticus
Anaerococcus_vaginalis
* Peptoniphilus_indolicus
SpeciesX
Anaerococcus_lactolyticus
Prevotella_timonensis
* Alloscardovia_omnicolens
* Finegoldia_magna
Lactobacillus_acidophilus
* Streptococcus_anginosus
Pseudoclitrobacter_arthropi
Bifidobacterium_longum
Eubacterium_rectale
Bacteroides_vulgatus
Dialister_invisus
B
Benign Endometrium
Controls
6000
5000
4000
3000
2000
1000
0
SpeciesX
Holdemanella_biformis
Eubacterium_rectale
Blautia_luti
Oscillibacter_unclassified
Faecalibacterium_prausnitzii
Phascolarctobacterium_succinatutens
Faecalibacterium_prausnitzii
Lactobacillus_crispatus
Dorea_longicatena
Streptococcus_salivarius
Bacteroides_vulgatus
Ruminococcus_faecis
Bacteroides_caccae
Atopobium_vaginae
Peptoniphilus_harei
Faecalibacterium_unclassified
Collinsella_aerofaciens
Blautia_wexlerae
Anaerococcus_unclassified
Benign FT
Controls
Read counts
40000
30000
20000
10000
5000
0
* Lactobacillus_crispatus
Anaerococcus_lactolyticus
Gardnerella_vaginalis
* Peptoniphilus_indolicus
Peptostreptococcus_anaerobius
* Prevotella_timonensis
* Lactobacillus_iners
Anaerococcus_lactolyticus
Anaerococcus_vaginalis
Eubacterium_rectale
Actinotignum_sanguinis
Holdemanella_biformis
* Finegoldia_magna
Corynebacterium_sundsvallense
* Corynebacterium_tuberculostearicum
SpeciesX
* Lactobacillus_gasseri
* Streptococcus_anginosus
Corynebacterium_xerosis
Blautia_luti
Benign FT
Controls
40000
30000
20000
10000
6000
4000
2000
0
SpeciesX
Holdemanella_biformis
Eubacterium_rectale
Blautia_luti
Oscillibacter_unclassified
Faecalibacterium_prausnitzii
Phascolarctobacterium_succinatutens
Faecalibacterium_prausnitzii
Lactobacillus_crispatus
Dorea_longicatena
Streptococcus_salivarius
Bacteroides_vulgatus
Ruminococcus_faecis
Bacteroides_caccae
Atopobium_vaginae
Peptoniphilus_harei
Faecalibacterium_unclassified
Collinsella_aerofaciens
Blautia_wexlerae
Anaerococcus_unclassified

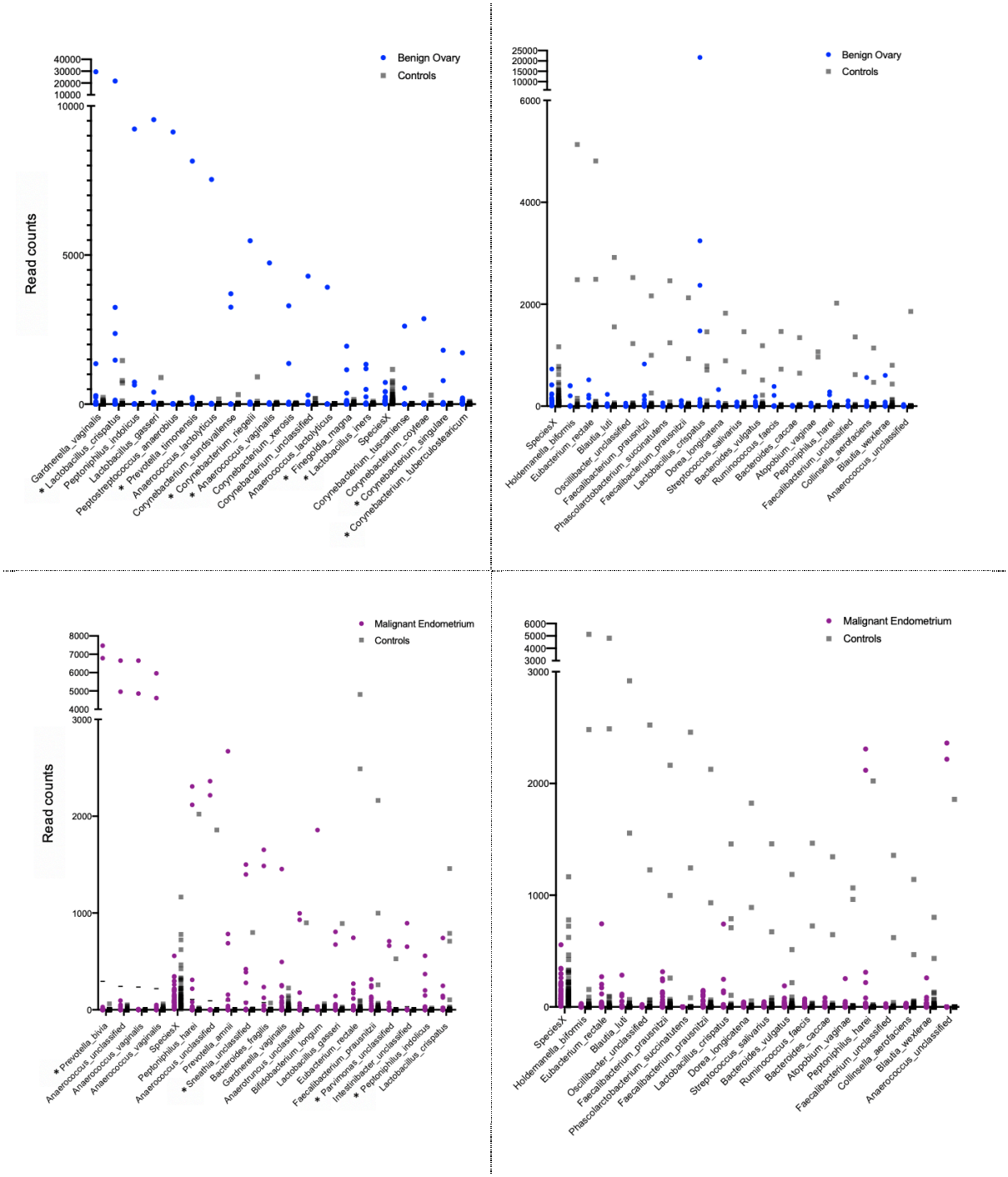


Figure 4.4. Top 20 most abundant bacterial species in low biomass samples and controls. Comparison of top 20 most abundant bacterial species found in low biomass samples versus controls (panel A) and top 20 bacterial species found in controls versus low biomass samples (panel B). Asterisks denote bacterial species that reached statistical significance using univariate analysis with Mann-Whitney/Kruskal Wallis test and *p* value cut-off 0.05.

Table 4.3. Species enriched in low bacterial biomass sites versus contaminant controls.

Name	P values*	Name	P values*
Benign endometrium			
Lactobacillus_crispatus	8.2893E-6	*Peptoniphilus_indolicus*	0.01016
Streptococcus_anginosus	1.257E-4	*Gardnerella_vaginalis*	0.012933
Lactobacillus_gasseri	4.6633E-4	*Comamonas_jiangduensis*	0.014224
Peptostreptococcus_anaerobius	8.5348E-4	*Romboutsia_unclassified*	0.014648
Alloscardovia_omnicolens	0.0014002	*Corynebacterium_faecale*	0.03665
Finegoldia_magna	0.0027741	*Blautia_faecis*	0.03796
Prevotella_timonensis	0.0031196		
Benign fallopian tube			
Cloacibacterium_rupense	1.5882E-6	*Staphylococcus_hominis*	0.008819
Comamonas_denitrificans	2.9511E-6	*Peptoniphilus_lacrimalis*	0.0090542
Azonexus_unclassified	8.8915E-6	*Peptoniphilus_harei*	0.012825
Corynebacterium_tuberculostearicus	2.1477E-5	*Lactobacillus_gasseri*	0.014153
Dialister_propionicifaciens	5.3929E-5	*Varibaculum_cambriense*	0.018753
Facklamia_hominis	6.053E-5	*Ezakiella_unclassified*	0.019851
Peptoniphilus_unclassified	6.0711E-5	*Corynebacterium_riegelii*	0.026934
Campylobacter_ureolyticus	6.0367E-4	*Lactobacillus_iners*	0.035608
Finegoldia_magna	8.3764E-4		
Lactobacillus_crispatus	8.4385E-4		
Peptoniphilus_indolicus	0.0014938		
Corynebacterium_unclassified	0.0023234		
Prevotella_timonensis	0.0030378		
Streptococcus anginosus	0.0054189		
Benign ovary			
Alicycliphilus_denitrificans	7.6565E-8	*Peptococcus_niger*	0.010236
Comamonas_denitrificans	5.9077E-6	*Peptoniphilus_harei*	0.011971
Corynebacterium_tuberculostearicus	1.0283E-5	*Varibaculum_cambriense*	0.02786
Azonexus_unclassified	1.5317E-5	*Dialister_invisus*	0.032777
Acinetobacter_guangdongensis	5.9321E-5	*Lactobacillus_iners*	0.033403
Streptococcus anginosus	8.1661E-5	*Anaerococcus_vaginalis*	0.033561
Dialister_propionicifaciens	3.5319E-4	*Flavobacterium_gillisiae*	0.038392
Prevotella_timonensis	9.5446E-4	*Staphylococcus_hominis*	0.038893

Lactobacillus_crispatus	0.0010095	*Prevotella_buccalis*	0.042004
Corynebacterium_riegelii	0.0017314	*Campylobacter_ureolyticus*	0.042706
Finegoldia_magna	0.0033297	*Clostridium_chromiireducens*	0.047336
Corynebacterium_singulare	0.0064919		
Catabacter_unclassified	0.0079931		
Malignant endometrium			
Prevotella_timonensis	5.1331E-5	*Prevotella_disiens*	0.01212
Finegoldia_magna	3.0225E-4	*Prevotella_bivia*	0.012861
Parvimonas_unclassified	4.3253E-4	*Sneathia_unclassified*	0.012914
Turicibacter_sanguinis	0.0011677	*Pseudocitrobacter_anthropi*	0.014047
Stenotrophomonas_maltophilia	0.002667	*Flavobacterium_swingsii*	0.018157
Phascolarctobacterium_faecium	0.051546	*Intestinimonas_unclassified*	0.022007
Flavonifractor_unclassified	0.0051483	*Peptococcus_niger*	0.023939
Ruminococcus_unclassified	0.0056931	*Peptoniphilus_lacrimalis*	0.026211
Dialister_propionicifaciens	0.0068012	*Peptostreptococcus_anaerobius*	0.032595
Lactobacillus_animalis	0.0075537	*Ruminococcus_torques*	0.041932
Peptoniphilus_indolicus	0.0086988	*Anaerococcus_obesiensis*	0.044989
Corynebacterium_unclassified	0.010064	*Campylobacter_ureolyticus*	0.047759

*Univariate analysis with Mann-Whitney/Kruskal Wallis test and *p* value cut-off 0.05.

4.3.1.5 Assessment of intra-patient contamination in laparoscopic procedures

Besides non-patient contamination introduced by the environment, intra-patient contamination can also occur across different anatomical sites during sample collection that could impact on findings from low biomass sites. During laparoscopy, a uterine manipulator is inserted inside the uterus and left *in situ* intra-operatively to facilitate surgical manoeuvres with the potential risk of microbial transfer from the cervix into the uterine cavity. In addition to this, the surgical specimen is collected through the vagina exposing the ovarian and fallopian tube surfaces to the heavily colonised vaginal mucosa. I therefore assessed whether practices adopted in laparoscopic hysterectomies (62.5% of benign cases, 91.9% of malignant cases) introduce intra-patient contamination in low bacterial biomass sites. I compared intra-individually the microbial composition of fallopian tubes and ovaries against higher vaginal samples to test for contamination during vaginal retrieval of specimen and lower/higher endometrium against cervical samples to identify contamination during uterine manipulation. I compared transabdominal (n=13) and laparoscopic (n=13) hysterectomies performed for either benign indications or endometrial malignancy at species level to increase taxonomic detail (Figure 4.5).

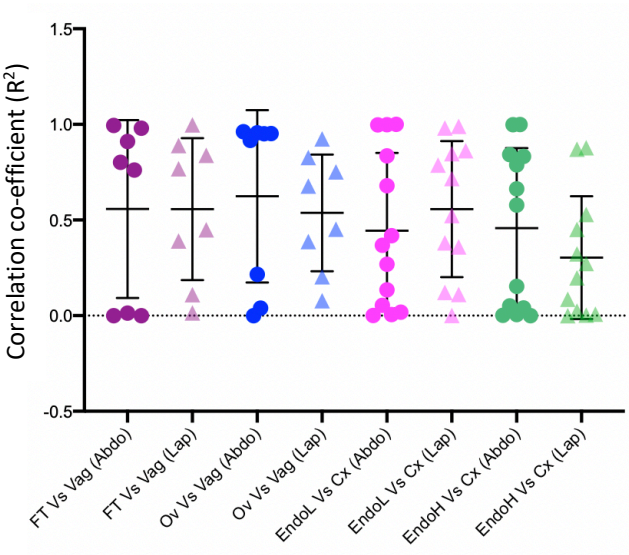


Figure 4.5. Comparison between laparoscopic and transabdominal procedures to determine potential contamination of sites of interest in laparoscopy during uterine manipulator insertion transcervically and vaginal retrieval of surgical specimen. Intraindividual calculation of microbial correlation co-efficient (R^2) at species level between fallopian tubes/ovaries and vagina or lower/higher endometrium and cervix in transabdominal and laparoscopic hysterectomies. Dots and triangles represent individual R^2 values for each patient. Lines represent mean with SD. *FT: Fallopian tube; Vag: Vagina; Ov: Ovary; EndoL: Endometrium Lower; EndoH: Endometrium Higher; Cx: Cervix; Abdo: Abdominally; Lap: Laparoscopically.*

In surgery performed transabdominally, the microbiota of fallopian tubes and ovaries were either highly correlated with vagina or almost completely uncorrelated. In contrast, a wide spread of correlation between the vaginal and upper reproductive tract samples was observed in laparoscopic procedures, likely indicative of a contamination in an estimated quarter of cases (2/8, 25%). Comparable correlation in the microbial compositional structure of lower endometrium and cervical samples was observed in both surgical approaches, which could be ascribed to the anatomical proximity of sites and expected microbial continuum. Conversely, higher endometrium versus cervix in transabdominal procedures was either strongly correlated in around half of the cases or uncorrelated in the remaining. In laparoscopy, a spread of correlation was observed, suggestive of potential microbial transfer from the cervix into the fundal endometrium in one third (4/12, 33%) of patients. These findings point towards potential intra-patient contamination in a proportion of laparoscopic procedures.

4.3.1.6 Microbial colonisation is less pronounced intracellularly compared to mucosal interfaces

After demonstrating the presence of bacterial genetic signatures in low biomass samples in a subset of benign and endometrial cancer patients, I next compared the microbiota composition of samples collected from epithelial surfaces with those extracted from a tissue biopsy. Paired swab-tissue samples were collected from 7 patients from low biomass sites and cervix. Total sequence read counts were considerably lower in tissue samples, except in patient EW171, and microbial composition coincided in only 3/10 of paired samples (Figure 4.6A). Interestingly, cervical biopsies were almost completely devoid of *L. crispatus*, a cervical epithelium commensal, while α- and β- diversity did not significantly differ between tissue and swab samples, but this may be due to small sample size (Figure 4.6B).

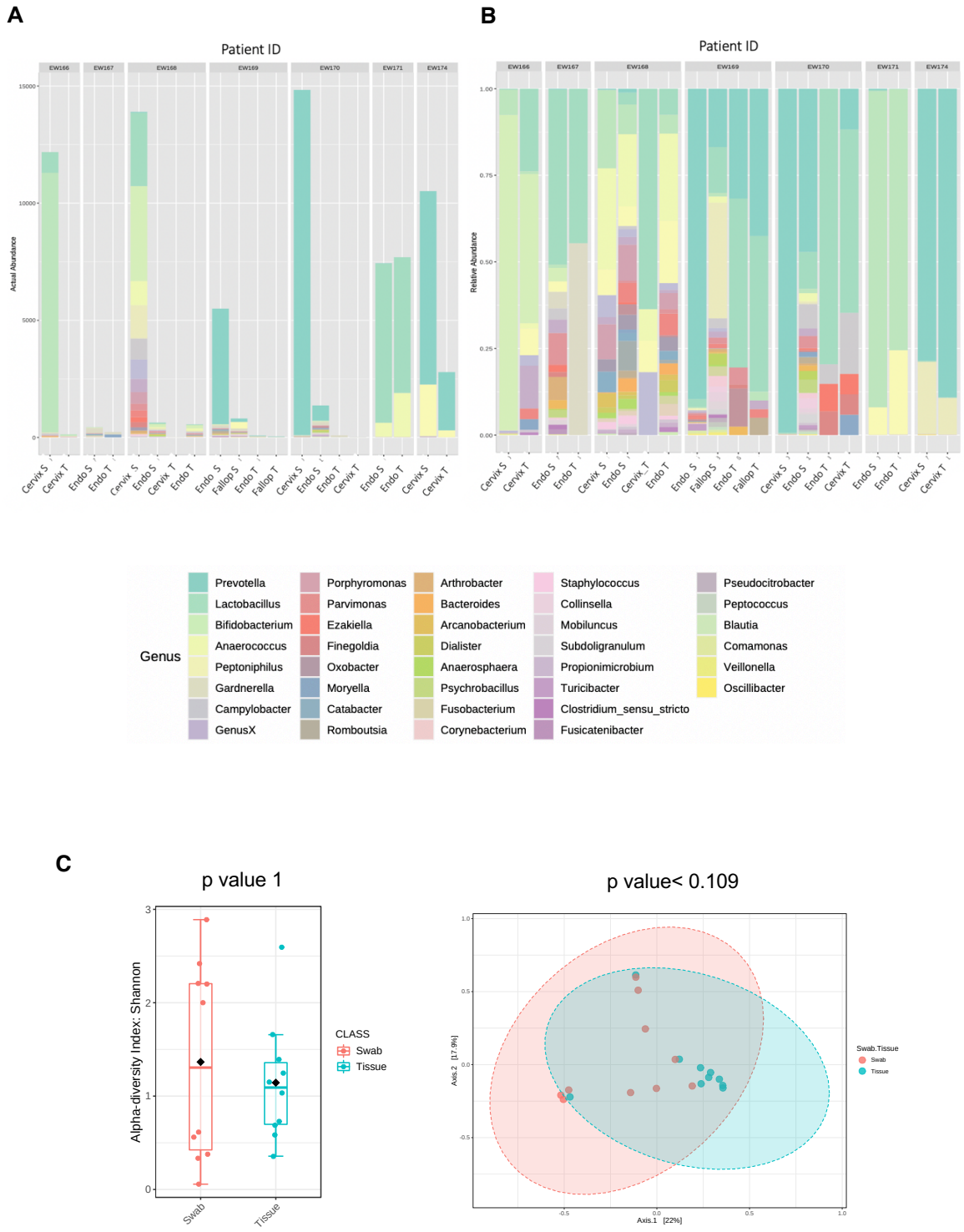


Figure 4.6. Pairwise comparison of microbial yield and signatures between swab and tissue samples from different sites (genera). A. Tissue actual abundance of microbes is considerably lower than swab and **B.** composition differs in the majority of cases. **C.** Shannon α-diversity and β-diversity did not reveal any significant differences between the two different sampling methods. *S: swab, T: tissue.*

4.3.2 Evidence for a microbial continuum along the female genital tract and correlations with rectal microbiota

4.3.2.1 Patient demographics and characteristics

The potential of microbial continuum in the reproductive tract was assessed in 16 benign and 16 endometrial cancer patients. Microbiome swabs were collected from the whole length of genital tract (vagina, cervix, endometrium, fallopian tubes, ovaries) in benign patients and from the vagina, cervix and endometrium of endometrial cancer patients. Patient and clinical characteristics are shown in Table 4.4.

Table 4.4. Patient and clinical characteristics of the female genital tract microbiota continuum cohort.

Patient/Clinical characteristics	Benign (n=16)	Endometrial ca (n=16)
Ethnicity, n/N (%)		
White	13/16 (81)	7/16 (44)
Asian	0/16 (0)	7/16 (44)
Black/African/Caribbean	3/16 (19)	1/16 (6)
Other	0/16 (0)	1/16 (6)
Age (years), n/N (%)		
18- 34	0/16 (0)	0/16 (0)
35- 49	1/16 (6)	1/16 (6)
50- 64	9/16 (56)	8/16 (50)
≥ 65	6/16 (38)	7/16 (44)
BMI status, n/N (%)		
Underweight (<18.5)	0/16 (0)	1/16 (6)
Normal (18.5- 24.9)	4/16 (25)	2/16 (13)
Overweight (25- 29.9)	4/16 (25)	6/16 (38)
Obese (≥ 30)	8/16 (50)	7/16 (44)
Parity, n/N (%)		
Nulliparous	2/16 (13)	5/16 (31)
Parous	14/16 (87)	11/16 (69)
Menopausal status, n/N (%)		
Premenopausal	1/16 (6)	1/16 (6)

Postmenopausal	15/16 (94)	15/16 (94)
Surgical approach		
Laparoscopic	9/16 (56)	15/16 (94)
Transabdominal	7/16 (44)	1/16 (6)

4.3.2.2 Microbial composition is highly correlated between higher and lower vagina but less correlated between fundal and lower endometrium

Comparison of the microbial profile of the higher 1/3 and lower 2/3 of vagina at genera and species level showed high intra-patient correlation ($R^2 \geq 0.7$) in 92% (genus and species) of benign patients and 86% (genus), 76% (species) of endometrial cancer patients (Figure 4.7). Similar analyses comparing higher (fundal) and lower endometrium demonstrated comparatively modest correlation in benign patients (genus 62%, species 57% high correlation) and even lower correlation in patients with endometrial malignancy (genus 42%, species 31% high correlation) (Figure 4.7). The lower microbial correlation of upper and lower malignant endometrium compared to benign endometrium was found to be significantly different (p=0.0007 genus; p=0.0003 species). These findings indicate that sampling the most accessible lower vagina is sufficient to predict the microbial structure of upper vagina in most cases, while bigger discrepancies occur between lower and fundal endometrium that are more pronounced among endometrial cancer patients.

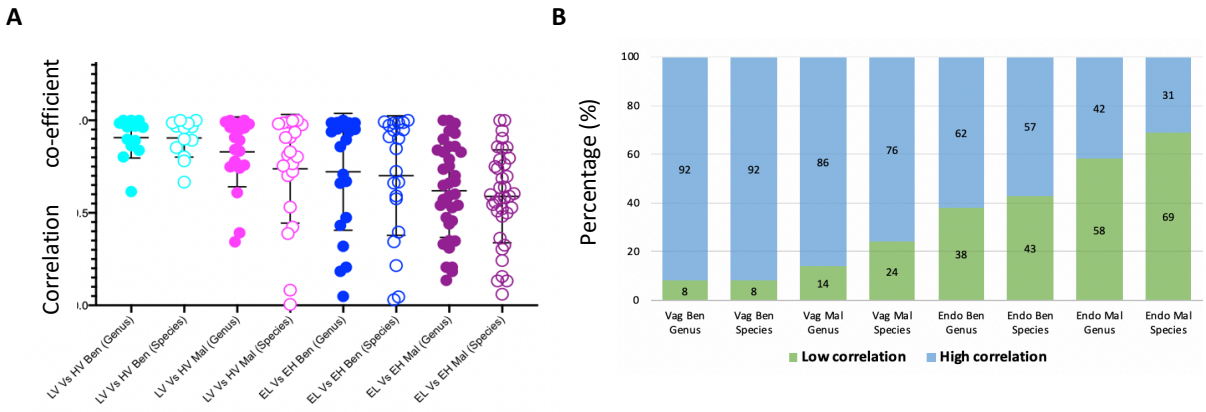


Figure 4.7. Microbiome correlation analysis at genera and species level in the lower and higher vagina/endometrium of benign and endometrial cancer patients. A. Intra-patient correlation co-efficient (R^2) of lower and higher vagina/endometrium at genera and species level for benign and

endometrial cancer patients. Dots and circles represent individual R^2 values for each patient. Lines represent mean with SD. **B.** Stacked bar chart illustrating microbial correlation along vaginal or endometrial length in benign and endometrial cancer patients. High correlation defined as $R^2 \geq 0.7$. *LV: Lower Vagina; HV: Higher Vagina; EL: Endometrium Lower; EH: Endometrium Higher; Ben: Benign; Mal: Malignant.*

4.3.2.3 The most abundant species of the lower genital tract also colonise the upper genital tract in benign patients

I next sought to determine whether there exists a continuum along the full length of the female genital tract in benign patients. Here, a continuum was defined as the presence of bacterial species in all sites of the lower and upper genital tract (vagina, cervix, endometrium, fallopian tube, ovary) at a relative abundance of at least 0.5%. Venn diagrams were used to depict patterns of overlapping colonisation among sites (Figures 4.8 and 4.9). In 75% (12/16) of benign patients, the most abundant species of the lower genital tract were also recovered from all sites of the upper genital tract, indicating that sampling of the easily accessible vagina could potentially be used as a proxy to predict upper genital tract microbial synthesis. In two patients the microbial continuum was disrupted at the level of fallopian tube or ovary with microbial concordance of the rest of sites, while for the remaining two patients the lower genital tract microbial composition was uncorrelated from the upper. In the endometrial cancer cohort, which was microbially more diverse that the benign cohort, a median of 7.2% of bacterial species were shared by the lower genital tract and endometrium (Figure 4.9B).

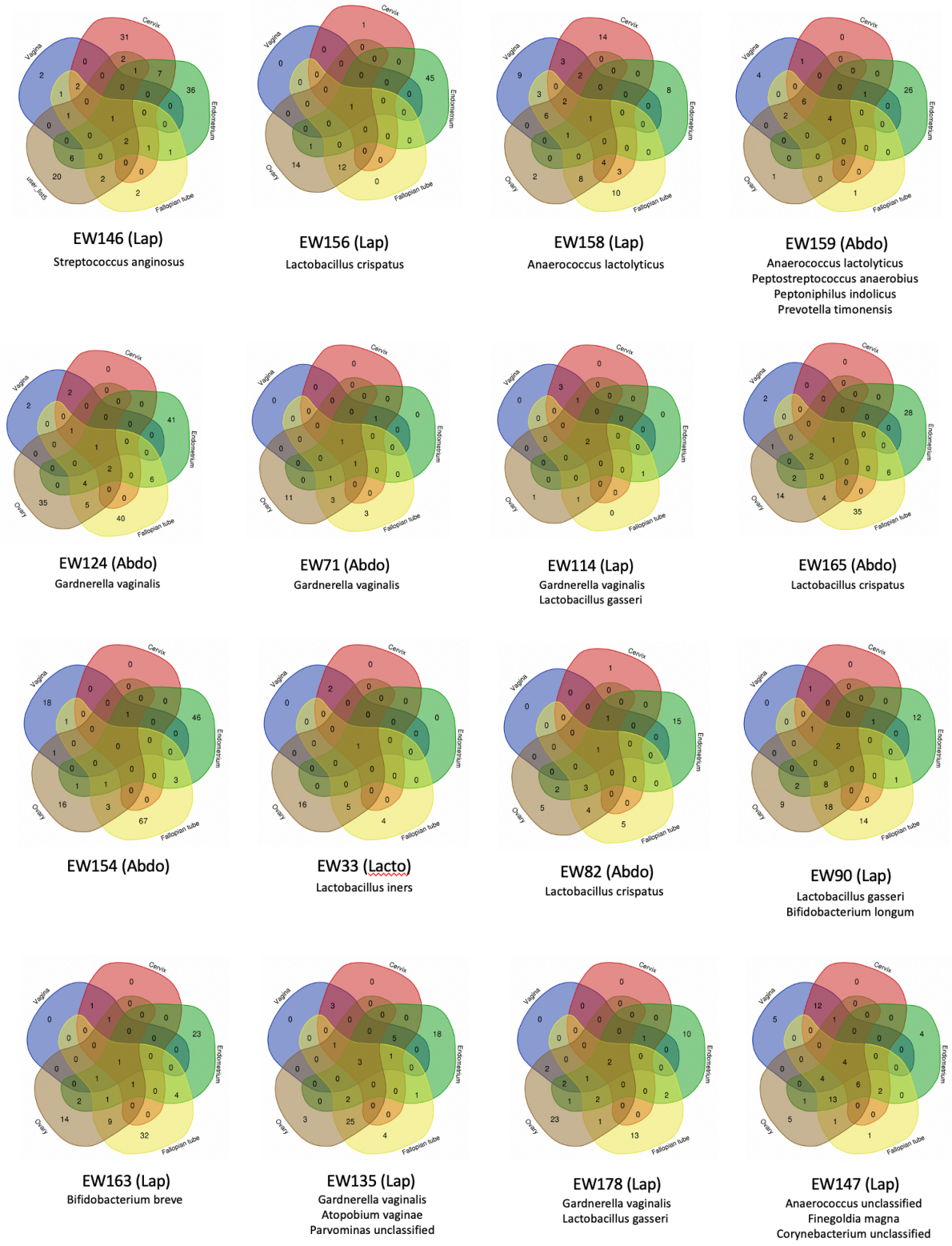
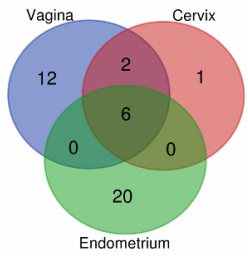
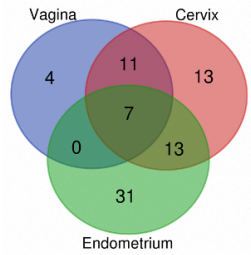


Figure 4.8. Microbial continuum along the female genital tract of benign patients. Venn diagrams illustrating microbial species shared by all sites of the lower and upper genital tract (vagina, cervix, endometrium, fallopian tube, ovary) in 16 patients with benign pathology. Only species with an at least 0.5% relative abundance were included. *Lap: Laparoscopic; Abdo: Abdominal.*

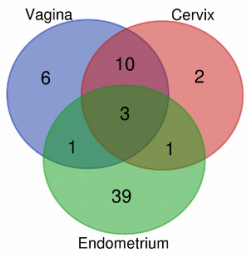
A



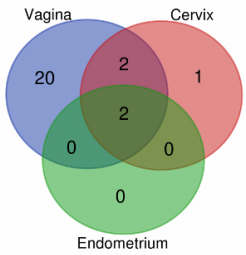
EW155 (Lap)



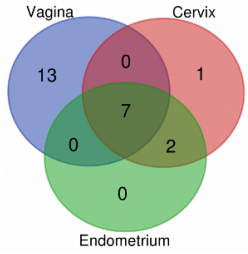
EW142 (Lap)



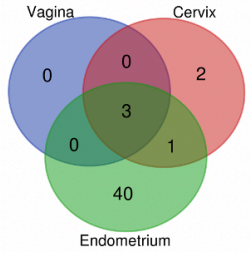
EW141 (Lap)



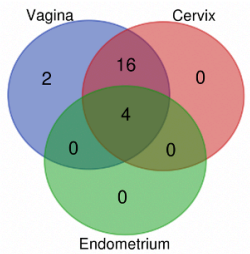
EW176 (Lap)



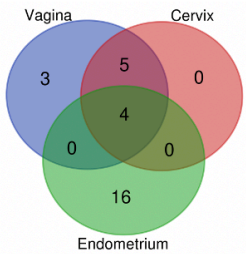
EW145 (Lap)



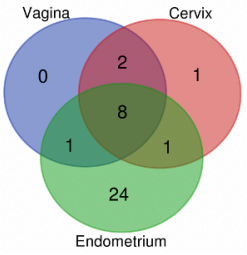
EW131 (Lap)



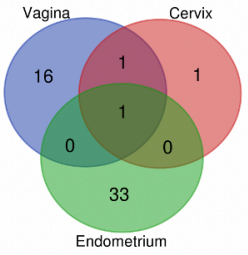
EW171 (Lap)



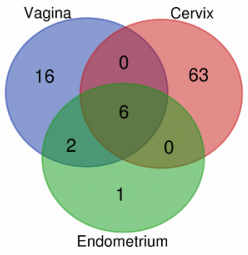
EW173 (Lap)



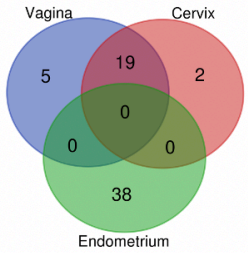
EW144 (Lap)



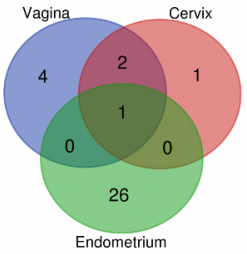
EW160 (Lap)



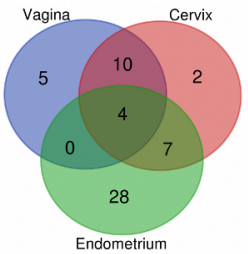
EW161 (Lap)



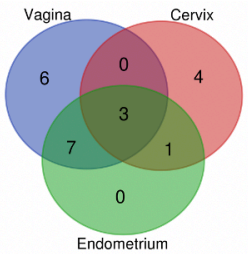
EW162 (Lap)



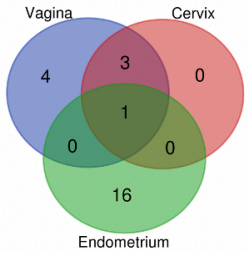
EW166 (Lap)



EW167 (Lap)



EW150 (Lap)



EW172 (Abdo)

B

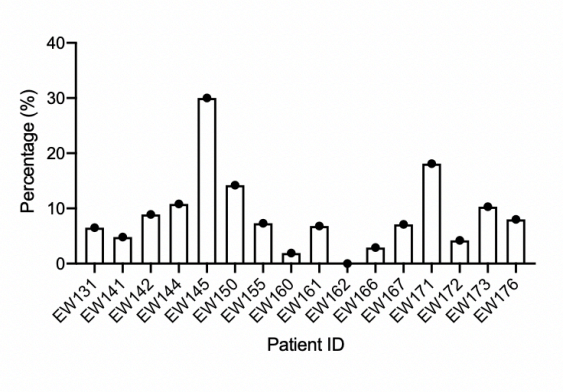
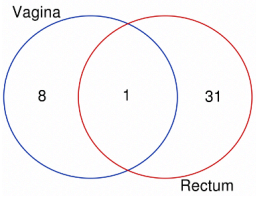


Figure 4.9. Microbial continuum along the female genital tract of endometrial cancer patients. A. Venn diagrams illustrating microbial species shared by the lower and upper genital tract (vagina, cervix, endometrium) in 16 patients with endometrial cancer. **B.** Proportion of overlapping bacterial species in all sites. Only species with an at least 0.5% relative abundance were included. *Lap: Laparoscopic; Abdo: Abdominal.* Microbial species shared by all sites: **EW155***: Prevotella amnii, Atopobium vaginae, Gardnerella vaginalis, Anaerotruncus* unclassified*, Megasphaera* unclassified*, Sneathia* unclassified*;* **EW142***: Finegoldia magna, Prevotella timonensis, Peptoniphilus indolicus, Streptococcus mitis, Catabacter* unclassified*, Peptoniphilus harei, Campylobacter ureolyticus;* **EW141***: Finegoldia magna, Veillonalla parvula, Prevotella timonensis;* **EW145***: Sneathia* unclassified*, Anaerococcus* unclassified*, Streptococcus agalactiae, Anaerococcus obesiensis, Anaerotruncus* unclassified*, Peptoniphilus harei, Parvimonas* unclassified*;* **EW176***: Anaerococcus* unclassified*, Anaerococcus vaginalis;* **EW171***: Lactobacillus gasseri, Peptoniphilus indolicus, Prevotella bivia, Peptoniphilus harei;* **EW173**: *Fusobacterium gonidiaformans, Pseudocitrobacter anthropic, Peptostreptococcus anaerobius, Bacteriodes fragilis;* **EW131***: Lactobacillus iners, Gardnerella vaginalis, Lactobacillus crispatus;* **EW144***: Anaerococcus* unclassified*, Anaerococcus vaginalis, Varibaculum anthropi, Finegoldia magna, Peptoniphilus harei, Propionimicrobium lymphophilum, Streptococcus anginosus, Alloscardovia omnicolens;* **EW160***: Gardnerella vaginalis;* **EW161***: Peptococcus* unclassified*, Subbdoligranulum* unclassified*, Bacteroides vulgatus, Species X, Bacteroides coprocola, Faecalibacterium prausnitzii;* **EW166***: Bifidobacterium breve;* **EW167***: Species X, Corynebacterium pyruviciproducens, Corynebacterium faecale, Psychrobacillus insolitus;* **EW150***: Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum;* **EW172**: *Lactobacillus crispatus.*

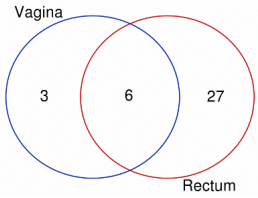
4.3.2.4 Vaginal microbiota are poorly correlated with rectal microbiota

Given their anatomic vicinity, I next investigated whether vaginal microbiota are correlated with rectal microbiota. Correlation in microbial composition between both anatomical sites was examined in 16 women with benign pathology and 26 women with endometrial cancer. Venn diagrams were used to illustrate shared resident microbial species intra-individually in samples collected from the lower third of vagina and rectum (Figures 4.10 and 4.11). Only sequencing reads of at least 0.5% relative abundance were included in the analysis. Rectal microbiota were more diverse than vaginal, and vaginal microbiota of endometrial cancer patients were more diverse than benign patients (median of bacterial species ≥ 0.5% relative abundance: benign vagina- 7.5, benign rectum- 32, malignant vagina- 19.5, malignant rectum- 33.5). The less diverse vaginal ecosystem of benign patients shared no common species with

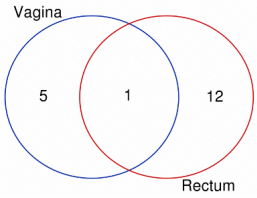
rectum in 44% (7/16) of cases, in 25% (4/16) of patients one bacterial species was shared and in 31% (5/16) of cases ≥ 2 bacterial species overlapped with a median of 4 OTUs. In endometrial cancer patients, 15% (4/26) of cases did not share any common species, 27% (7/26) shared one bacterial species, while 58% (15/26) had ≥ 2 bacterial species in common with a median of 5 OTUs.



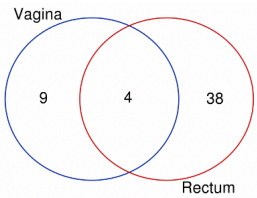
EW120 (Ben)
Pseudocitrobacter_anthropi



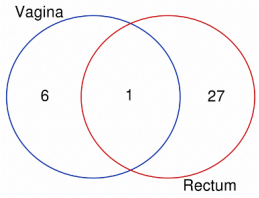
EW121 (Ben)
Prevotella_bivia Anaerophaga_unclassified
Gardnerella_vaginalis
Peptostreptococcus_anaerobius
Prevotella_timonensis Lactobacillus_iners



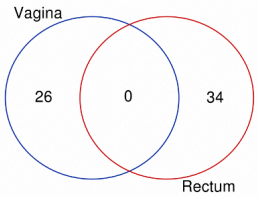
EW124 (Ben)
Enterococcus_hirae



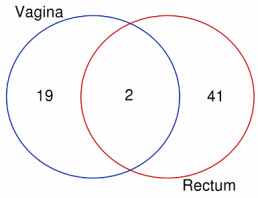
EW135 (Ben)
Gardnerella_vaginalis
Anaerotruncus_unclassified
Prevotella_timonensis
Dialister_unclassified



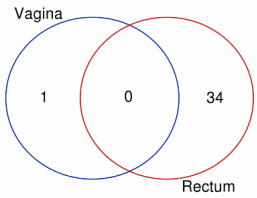
EW146 (Ben)
Streptococcus_anginosus



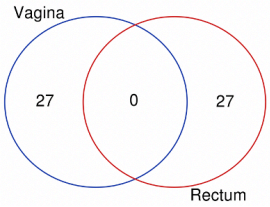
EW147 (Ben)



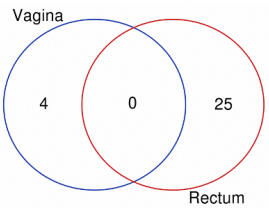
EW154 (Ben)
Peptoniphilus_harei
Peptoniphilus_lacrimalis



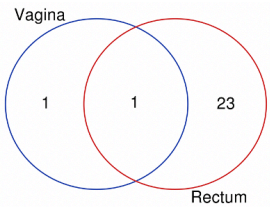
EW156 (Ben)



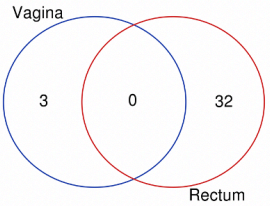
EW158 (Ben)



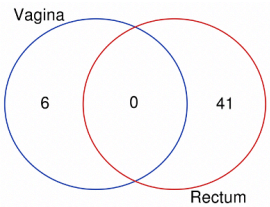
EW163 (Ben)



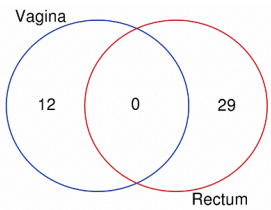
EW165 (Ben)
Streptococcus_anginosus



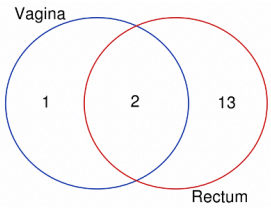
EW170 (Ben)



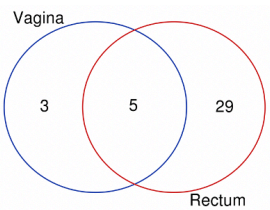
EW169 (Ben)



EW173 (Ben)

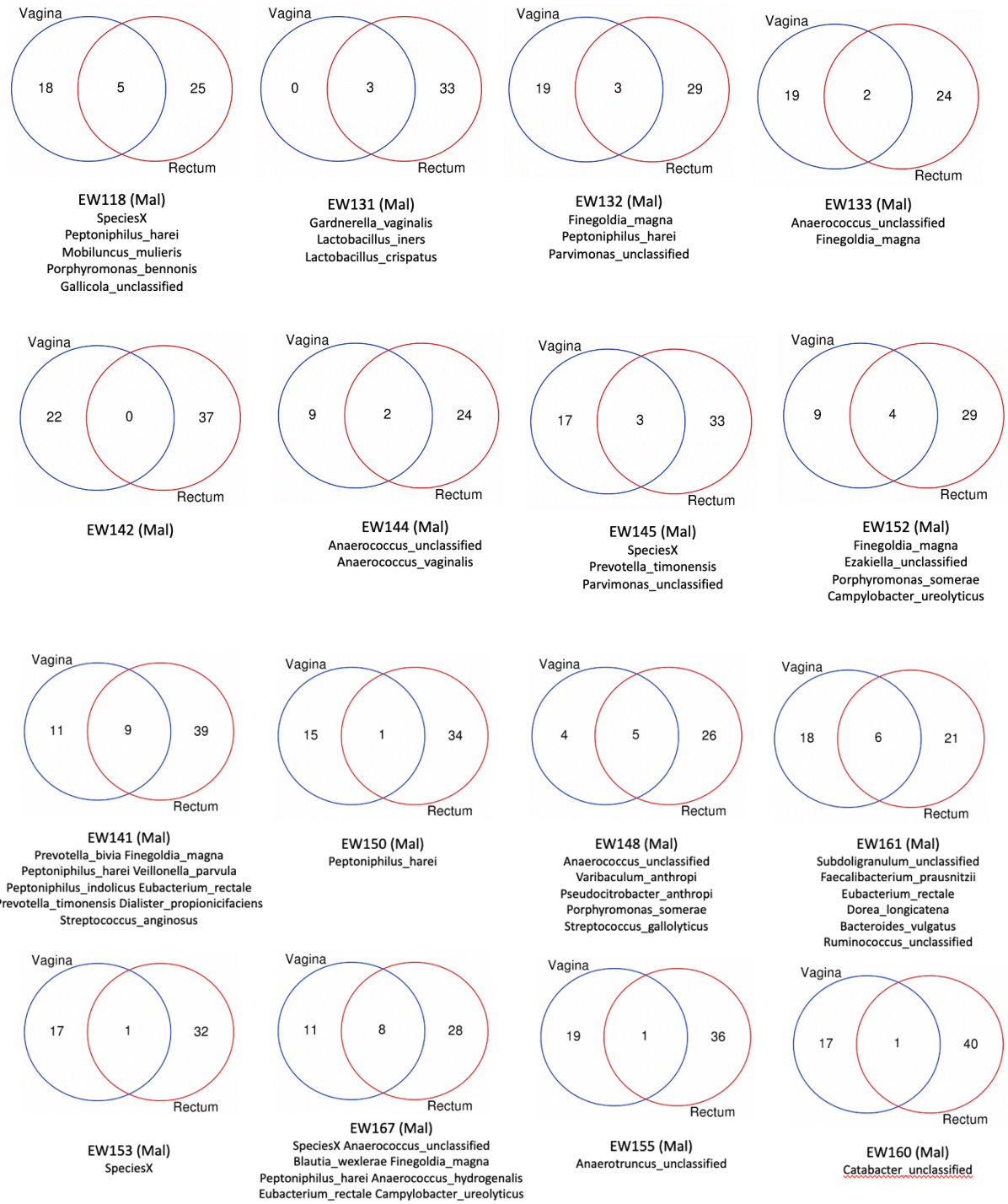


EW174 (Ben)
Lactobacillus_gasseri
Gardnerella_vaginalis



EW178 (Ben)
Prevotella_bivia
Streptococcus_agalactiae
Finegoldia_magna
Peptostreptococcus_anaerobius
Streptococcus_anginosus

Figure 4.10. Intra-individual correlation between vaginal-rectal microbiota of benign patients. Venn diagrams illustrating microbial species shared by the lower third of vagina and rectum in 16 patients with benign pathology. Only species with an at least 0.5% relative abundance were included. *Ben: Benign.*



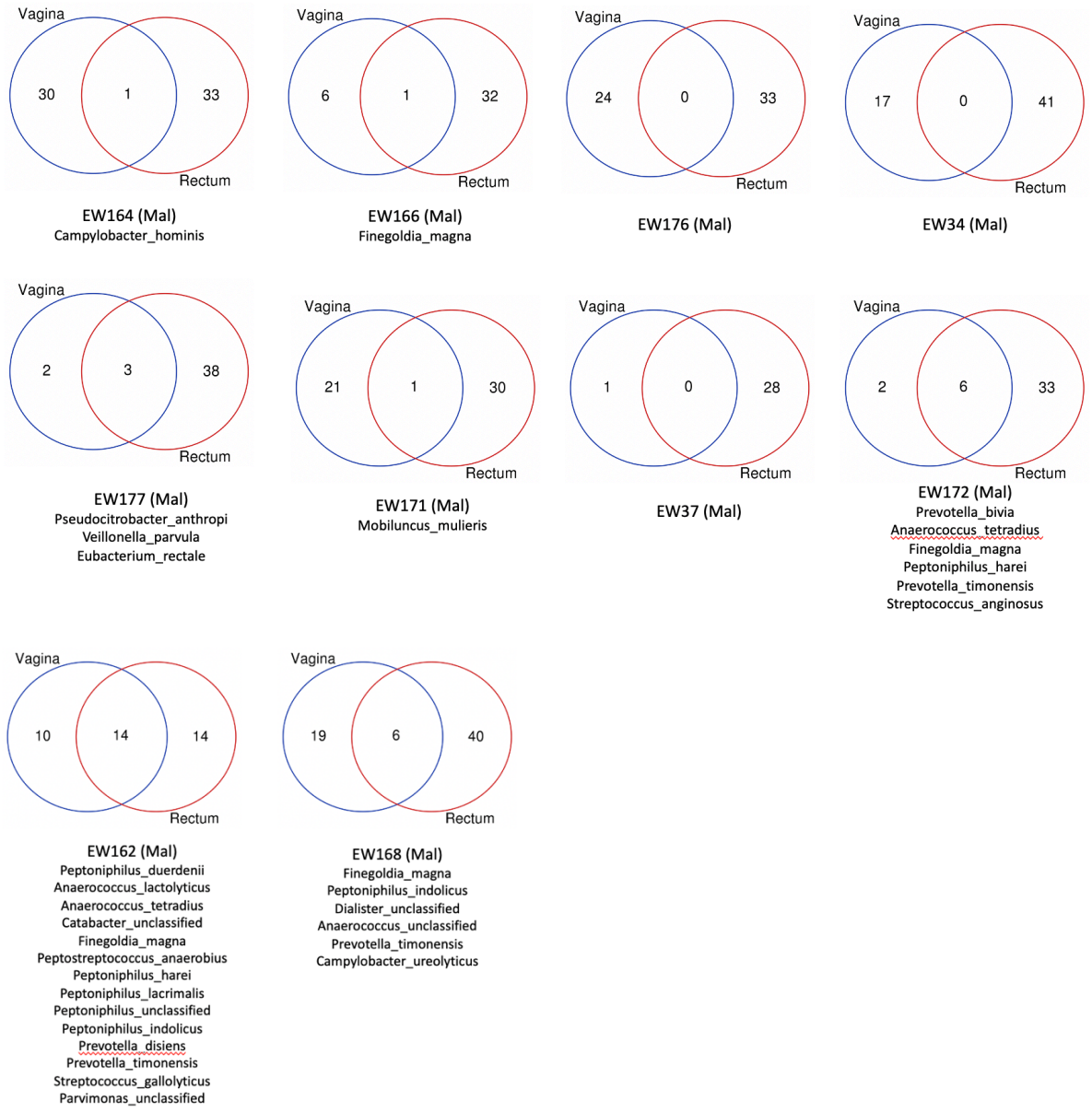


Figure 4.11. Intra-individual correlation between vaginal-rectal microbiota of endometrial cancer patients. Venn diagrams illustrating microbial species shared by the lower third of vagina and rectum in 26 women with endometrial cancer. Only species with an at least 0.5% relative abundance were included. *Mal: Malignant.*

4.5 Discussion

In this chapter, I aimed to investigate the presence of a genuine microbial fingerprint in the upper female genital tract of benign and endometrial cancer patients. Upper genital tract sterility is still debated given that female genital tract metagenomic studies are commonly hampered by contamination from both patient and non-patient sources. Absolute sterility is challenging to maintain during sample collection and processing and encompasses environmental and cross- (between samples) contamination [343]. This, in conjunction with the increased sensitivity of next-generation sequencing technologies can lead to the detection of microbial signals that represent background noise obscuring actual microbial signatures. Even though this is highly unlikely to significantly affect sites of high microbial abundance, such as the vagina, cervix and rectum, it can distort findings from low bacterial biomass sites, like the endometrium, fallopian tubes and ovaries. I attempted to address this through integration of four different sets of technical controls that accounted for air and reagent/equipment contaminants ("kitome") that can occur during sample collection or during DNA extraction and 16S rRNA gene sequencing. This approach led to the discarding of approximately 15% of total sequencing data that was attributed to background DNA contamination from these sources.

However, it was also important to attempt to address potential patient sources of contamination derived from sampling practices adopted in laparoscopic hysterectomies, such as the insertion of a uterine manipulator and the vaginal retrieval of surgical specimen intra-operatively as well as the transcervical collection of endometrial samples. By comparing intra-individually, the microbial composition across different sites between thirteen transabdominal and thirteen laparoscopic hysterectomies, likely intra-patient contamination was observed in up to 1/3 of samples collected using laparoscopic procedures, secondary to instrumentation and specimen collection practices. In contrast to these findings, a previous report comparing endometrial bacterial distribution between transcervical and transuterine

collection methods reported high bacterial similarity between the two methods, indicating low contamination [141].

In addition to composition comparisons between patient samples and controls, I also used quantitative real time PCR to compare bacterial load between sample types. As expected, this data showed that bacterial abundances of endometrial, fallopian tube and ovarian samples are significantly lower (2-4 $\log_{10}$) than vaginal and cervical samples and comparable to bacterial abundances estimated in negative controls. These findings are consistent with Mitchell et al., who reported that mean bacterial quantities in the endometrium and upper endocervix are lower than vaginal levels by 2-4 $\log_{10}$ rRNA gene copies [142], which was further corroborated by the Chen study showing that vaginal sites contained about four orders of magnitude more bacteria (10^{10}-10^{11}) than the endometrium and peritoneal fluid, still exhibiting much lower cycle threshold (Ct values) than negative controls [141]. Another study confirmed high bacterial signals in vagina and comparable bacterial load in cervix and endometrium with most cervical and endometrial samples (60-72%) exceeding the bacterial copies of blank controls [448].

Furthermore, compositional differences in the residual microbial signals from patient and control samples were identified. In benign patients, several endometrial bacterial species were enriched compared to controls, including *Lactobacillus crispatus, Streptococcus anginosus, Lactobacillus gasseri, Peptostreptococcus anaerobius, Prevotella timonensis*, *Gardnerella vaginalis*; in fallopian tubes *Cloacibacterium rupense*, *Comamonas denitrificans*, *Corynebacterium tuberculostearicus, Dialister propionicifaciens, Lactobacillus crispatus*; in the ovary *Alicycliphilus denitrificans, Comamonas denitrificans*, *Corynebacterium tuberculostearicus, Streptococcus anginosus* and *Lactobacillus crispatus* among others. The most common endometrial species reported in the Mitchell et al. study were *L. iners* (45%), *Prevotella* spp. (33%) and *L. crispatus* (33%) [142], while in the Chen study *Lactobacillus, Vagococcus, Acinetobacter, Pseudomonas*, which were also identified in the fallopian tube [141]. An additional study by Winters *et al.* identified *Acinetobacter, Pseudomonas,*

Comamonadaceae and *Cloacibacterium* in endometrial samples, while *L. crispatus* and *L. iners* were rarely observed [448]. In endometrial cancer patients, *Prevotella timonensis, Finegoldia magna, Peptoniphilus indolicus, Prevotella bivia, Peptoniphilus lacrimalis* and *Anaerococcus obesiensis* were amongst the species that were found significantly enriched in the endometrial samples compared to controls. Scepticism still remains on what to consider a genuine microbial signature in the low biomass sites, since a few of the species identified by our and other studies are known contaminants despite the integration of appropriate controls.

Overall, a microbiota signature above background contamination was observed in 62% of benign endometrium, 50% of malignant endometrium, 85% of benign fallopian tube and 95% of benign ovary. Winters et al. also reported a 60% (15/25) bacterial recovery above background DNA controls from the endometrium of women with benign pathology, mainly fibroids, undergoing transabdominal hysterectomy [448]. Mitchell and colleagues concluded that 95% (55/58) of non-cancer patients having total laparoscopic or laparoscopically-assisted vaginal hysterectomy without an intracervical manipulator had endometrial colonisation with at least one species [142], while Chen et al. enrolled 95 reproductive age women and isolated bacteria from the endometrium, fallopian tubes and peritoneal fluid during either laparotomy or laparoscopy [141]. Notably, our results are predominantly deduced from post-menopausal women >50 years of age, while the aforementioned reports involved mainly premenopausal women <50 years of age. This may account for some of the discrepancies observed between studies alongside differences in the inclusion of negative controls but regardless, the data suggests that a microbiota signature is detectable in the upper reproductive tract of a proportion of women regardless of age and menopausal status.

Comparison of microbiota profiles from tissue biopsies of the genital tract and paired swab samples identified both compositional and biomass differences, with the former tending to have comparably lower bacterial biomass. This may simply reflect the relatively small proportion of mucosal surface area contained within a tissue biopsy.

Compositionally, microbial profiles were only similar in less than one third of swab-tissue pairs, implying that microbiota residing deeper in tissue may be different from those colonising the mucosal surface. Interestingly, cervical biopsies were almost completely devoid of *L. crispatus*, a cervical epithelium commensal, also suggesting that some microbes may reside exclusively on mucosal surfaces, while others penetrate cells or are transferred to deeper structures via bloodstream. These findings are in agreement with similar studies comparing microbiota in swabs versus tissue biopsies from skin [463] and rectum [464] showing differences in diversity and taxonomic composition between the two sample types. However, in paired swabs-biopsies from oesophageal and gastric cancer patients, comparable α-diversity and dominant bacterial genera were reported [465].

Investigation of a potential microbial continuum within the female genital tract was next explored in sixteen benign and sixteen endometrial cancer patients. A systematic and rigorous collection of patient-matched samples across all FGT locations was undertaken, including careful consideration of potential sources of contamination derived from the theatre and the pathology laboratories where collection and processing of samples occurred. This valuable matched sample cohort included samples from the lower two thirds of vagina, higher one third of vagina, cervical os, lower half of endometrium, fundal endometrium, fallopian tubes and ovaries for each patient. The microbial composition was highly correlated (correlation co-efficient $R^2>0.7$) between higher and lower vagina in both benign and endometrial cancer patients but less correlated between fundal and lower endometrium (correlation co-efficient $R^2<0.7$), especially in endometrial cancer patients. Next, I interrogated the microbial continuum along the whole length of the female genital tract and found that in 75% (12/16) of benign patients the most abundant species of the lower genital tract (ie., *L. crispatus, L. iners, L. gasseri, G. vaginalis, S. anginosus,* and *B. breve*) were also detectable (>0.5% relative abundance) from all sites of the upper genital tract. These findings support the notion of bacterial colonisation through ascension for some of the most abundant species of the lower genital tract in benign patients. However, in the endometrial cancer cohort, which was microbially more diverse than the benign

cohort, a median of 7.2% of bacterial species was shared by the lower genital tract and endometrium, suggesting that upper genital tract microbial inferences cannot be based on vaginal bacterial composition.

The concept of the rectal microbiota seeding the female genital tract has been suggested by studies comparing the microbial composition of both sites and demonstrating up to 44% (28/63) overlap of bacterial species between rectum and vagina, including *L. crispatus*, *L. gasseri* and *L. jensenii* [194 462]. In contrast to these findings, the results present in this chapter indicate that in more than two thirds of women with benign disease, none or one microbial species is shared between the vagina and rectum, while in the vagina of endometrial cancer patients, which is microbially more diverse compared to the vagina of benign controls, more than half of patients have ≥ 2 bacterial species in common with rectum, still accounting for only 15% sharedness of rectal microbiota with vagina. Whether shared microbiota between non-communicating anatomical structures denote local translocation of microbes, haematogenous dissemination or separate mechanisms of colonisation remains elusive.

Contemporary microbiome research mandates stringent consideration and accounting for potential sources of contamination, especially when low biomass microbiomes are interrogated. The adverse effect of reagent and laboratory contaminants, coupled with intra-patient contamination during sample collection can lead to spurious conclusions. After establishing true microbial signatures, enquiring about the intra- and inter-organ phylogenetic affiliations of host microbiota warrants investigation. Determining microbial gradients throughout human tissue from epithelial surfaces to muscle layers and serous membranes and microbial associations between different anatomical sites could shed light on microbial origins and mechanisms of microbial circulation.

4.6 Conclusions

In summary, this is the first study to address the presence of genuine microbial communities in low biomass sites of the genital tract of both benign and endometrial cancer patients. The contamination-aware experimental design and analysis led to the conclusion that a subset of patients harbours microbiota in the upper genital tract that are quantitatively and compositionally distinguishable from background contaminants. The data also suggests caution should be exercised when analysing data from laparoscopic procedures because the insertion of a uterine manipulator intra-operatively and vaginal recovery of surgical specimen could introduce cross-site contamination intra-individually in some patients. Stronger microbial signals with higher abundances and enrichment of several taxa are recovered from swabs of the female genital tract compared to respective tissue biopsies, indicating that epithelial surfaces are more heavily colonised. A microbial continuum from vagina to ovaries was demonstrated for a limited number of bacterial species that are highly abundant in the vagina of patients with benign pathology. In contrast, evidence for microbial sharedness along the genital tract in endometrial cancer patients was poor. Lastly, vaginal microbiota were uncorrelated with rectal microbiota for most benign patients, while endometrial cancer patients demonstrated a slightly increased, yet still limited, number of phylogenetic affiliations.

4.7 Statement of Contribution

Processing of sequencing data with Decontam package for identification of contaminant sequences was performed by Dr Sherrianne Ng.

CHAPTER 5.

Comparison of female genital tract and rectal microbiota in endometrial cancer patients and benign controls

---

Content from this chapter is currently under preparation as:

Semertzidou A, Smith A, Brosens J, Marchesi J, Bennett P, MacIntyre D and Kyrgiou M. The role of genital tract microbiota continuum in endometrial cancer. *(Under preparation)*

5.1 Introduction

Deviation of healthy microbiome signatures is a characteristic of several disease states. The oncobiome, a term coined to describe the field of research investigating the role of the microbiome in human cancer development, has seen exponential growth and holds promise that manipulation of host-associated microbiota could impact on cancer pathogenesis, progression and response to anti-cancer treatments.

In gynaecological oncology research, the biggest body of evidence supporting a role of dysbiotic microenvironments and pathology pertains to cervical dysplasia and cancer. Almost all cross-sectional studies undertaken to date have observed a consistent microbial signature in cervicovaginal microbiota of women with HPV infection, cervical intraepithelial neoplasia (CIN) and invasive cervical carcinoma characterised by *Lactobacillus* depletion, high diversity and high relative abundance of *Gardnerella, Sneathia, Megasphaera, Atopobium, Bacteroides* and *Prevotella* [466]. Two recent systematic reviews and meta-analyses have concluded that *L. crispatus* depletion associates with hrHPV and CIN [128 310], while *L. iners* dominance in cervicovaginal microbiota correlates with 2-3 times higher odds of hrHPV prevalence and CIN, as well as 3-5 times higher odds of any prevalent HPV in comparison to *L. crispatus* [310]. Our group has shown in a longitudinal study that *Lactobacillus*-dominant vaginal microbiota associate with increased regression of untreated CIN2 at 12 months [131].

Associations of genital tract microbiota with endometrial and ovarian cancer have been more sparsely investigated owing to the reduced sampling accessibility of internal organs and the hurdles of evaluating low-biomass, contamination-prone anatomical sites. Epidemiologic studies have associated pelvic inflammatory disease, caused by bacteria, with an increased risk for ovarian cancer [467-471], while microbiome studies have acknowledged deviations from healthy and benign controls in the cervicovaginal [136 137], ovarian [159-162 472], peritoneal [165], gut [187] and serum [166] microbiota of ovarian cancer patients.

In a recent observational study, PID patients were found to have a 1.79-fold higher risk of developing endometrial cancer than the non-PID patients following adjustment for potential risk factors [24]. Only a small number of recently conducted studies have started to investigate the potential existence of endometrial cancer-associated microbiota. Two studies by the same group reported an enrichment of seventeen taxa in the genital tract of endometrial cancer patients, with *Porphyromonas somerae* having the strongest association with the disease, as confirmed by targeted qPCR [158] [308]. The authors also demonstrated that risk factors for endometrial cancer (menopause, obesity) and high vaginal pH increase the diversity of lower tract and uterine microbiota [308]. A 2020 study reported decreased α-diversity in endometrial cancer tissue and enrichment of the *Micrococcus* genus in 25 endometrial cancer patients compared to 25 patients with benign uterine pathology [473], while a 2021 study assessed differences in bacterial, archaea, and viral transcript (BAVT) expression between 62 endometrioid endometrial cancers, 112 high-grade serous ovarian cancers and 12 normal fallopian tubes [474]. This case-control study revealed that 93 BAVTs are differentially expressed between HGSC and EEC, BAVT expressions in normal tissue were the highest, followed by endometrial and ovarian cancer samples and that human loci (genes, lncRNAS) harbour genetic material from these microorganisms.

The endometrial oncobiome still remains poorly characterised. Further cross-sectional and longitudinal studies are required to shed light on the disparities between endometrial eubiosis and oncobiosis, the temporal microbial transitions in women with risk factors predisposing to endometrial cancer and finally the functionality of onco-signatures that could reveal actionable features to exploit for therapeutic benefit.

5.2 Aim

- To characterise and compare the female genital tract and rectal microbiota in women with and without endometrial cancer and explore differences in bacterial composition according to disease characteristics (histological type, grade).

5.3 Results

5.3.1 Patient demographic and clinical characteristics

Sixty-one women undergoing laparoscopic or open hysterectomy were prospectively recruited; 37 had endometrial cancer and 24 were benign controls. In total, 178 benign, 207 malignant and 51 technical control samples were sequenced and analysed. Patient and clinical characteristics are shown in Table 5.1.

The majority of patients were of white background (37/61, 60.7%), obese (BMI≥ 30, 26/61, 42.6%), non-diabetic (47/61, 77%), parous (46/61, 75.4%), post-menopausal (56/61, 91.8%), had never used hormone replacement therapy (48/61, 78.7%), had never smoked (38/61, 62.3%) and had not used antibiotics within the last four weeks (52/61, 85.2%). Oral contraceptive use and current coffee consumption were more common in the benign arm (16/24, 66.7% and 13/24, 54.2% respectively) than endometrial cancer patients (16/37, 43.2% and 15/37, 40.5% respectively).

The two groups were largely comparable with the exception of age (endometrial cancer ≥65, 56.8%; benign 50-64, 58.3%, p= 0.0077). The majority of endometrial cancers were endometrioid tumours (30/37, 81%) and of stage I (31/37, 83.8%).

Table 5.1. Patient demographic and clinical characteristics of the endometrial cancer cohort and benign controls.

Patient Characteristics	Benign (n=24)	Endometrial ca (n=37)	Total (n=61)	OR (95% CI) / P-value	*P* value* (Benign vs Cancer)
Ethnicity, n/N (%)					
White	17/24 (70.8)	20/37 (54.1)	37/61 (60.7)		0.1156
Asian	1/24 (4.2)	10/37 (27)	11/61 (18)	0.12 (0.01, 1.01)/ 0.035	
Black/African/Caribbean	5/24 (20.8)	5/37 (13.5)	10/61 (16.4)	1.18 (0.29, 4.76)/ 1.00	
Other	1/24 (4.2)	2/37 (5.4)	3/61 (4.9)	0.59 (0.05, 7.07)/ 1.00	
Age (years), n/N (%)					
18- 34	1/24 (4.2)	0/37 (0)	1/61 (1.7)		**0.0077**
35- 49	4/24 (16.7)	1/37 (2.7)	5/61 (8.2)	1.00 (0.02, 40.28)/ 1.00	
50- 64	14/24 (58.3)	15/37 (40.5)	29/61 (47.5)	0.31 (0.01, 8.28)/ 0.484	
≥ 65	5/24 (20.8)	21/37 (56.8)	26/61 (42.6)	0.09 (0.003, 2.39)/ 0.064	
BMI status, n/N (%)					
Underweight (<18.5)	1/24 (4.2)	2/37 (5.4)	3/61 (4.9)	0.50 (0.04, 7.10)/ 1.00	0.8817
Normal (18.5- 24.9)	6/24 (25)	6/37 (16.2)	12/61 (19.7)		
Overweight (25- 29.9)	7/24 (29.1)	13/37 (35.2)	20/61 (32.8)	0.59 (0.05, 7.07)/ 0.473	
Obese (≥ 30)	10/24 (41.7)	16/37 (43.2)	26/61 (42.6)	0.54 (0.13, 2.31)/ 1.00	
Parity, n/N (%)					
Nulliparous	5/24 (20.8)	10/37 (27)	15/61 (24.6)		0.583
Parous	19/24 (79.2)	27/37 (73)	46/61 (75.4)	1.41 (0.41, 4.78)	
Menopausal status, n/N (%)					
Premenopausal	3/24 (12.5)	2/37 (5.4)	5/61 (8.2)		0.373
Postmenopausal	21/24 (87.5)	35/37 (94.6)	56/61 (91.8)	0.40 (0.06, 2.59)	
Contraceptive pill use, n/N (%)					
Ever users	16/24 (66.7)	16/37 (43.2)	32/61 (52.5)	2.63 (0.90, 7.65)	0.735
Never users	8/24 (33.3)	21/37 (56.8)	29/61 (47.5)		
HRT use, n/N (%)					
Ever users	8/24 (33.3)	5/37 (13.5)	13/61 (21.3)	3.20 (0.90, 11.38)	0.065

Never users	16/24 (66.7)	32/37 (86.5)	48/61 (78.7)		
Diabetes status, n/N (%)					
Diabetic	5/24 (20.8)	9/37 (24.3)	14/61 (23)	0.82 (0.24, 2.83)	0.751
Nondiabetic	19/24 (79.2)	28/37 (75.7)	47/61 (77)		
Smoking, n/N (%)					
Ever smokers	11/24 (45.8)	12/37 (32.4)	23/61 (37.7)	1.76 (0.61, 5.08)	0.291
Never smokers	13/24 (54.2)	25/37 (67.6)	38/61 (62.3)		
Current coffee consumption, n/N (%)					
Yes	13/24 (54.2)	15/37 (40.5)	28/61 (45.9)	1.73 (0.61, 4.89)	0.297
No	11/24 (45.8)	22/37 (59.5)	33/61 (54.1)		
Antibiotic use last 4 weeks, n/N (%)					
Yes	5/24 (20.8)	4/37 (10.8)	9/61 (14.8)	2.17 (0.52, 9.08)	0.298
No	19/24 (79.2)	33/37 (89.2)	52/61 (85.2)		
Surgical approach					
Laparoscopic	15/24 (62.5)	34/37 (91.9)	49/61 (80.3)		
Transabdominal	9/24 (37.5)	3/37 (8.1)	11/61 (18)		
Histological type, n/N (%)					
Endometrioid	NA	30/37 (81)			NA
Serous	NA	3/37 (8)			
Clear cell	NA	3/37 (8)			
Carcinosarcoma	NA	1/37 (3)			
FIGO Grade, n/N (%)					
I	NA	12/37 (32.4)			NA
II	NA	11/37 (29.7)			
III	NA	14/37 (37.8)			
FIGO Stage 2009, n/N (%)					
IA	NA	26/37 (70.3)			NA
IB	NA	5/37 (13.5)			
II	NA	3/37 (8.1)			
IIIA	NA	1/37 (2.7)			
IIIC	NA	1/37 (2.7)			
IV	NA	1/37 (2.7)			

*Statistical significance (*p* value) assessed by Fisher's exact test.

5.3.2 Cervicovaginal and rectal bacterial load is reduced in endometrial cancer

To compare the overall bacterial load in endometrial cancer patients versus benign controls at multiple locations, qPCR of the 16S rRNA gene was performed. Bacterial copies from the vagina (both lower 2/3 and higher 1/3), cervix and rectum of endometrial cancer patients were found to be between 1-2 $\log_{10}$ lower than benign patients with the strongest differences observed in the higher vagina and cervix (EC versus Benign controls, Lower Vagina: p-value 0.0159; Higher Vagina: p-value 0.0031; Cervix: p-value 0.0018; Rectum: p-value 0.0408). No significant differences were noted in the endometrium, fundal or lower, of women with or without malignancy, where bacterial quantities were comparable to those of controls (Figure 5.1).

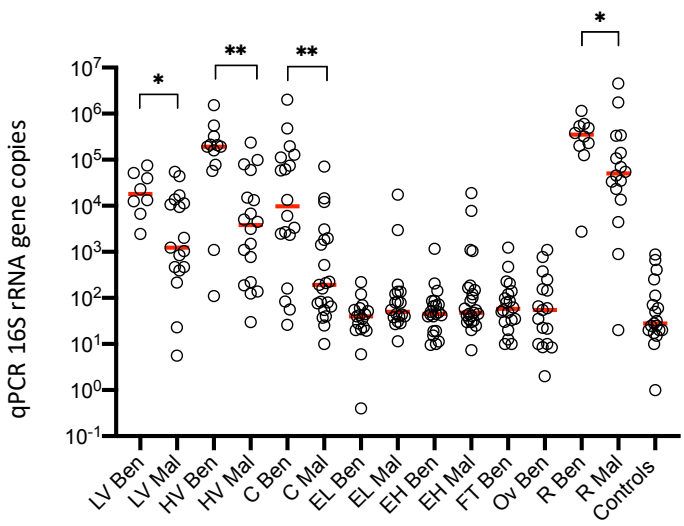


Figure 5.1. qPCR bacterial load at different locations in benign, endometrial cancer patients and controls. Red line represents median. *Ben: Benign, Mal: Malignant, LV: Lower Vagina, HV: Higher Vagina, C: Cervix, EL: Endometrium Lower, EH: Endometrium Higher, FT: Fallopian tube, Ov: Ovary, R: Rectum.* * p-value < 0.05, ** p-value < 0.005.

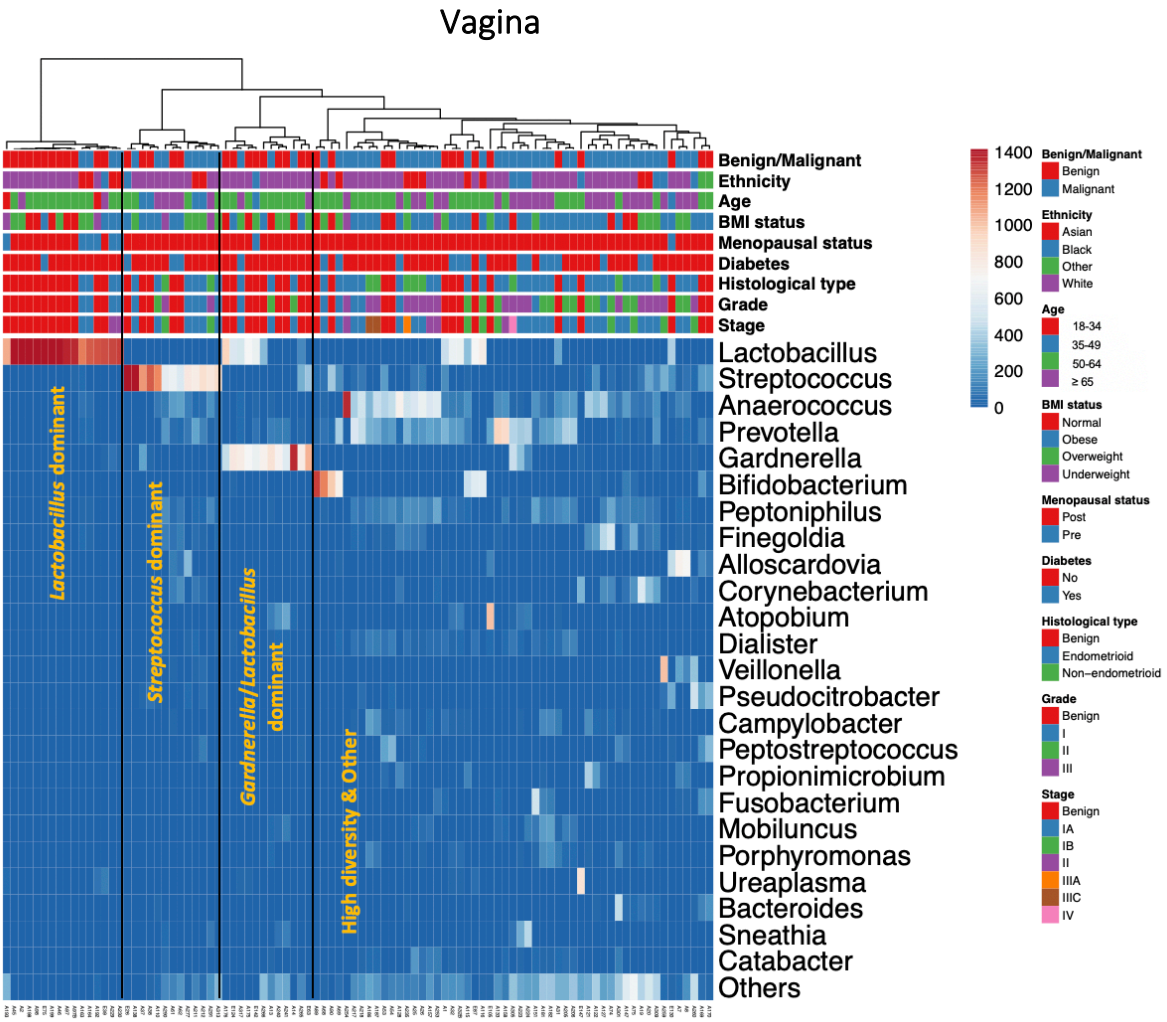
5.3.3 *Lactobacillus* depletion and high microbial diversity along the female genital tract are characteristic of endometrial cancer patients compared to benign controls

Compositional differences between benign and endometrial cancer patients were investigated by comparing the community structure at different anatomical sites (vagina, cervix, endometrium). The fallopian and ovarian samples were excluded from comparative analysis as I thought they are less likely to contribute to endometrial carcinogenesis. Five vaginal, ten cervical and twenty-five endometrial samples were excluded from analysis due to low read counts. Subsequently, OTUs were randomly sub-sampled to the lowest common read count for each site to avoid sequencing bias. This technique retained 96% of OTU counts and still provided coverage of greater than 95% for all samples**.**

Hierarchical clustering analysis (HCA) at genera level revealed three distinct clusters of *Lactobacillus*- dominance (≥75% relative abundance), *Gardnerella/Lactobacillus*-dominance (≥ 50%/40%) and High diversity & Other at all sites examined with an additional *Streptococcus* (≥36%) cluster for vagina (Figures 5.2A, 5.3A and 5.4A). The High diversity & Other cluster was characterised by *Lactobacillus* depletion and included samples with higher Shannon α-diversity than other clusters, but also a small number of samples dominated by one or two bacteria that were not prevalent in other samples (Figure 5.5). The rates and frequency of the different clusters were compared between patients with and without endometrial cancer (Figures 5.2B, 5.3B and 5.4B). *Lactobacillus*- and *Gardnerella/Lactobacillus* dominance were significantly higher among benign patients (vagina 30% versus 22.5%, p= 0.000485; cervix 45.5% versus 18.1%, p= 0.003821; endometrium 41% versus 20.6%, p< 0.00001 respectively), while High diversity & Other was the most prevalent cluster among endometrial cancer patients (vagina 72.2%, p= 0.000485; cervix 82.1%, p= 0.003821; endometrium 87.8%, p< 0.00001), which was also accompanied by *Lactobacillus* depletion. For endometrial clusters, previous sub-analysis based on surgical method in Chapter 4 showed that the insertion of a uterine manipulator through the cervix in laparoscopic procedures (91.9% of EC cases vs 62.5% of benign cases) may cause contamination

from the cervix in 1/4-1/3 of samples. Nevertheless, EC endometrial samples, derived predominantly from laparoscopic procedures, also exhibited *Lactobacillus* depletion.

A

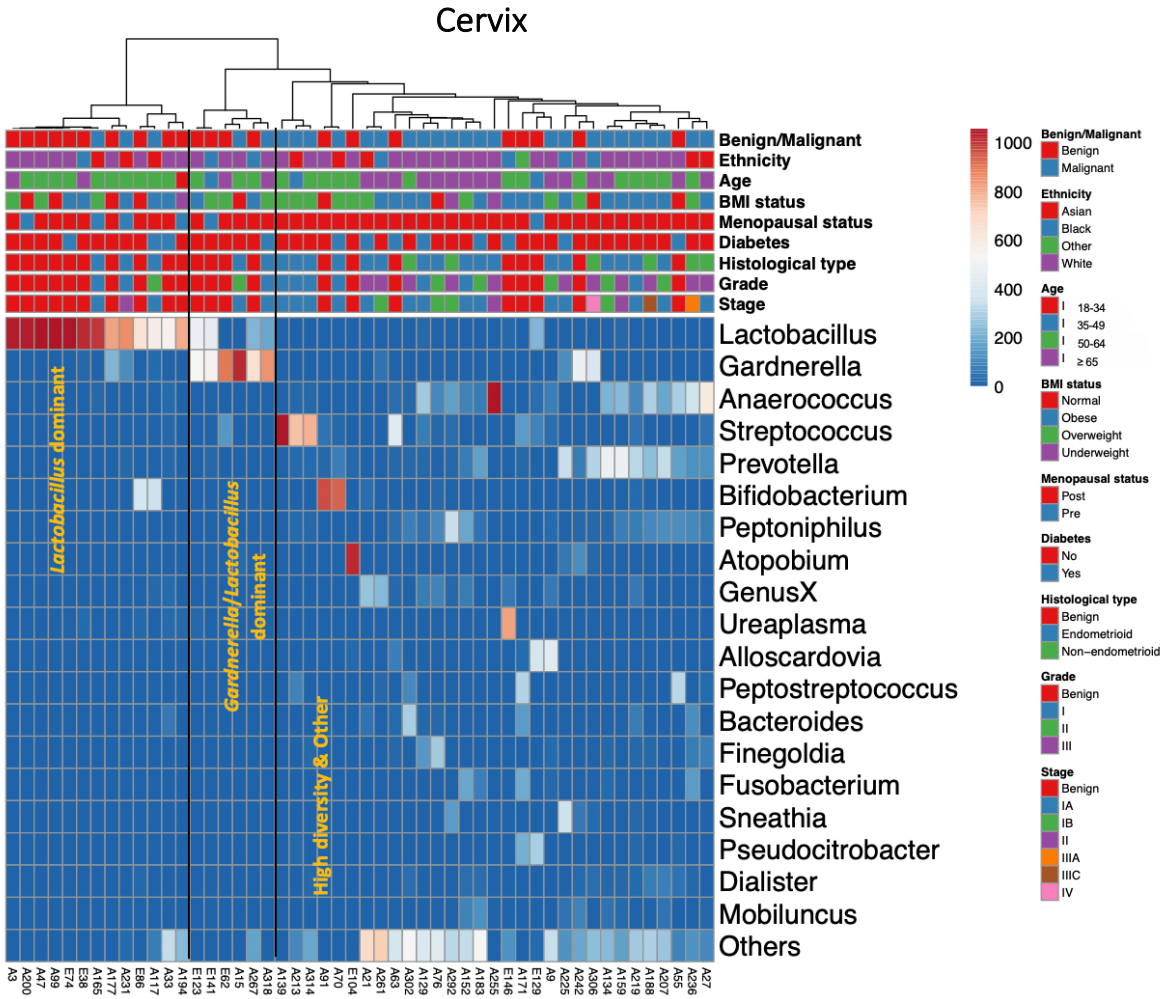


B

Vagina	Lactobacillus dominant n/N (%)	Streptococcus dominant n/N (%)	Gardnerella/Lactobacillus dominant n/N (%)	High diversity & Other n/N (%)	Total
Benign	12/40 (30)	5/40 (12.5)	9/40 (22.5)	14/40 (35)	40/40 (100)
Endometrial cancer	4/54 (7.4)	8/54 (14.8)	3/54 (5.6)	39/54 (72.2)	54/54 (100)
Total	16/94 (17)	13/94 (13.8)	12/94 (12.8)	53/94 (56.4)	
P value					0.000485

Figure 5.2. Vaginal microbiome composition at genus level according to presence or absence of endometrial malignancy. A. Hierarchical clustering analysis per endometrial malignancy, ethnicity, age, BMI status, menopausal status, diabetes, histological type, grade and FIGO stage of disease using Euclidean distance and Ward linkage of the 25 most commonly identified genera. Data were rarefied prior to analysis. **B.** Four microbial genera clusters are observed: *Lactobacillus*-dominant, *Streptococcus*-dominant, *Gardnerella/Lactobacillus*-dominant and High diversity & Other (*p* 0.000485). Women with benign conditions are predominantly *Lactobacillus* or *Gardnerella/Lactobacillus*-dominant, while women with endometrial cancer exhibit mostly high bacterial diversity and *Lactobacillus* depletion. *p* value was calculated by chi-squared test. *BMI: Body mass index*.

A

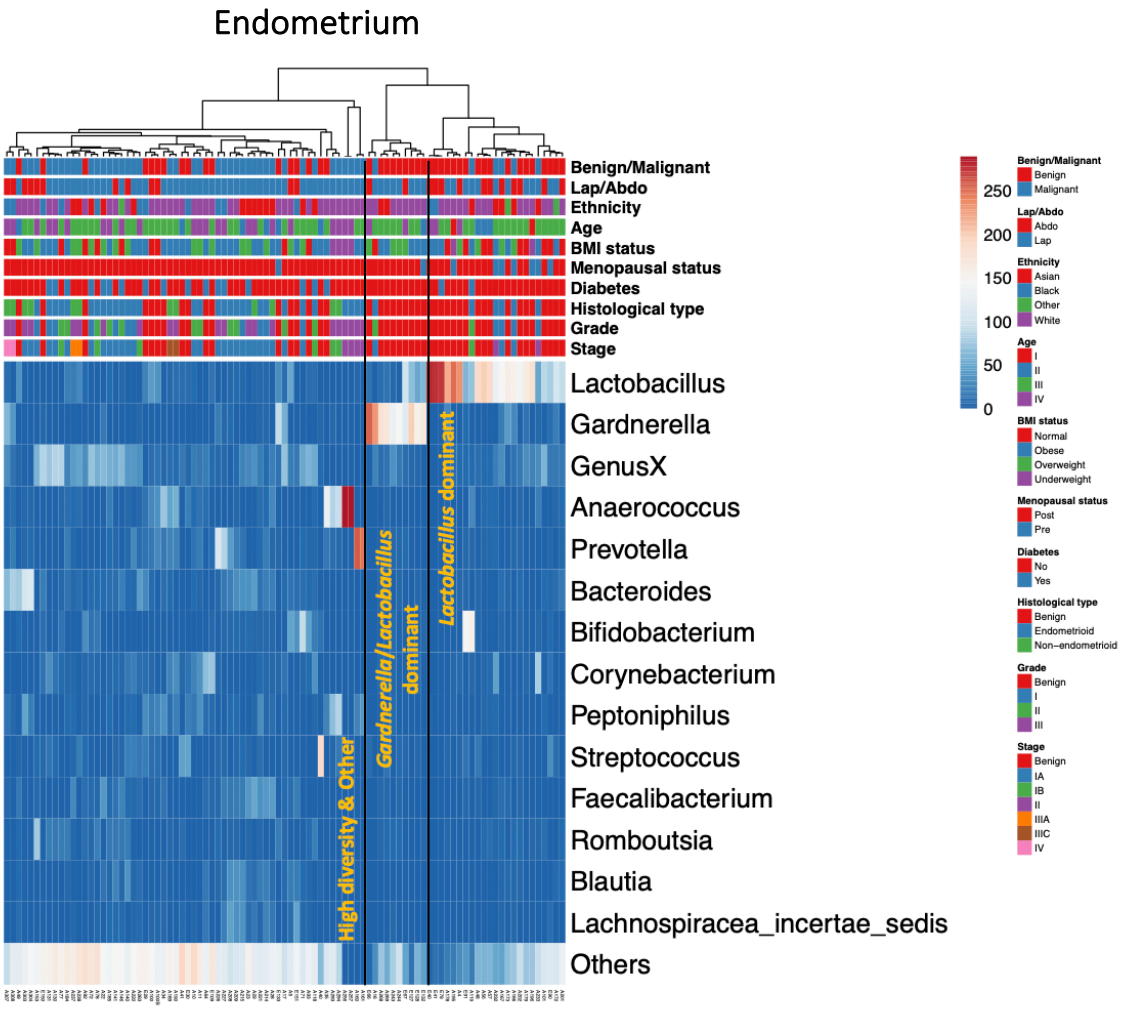


B

Cervix	**Lactobacillus dominant** n/N (%)	**Gardnerella/Lactobacillus dominant** n/N (%)	**High diversity & Other** n/N (%)	**Total** n/N (%)
Benign	10/22 (45.5)	4/22 (18.1)	8/22 (36.4)	22/22 (100)
Endometrial cancer	3/28 (10.7)	2/28 (7.1)	23/28 (82.1)	28/28 (100)
Total	13/50 (26)	6/50 (12)	31/50 (62)	50/50 (100)
P value				0.003821

Figure 5.3. Cervical microbiome composition at genus level according to presence or absence of endometrial malignancy. A. Hierarchical clustering analysis per endometrial malignancy, ethnicity, age, BMI status, menopausal status, diabetes, histological type, grade and FIGO stage of disease using Euclidean distance and Ward linkage of the 20 most commonly identified genera. Data were rarefied prior to analysis. **B.** Three microbial genera clusters are observed: *Lactobacillus*-dominant, *Gardnerella/Lactobacillus*-dominant and High diversity & Other (*p* 0.003821). Women with benign conditions are predominantly *Lactobacillus*-dominant, while women with endometrial cancer exhibit mostly high bacterial diversity and *Lactobacillus* depletion. *p* value was calculated by chi-squared test. *BMI: Body mass index*.

A



B

Endometrium	Lactobacillus dominant n/N (%)	Gardnerella/Lactobacillus dominant n/N (%)	High diversity & Other n/N (%)	Total n/N (%)
Benign	18/44 (41)	9/44 (20.6)	17/44 (38.6)	44/44 (100.2)
Endometrial cancer	5/49 (10.2)	1/49 (2)	43/49 (87.8)	49/49 (100)
Total	23/93 (24.7)	10/93 (10.8)	60/93 (64.5)	93/93 (100)
P value				< 0.00001

Figure 5.4. Endometrial microbiome composition at genus level according to presence or absence of endometrial malignancy. A. Hierarchical clustering analysis per endometrial malignancy, ethnicity, age, BMI status, menopausal status, diabetes, histological type, grade and FIGO stage of disease using Euclidean distance and Ward linkage of the 15 most commonly identified genera. Data were rarefied prior to analysis. **B.** Three microbial genera clusters are observed: *Lactobacillus*-dominant, *Gardnerella/Lactobacillus*-dominant and High diversity & Other (p <0.00001). Women with benign conditions are predominantly *Lactobacillus*-dominant, while women with endometrial cancer exhibit mostly high bacterial diversity and *Lactobacillus* depletion. p value was calculated by chi-squared test. *BMI: Body mass index*; *Lap: Laparoscopically; Abdo: Abdominally.*

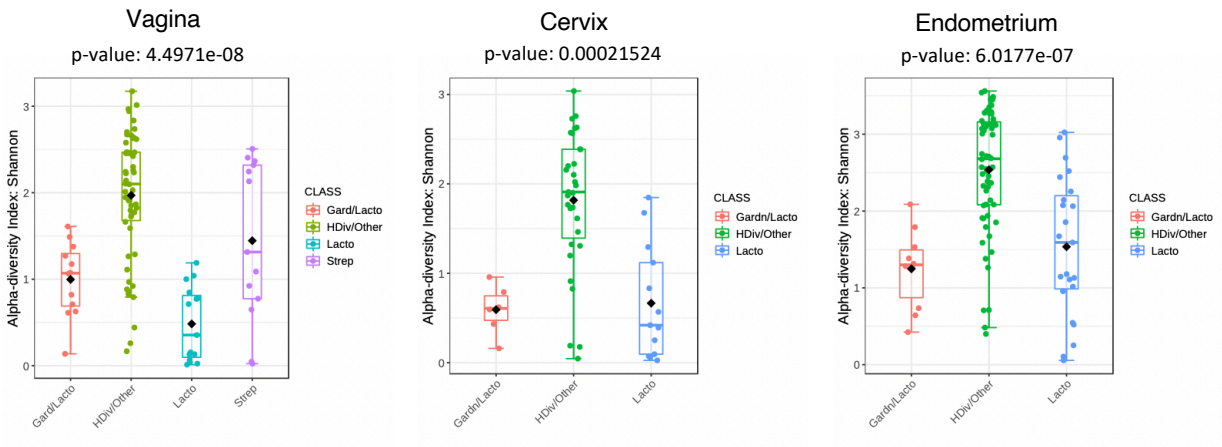


Figure 5.5. Shannon α-diversity among microbial clusters identified in different anatomical sites (species). Lacto: *Lactobacillus*; Gardn/Lacto: *Gardnerella/Lactobacillus*; Strep: *Streptococcus*; HDiv/Other: High diversity & Other.

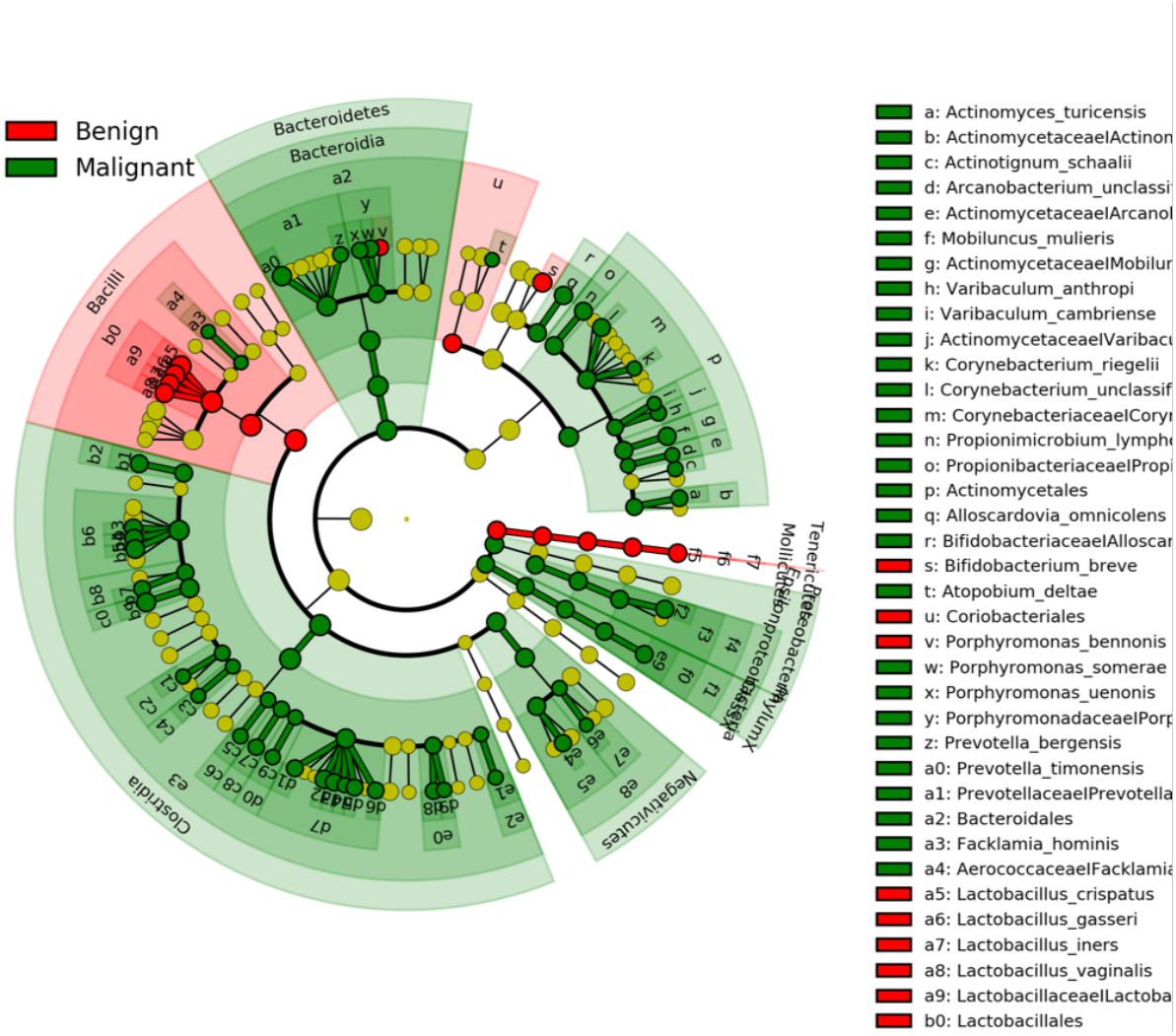
At species level, *Lactobacillus crispatus* accounted for the majority of *Lactobacillus* abundance at all sites (vagina, cervix, endometrium), while *Gardnerella vaginalis* was the second most abundant bacterial species across all samples. Interestingly, *L. crispatus* and *G. vaginalis* were observed to not co-occur in patients suggesting they are mutually exclusive microbes. In contrast, *G. vaginalis* was observed to co-occur with other *Lactobacillus* species, like *L. gasseri* and *L. iners*.

5.3.4 Identification of a microbial signature in the upper and lower genital tract in endometrial cancer

Linear discriminant analysis (LDA) effect size (LEfSe) modelling was used to identify differences in microbiota composition of endometrial cancer patients versus benign controls (Figure 5.6). In benign patients, an over-representation of *Lactobacillus* species, *L. crispatus*, *L. gasseri*, *L. iners* and *L. vaginalis*, was observed at all sites examined (vagina, cervix, endometrium) and *Bifidobacterium breve* in vagina and cervix. Conversely, in endometrial cancer patients, an enrichment of several microbes was observed at all sites, including *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus* species.

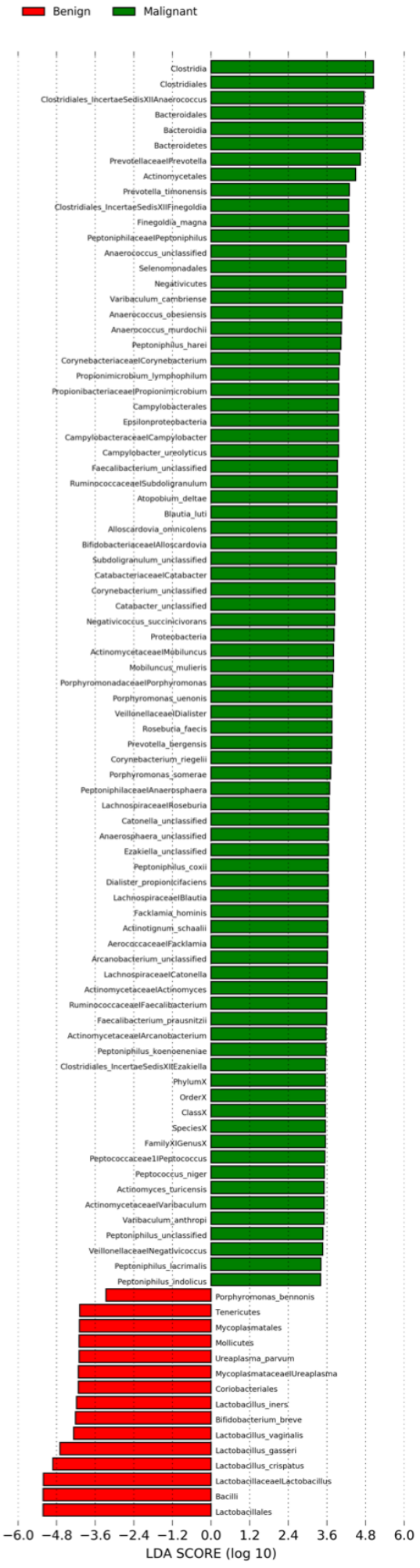
A

Vagina


Benign
Malignant
Bacteroidetes
Bacteroidia
Bacilli
Clostridia
Negativicutes
a: Actinomyces_turicensis
b: ActinomycetaceaeIActinon
c: Actinotignum_schaalii
d: Arcanobacterium_unclassi
e: ActinomycetaceaeIArcanol
f: Mobiluncus_mulieris
g: ActinomycetaceaeIMobilur
h: Varibaculum_anthropi
i: Varibaculum_cambriense
j: ActinomycetaceaeIVaribacu
k: Corynebacterium_riegelii
l: Corynebacterium_unclassif
m: CorynebacteriaceaeICoryn
n: Propionimicrobium_lympho
o: PropionibacteriaceaeIPropi
p: Actinomycetales
q: Alloscardovia_omnicolens
r: BifidobacteriaceaeIAlloscar
s: Bifidobacterium_breve
t: Atopobium_deltae
u: Coriobacteriales
v: Porphyromonas_bennonis
w: Porphyromonas_somerae
x: Porphyromonas_uenonis
y: PorphyromonadaceaeIPorp
z: Prevotella_bergensis
a0: Prevotella_timonensis
a1: PrevotellaceaeIPrevotella
a2: Bacteroidales
a3: Facklamia_hominis
a4: AerococcaceaeIFacklamia
a5: Lactobacillus_crispatus
a6: Lactobacillus_gasseri
a7: Lactobacillus_iners
a8: Lactobacillus_vaginalis
a9: LactobacillaceaeILactoba
b0: Lactobacillales

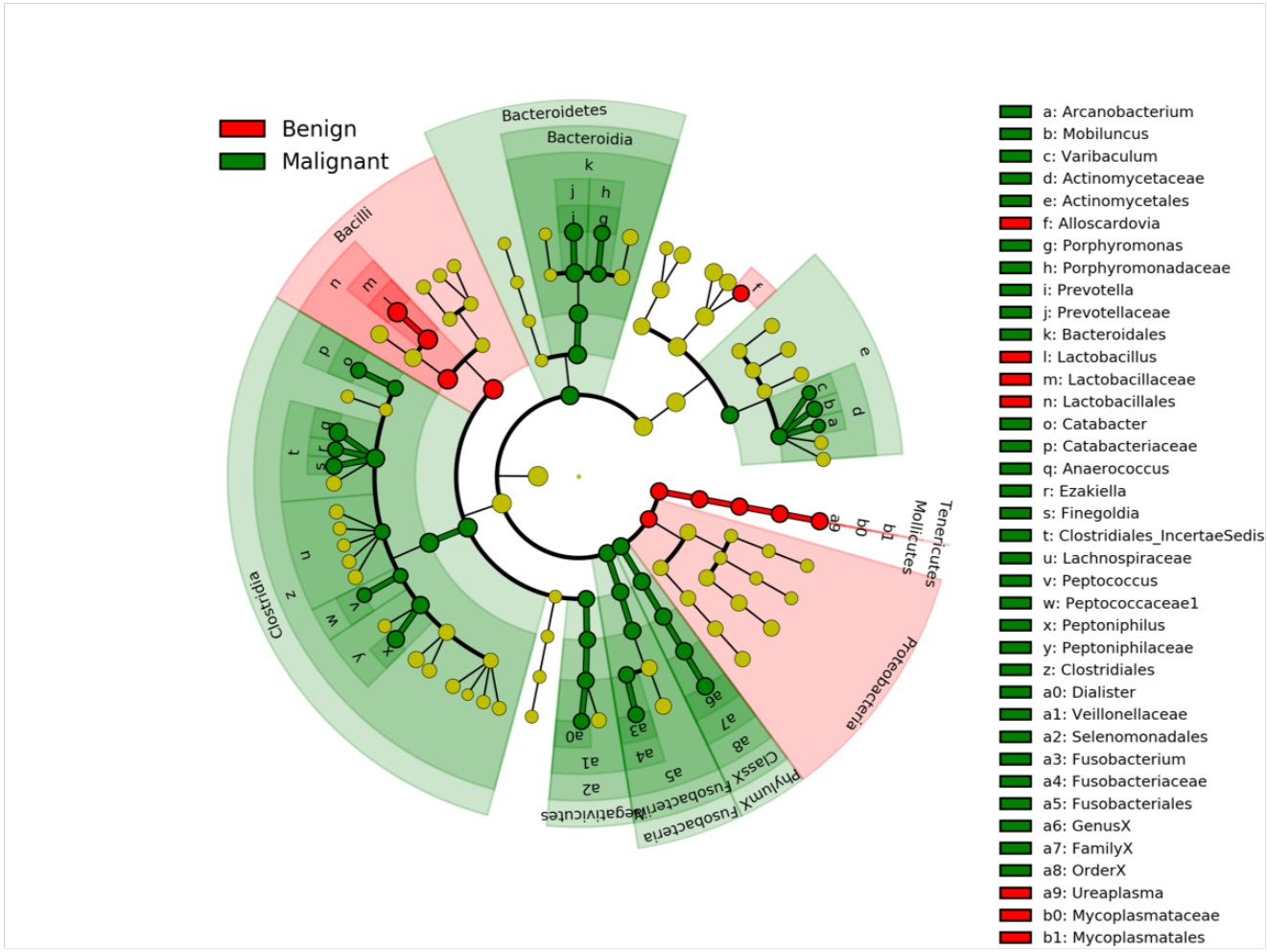
B

Vagina

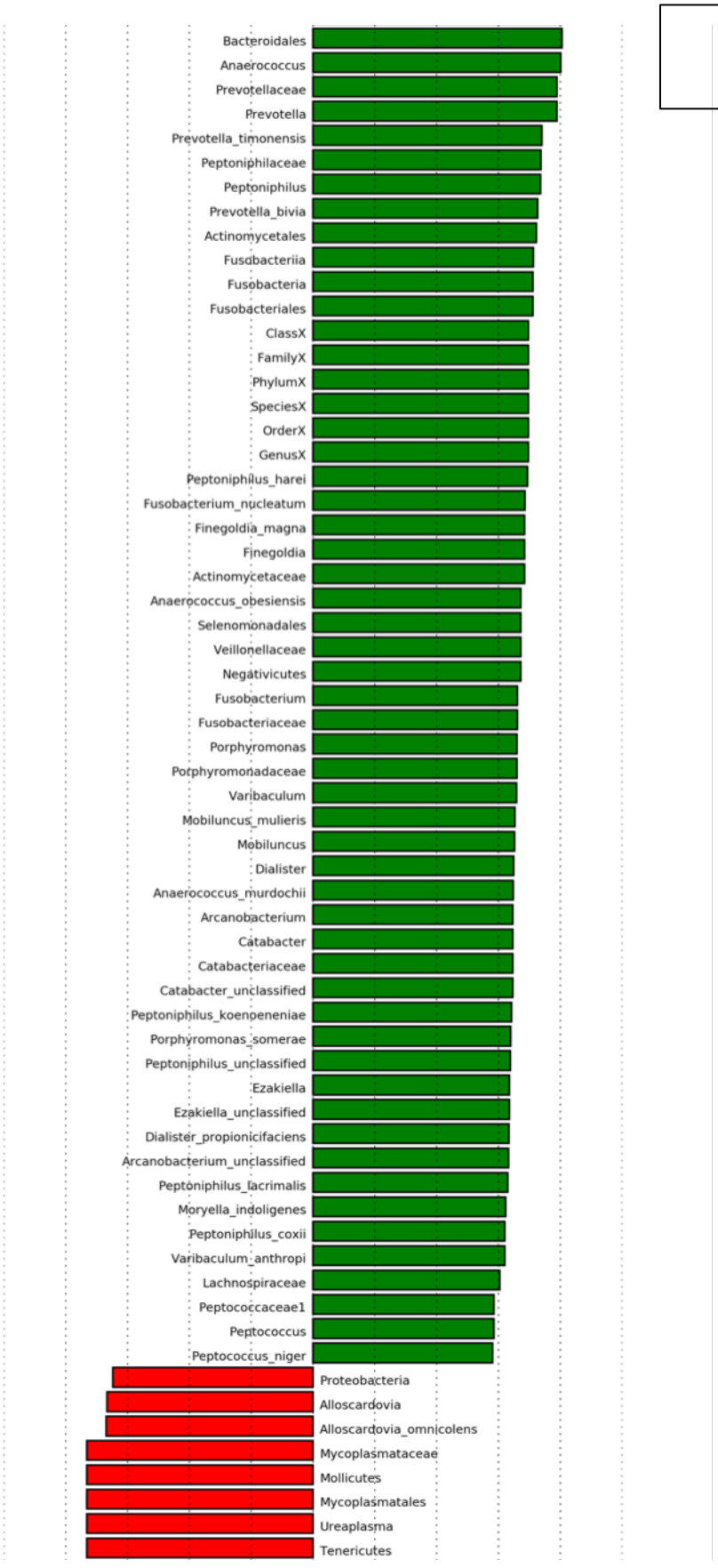

Benign
Malignant
Clostridia
Clostridiales
Clostridiales_IncertaeSedisXIIAnaerococcus
Bacteroidales
Bacteroidia
Bacteroidetes
PrevotellaceaeIPrevotella
Actinomycetales
Prevotella_timonensis
Clostridiales_IncertaeSedisXIIFinegoldia
Finegoldia_magna
PeptoniphilaceaeIPeptoniphilus
Anaerococcus_unclassified
Selenomonadales
Negativicutes
Varibaculum_cambriense
Anaerococcus_obesiensis
Anaerococcus_murdochii
Peptoniphilus_harei
CorynebacteriaceaeICorynebacterium
Propionimicrobium_lymphophilum
PropionibacteriaceaeIPropionimicrobium
Campylobacterales
Epsilonproteobacteria
CampylobacteraceaeICampylobacter
Campylobacter_ureolyticus
Faecalibacterium_unclassified
RuminococcaceaeISubdoligranulum
Atopobium_deltae
Blautia_luti
Alloscardovia_omnicolens
BifidobacteriaceaeIAlloscardovia
Subdoligranulum_unclassified
CatabacteriaceaeICatabacter
Corynebacterium_unclassified
Catabacter_unclassified
Negativicoccus_succinicivorans
Proteobacteria
ActinomycetaceaeIMobiluncus
Mobiluncus_mulieris
PorphyromonadaceaeIPorphyromonas
Porphyromonas_uenonis
VeillonellaceaeIDialister
Roseburia_faecis
Prevotella_bergensis
Corynebacterium_riegelii
Porphyromonas_somerae
PeptoniphilaceaeIAnaerosphaera
LachnospiraceaeIRoseburia
Catonella_unclassified
Anaerosphaera_unclassified
Ezakiella_unclassified
Peptoniphilus_coxii
Dialister_propionicifaciens
LachnospiraceaeIBlautia
Facklamia_hominis
Actinotignum_schaalii
AerococcaceaeIFacklamia
Arcanobacterium_unclassified
LachnospiraceaeICatonella
ActinomycetaceaeIActinomyces
RuminococcaceaeIFaecalibacterium
Faecalibacterium_prausnitzii
ActinomycetaceaeIArcanobacterium
Peptoniphilus_koenoeneniae
Clostridiales_IncertaeSedisXIIEzakiella
PhylumX
OrderX
ClassX
SpeciesX
FamilyXIGenusX
Peptococcaceae1IPeptococcus
Peptococcus_niger
Actinomyces_turicensis
ActinomycetaceaeIVaribaculum
Varibaculum_anthropi
Peptoniphilus_unclassified
VeillonellaceaeINegativicoccus
Peptoniphilus_lacrimalis
Peptoniphilus_indolicus
Porphyromonas_bennonis
Tenericutes
Mycoplasmatales
Mollicutes
Ureaplasma_parvum
MycoplasmataceaeIUreaplasma
Coriobacteriales
Lactobacillus_iners
Bifidobacterium_breve
Lactobacillus_vaginalis
Lactobacillus_gasseri
Lactobacillus_crispatus
LactobacillaceaeILactobacillus
Bacilli
Lactobacillales
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

A

Cervix

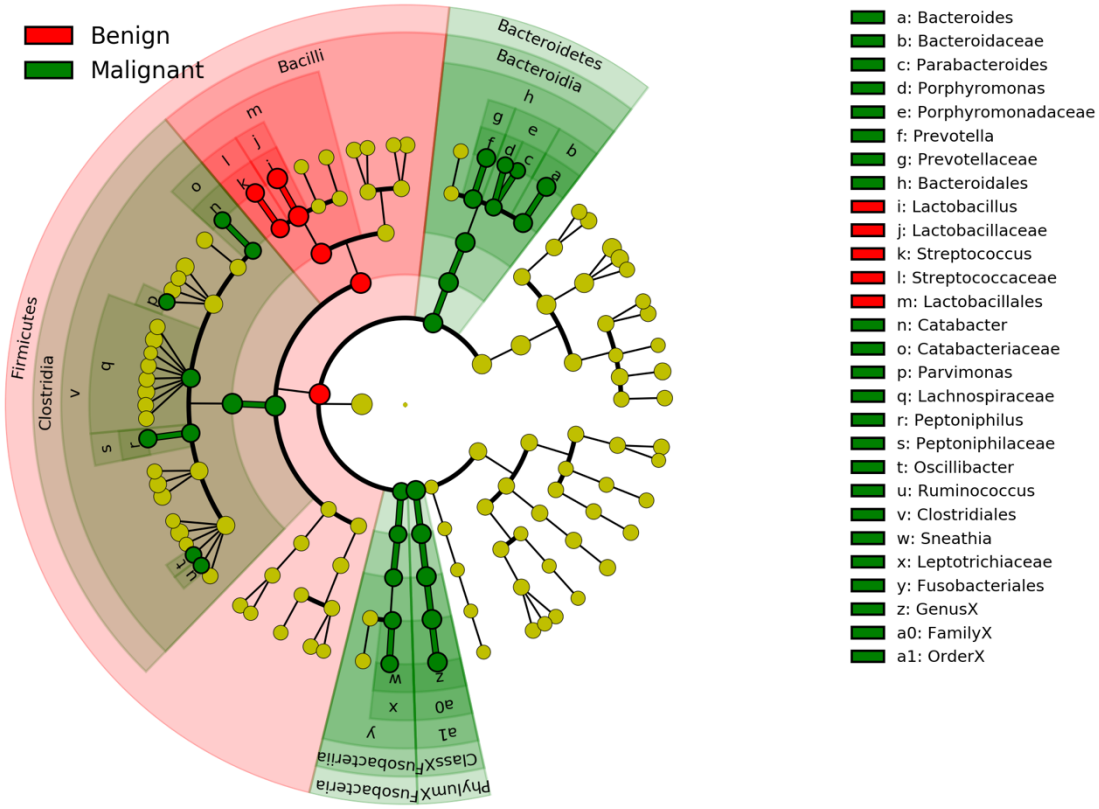

Benign
Malignant
Bacteroidetes
Bacteroidia
Bacilli
Clostridia
Tenericutes
Mollicutes
Proteobacteria
Phylum X Fusobacteria
Class X Fusobacteria
Negativicutes
a: Arcanobacterium
b: Mobiluncus
c: Varibaculum
d: Actinomycetaceae
e: Actinomycetales
f: Alloscardovia
g: Porphyromonas
h: Porphyromonadaceae
i: Prevotella
j: Prevotellaceae
k: Bacteroidales
l: Lactobacillus
m: Lactobacillaceae
n: Lactobacillales
o: Catabacter
p: Catabacteriaceae
q: Anaerococcus
r: Ezakiella
s: Finegoldia
t: Clostridiales_IncertaeSedis
u: Lachnospiraceae
v: Peptococcus
w: Peptococcaceae1
x: Peptoniphilus
y: Peptoniphilaceae
z: Clostridiales
a0: Dialister
a1: Veillonellaceae
a2: Selenomonadales
a3: Fusobacterium
a4: Fusobacteriaceae
a5: Fusobacteriales
a6: GenusX
a7: FamilyX
a8: OrderX
a9: Ureaplasma
b0: Mycoplasmataceae
b1: Mycoplasmatales

B


Cervix
Bacteroidales
Anaerococcus
Prevotellaceae
Prevotella
Prevotella_timonensis
Peptoniphilaceae
Peptoniphilus
Prevotella_bivia
Actinomycetales
Fusobacteriia
Fusobacteria
Fusobacteriales
ClassX
FamilyX
PhylumX
SpeciesX
OrderX
GenusX
Peptoniphilus_harei
Fusobacterium_nucleatum
Finegoldia_magna
Finegoldia
Actinomycetaceae
Anaerococcus_obesiensis
Selenomonadales
Veillonellaceae
Negativicutes
Fusobacterium
Fusobacteriaceae
Porphyromonas
Porphyromonadaceae
Varibaculum
Mobiluncus_mulieris
Mobiluncus
Dialister
Anaerococcus_murdochii
Arcanobacterium
Catabacter
Catabacteriaceae
Catabacter_unclassified
Peptoniphilus_koenoeneniae
Porphyromonas_somerae
Peptoniphilus_unclassified
Ezakiella
Ezakiella_unclassified
Dialister_propionicifaciens
Arcanobacterium_unclassified
Peptoniphilus_lacrimalis
Moryella_indoligenes
Peptoniphilus_coxii
Varibaculum_anthropi
Lachnospiraceae
Peptococcaceae1
Peptococcus
Peptococcus_niger
Proteobacteria
Alloscardovia
Alloscardovia_omnicolens
Mycoplasmataceae
Mollicutes
Mycoplasmatales
Ureaplasma
Tenericutes

A

Endometrium



B

Endometrium

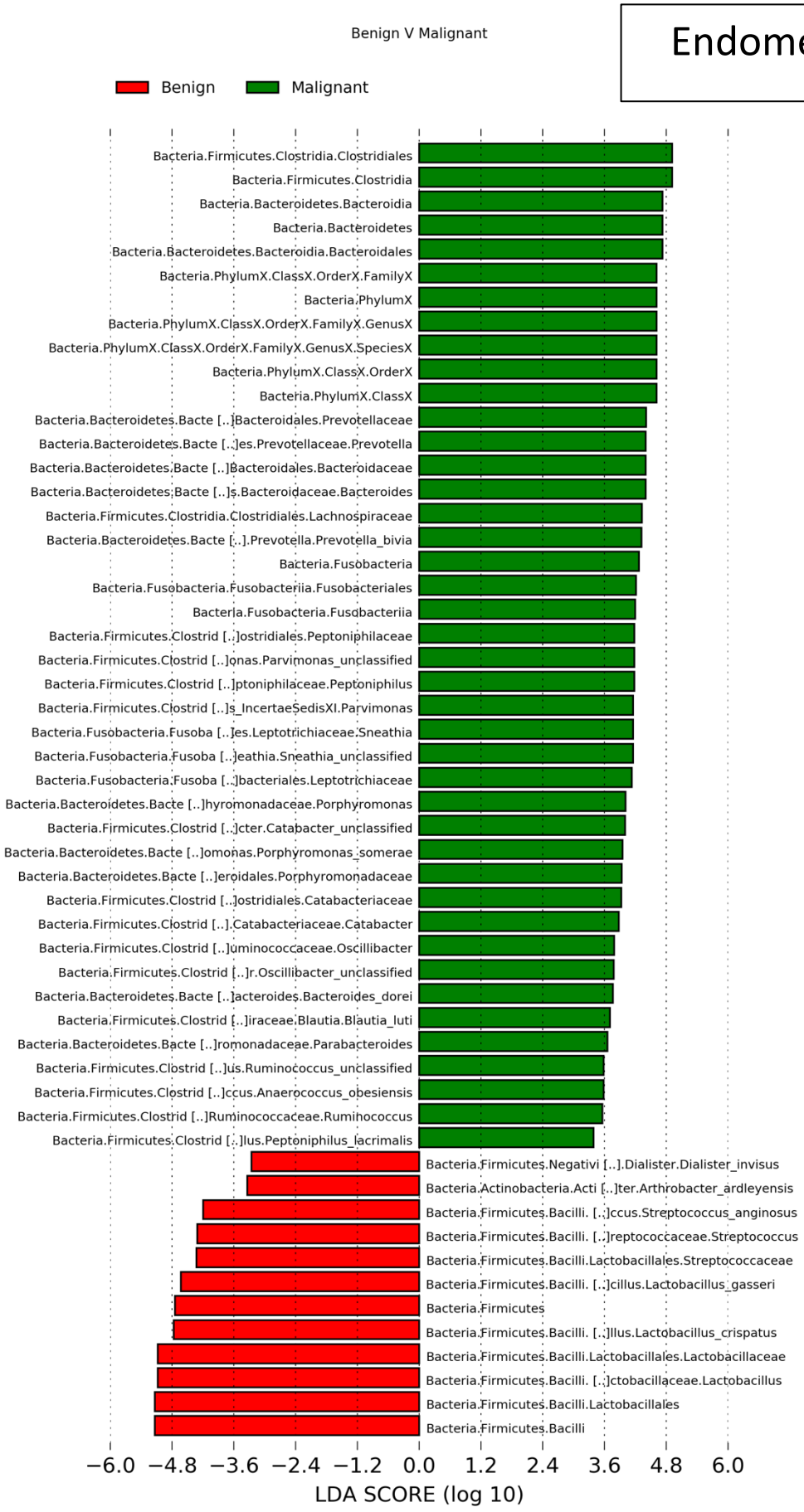


Figure 5.6. Significantly enriched taxa among patients with and without endometrial cancer in the upper and lower female genital tract. A. Cladograms representing taxa with different abundances between the comparison groups per site. **B.** Histograms of the LDA scores computed for features differentially abundant between endometrial cancer and benign patients. *LDA score: Linear discriminant analysis score.*

5.3.5 The rectum of endometrial cancer patients is depleted in site-specific commensals compared to benign controls

Analysis of rectal microbiota in 27 patients with and 18 patients without endometrial cancer was limited to phyla level due to high species diversity. *Firmicutes* (p= 0.002), *Bacteroidetes* (p= 0.002), *Actinobacteria* (p= 0.002) and *Proteobacteria* (p= 0.007) were found to be significantly depleted in endometrial cancer patients compared to controls and this association withstood FDR (False Discovery Rate) correction that accounts for false positive features (Figure 5.7).

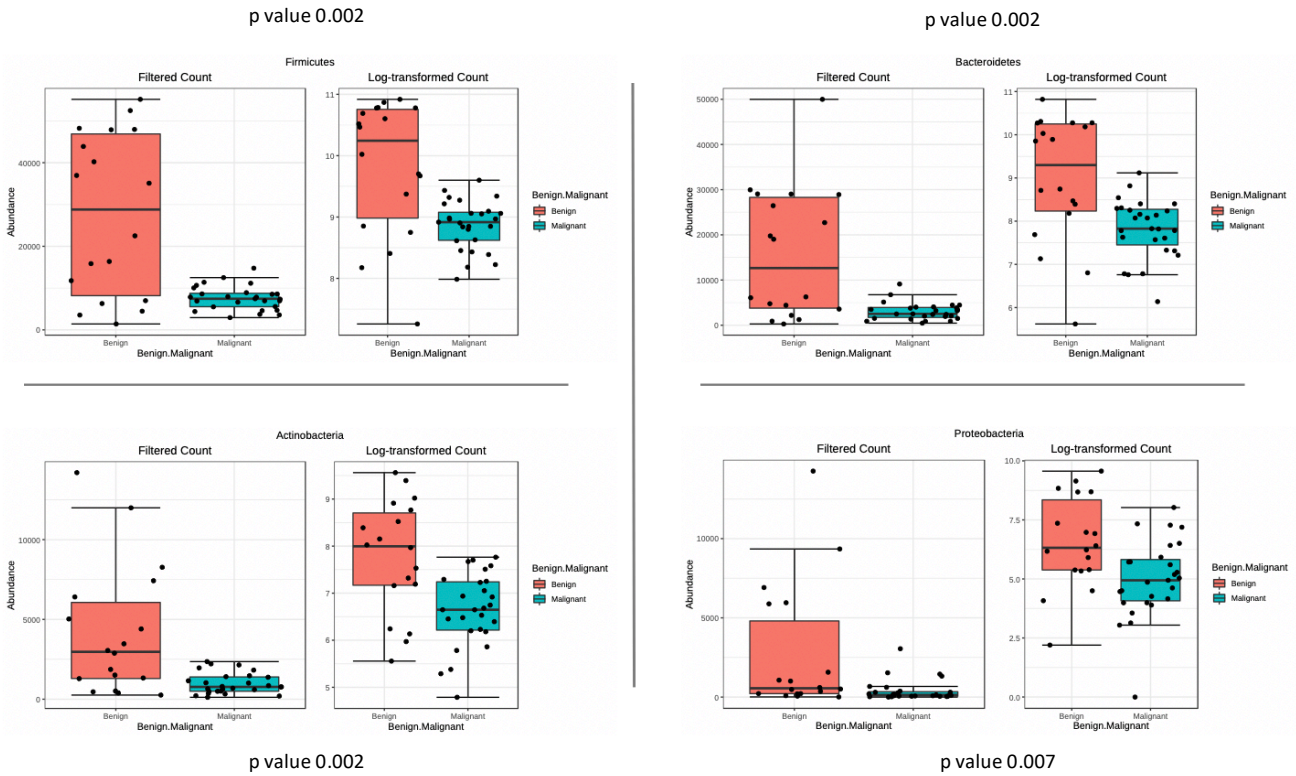


Figure 5.7. Comparison of microbial composition in the rectum of women with and without endometrial cancer at phylum level. The rectum of women with endometrial cancer displays

decreased colonisation with *Firmicutes* (p= 0.002), *Bacteroidetes* (p= 0.002), *Actinobacteria* (p= 0.002) and *Proteobacteria* (p= 0.007) compared to benign controls.

5.3.6 Lower and upper genital tract α- and β-diversity in endometrial cancer patients is significantly increased compared to benign controls

I next examined the α- (within samples) and β- (between samples) diversity in the microbial community composition among patients with and without endometrial cancer (Figure 5.8). Patients with endometrial cancer displayed higher Shannon α-diversity than patients without endometrial cancer both at genera and species level (vagina p= 7.5763e-05 and 2.6118e-06; cervix p= 0.006 and 0.003; endometrium p= 0.006 and 0.005 respectively). Similarly, β-diversity using the Bray-Curtis index and PERMANOVA statistical test was significantly different between benign and endometrial cancer patients in all anatomical sites examined at genera and species level (vagina p< 0.001; cervix p< 0.001 and <0.002 respectively; endometrium p< 0.001). On the other hand, α- and β-diversity were similar for rectum in benign and endometrial cancer patients (α-diversity: p= 0.455 genus and 0.235 species; β-diversity: p< 0.359 genus and < 0.306 species).

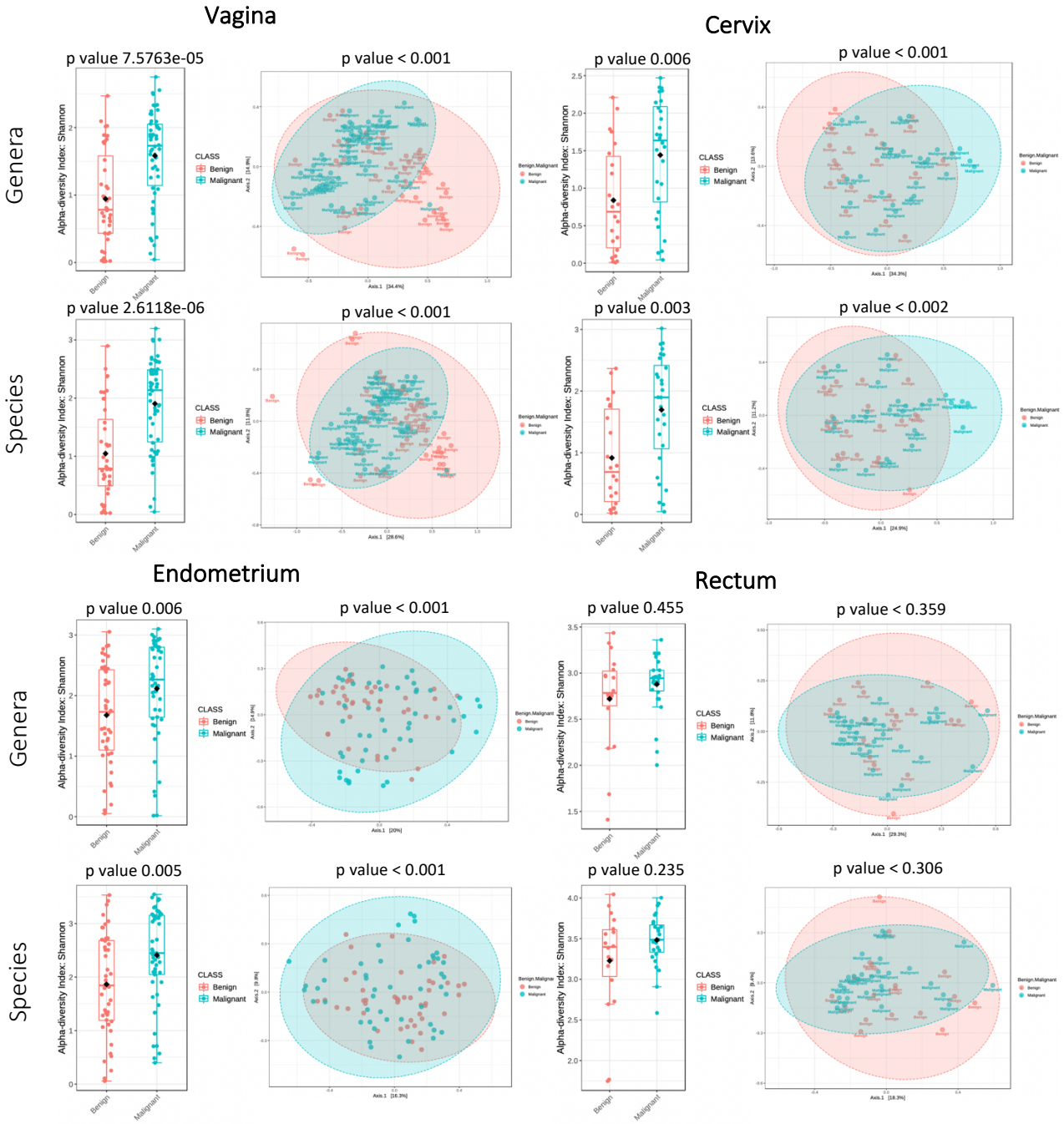


Figure 5.8. Comparison of microbiome Shannon α-diversity and β-diversity between benign and endometrial cancer patients per anatomical site at genera and species level. α- and β-diversity differed significantly between patients with and without endometrial cancer in the upper and lower female genital tract but not in the rectum. β-diversity was calculated using the Bray-Curtis index and PERMANOVA statistical test.

5.3.7 Different histotypes and grades of endometrial cancer are not associated with significant differences in microbial composition in the upper and lower genital tract

The next series of analyses were designed to address whether the histological types and grades of endometrial cancer are associated with different microbial fingerprints. A total of 30 endometrioid and 7 non-endometrioid (3 serous, 3 clear cell, 1 carcinosarcoma) tumours were included in the analysis, of which 12 were grade I, 11 grade II and 14 grade III. PCA plots were used to provide an overview of microbial similarity among different endometrial cancer histotypes and grades (Figure 5.9). With regards to histological type, some divergence of microbial signals was observed between endometrioid and non-endometrioid tumours only in the cervix, while the vagina and endometrium displayed extensive microbial overlap between the two histological types. Of note, microbial structure differed more between benign samples and non-endometrioid tumours in the vagina and cervix than benign and endometrioid endometrial cancer samples (Figure 5.9A). More specifically, a trend decrease in *L. crispatus* and *B. breve* levels was observed in non-endometrioid tumours but this did not reach statistical significance (Figure 5.10A), perhaps due to under-sampling of non-endometrioid tumours. The mean Shannon α-diversity index did not significantly differ between the two histological groups at genera level (p= 0.084 vagina; p= 0.365 cervix; p=0.936 endometrium), but β-diversity was different in the vagina and endometrium (p< 0.046 vagina; p< 0.069 cervix; p< 0.001 endometrium) (Figure 5.11A).

Examination of PCA plots coloured on the basis of grade revealed a slight degree of divergence between grade I and grade III cancers, while grade II tumours shared many microbial features with both grade I and grade III samples (Figure 5.9B). A trend depletion in relative abundance of *Lactobacillus* (*L. crispatus*) and *Bifidobacterium* (*B. breve*) and increase of *Anaerococcus* (*A. lactolyticus*) with increasing grade in the vagina, cervix and endometrium was observed but this did not reach statistical significance (Figure 5.10B). Shannon α-diversity and β-diversity were significantly

different in the vagina (p= 0.006 and p< 0.007 respectively) but not in the cervix (p= 0.368 and p< 0.191 respectively) among different grades at the genera level. In the vagina, grade I lesions displayed the lowest α-diversity, whereas this was similar between grade II and III tumours. In the endometrium, no differences between grades were noted for Shannon α-diversity (p= 0.23) but β-diversity was significantly different (p< 0.001) (Figure 5.11B). The discrepancies in diversities between sites might be attributable to the higher number of vaginal and endometrial than cervical samples included in the analysis, given that the vagina and endometrium were sampled both on their lower and higher part.

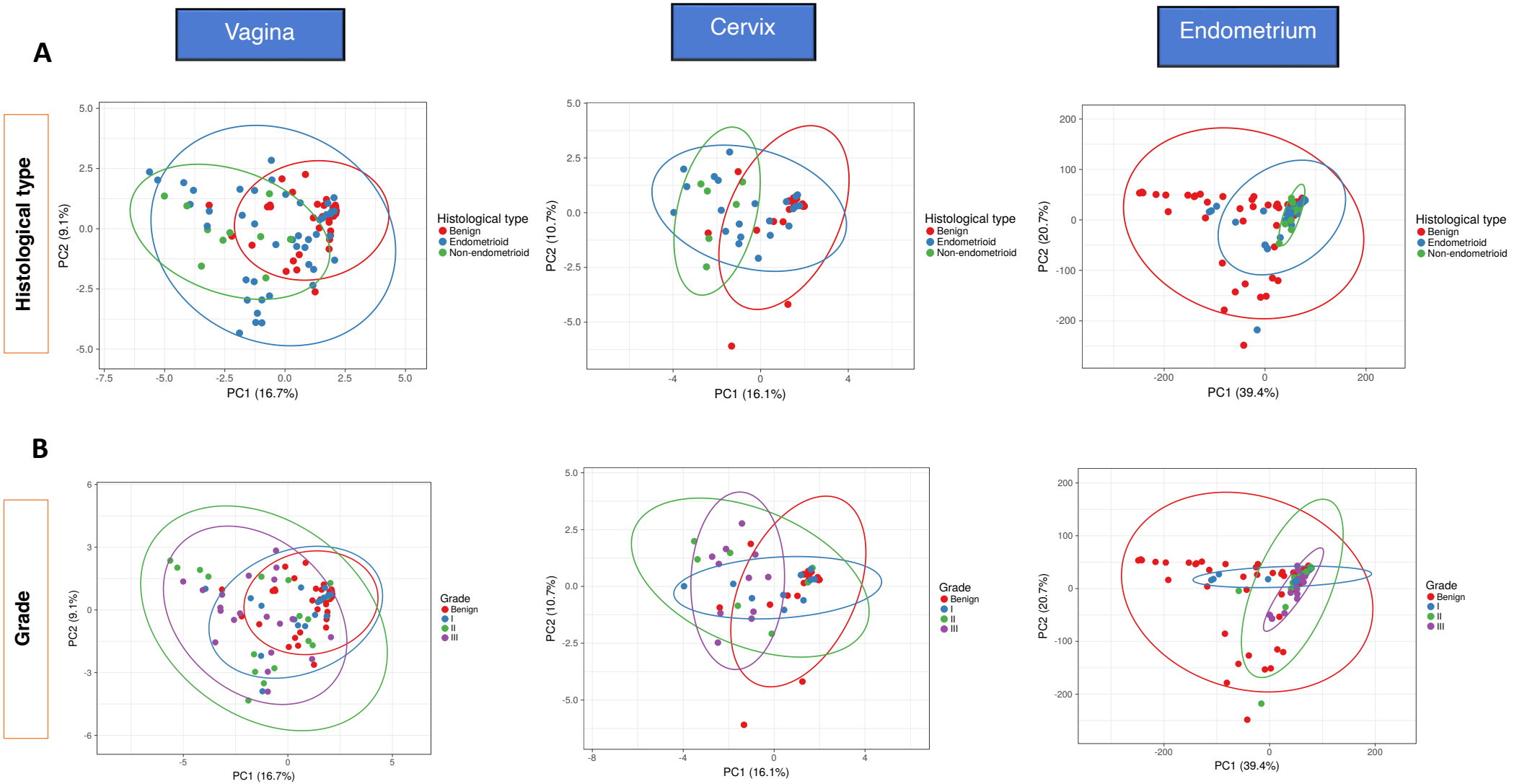


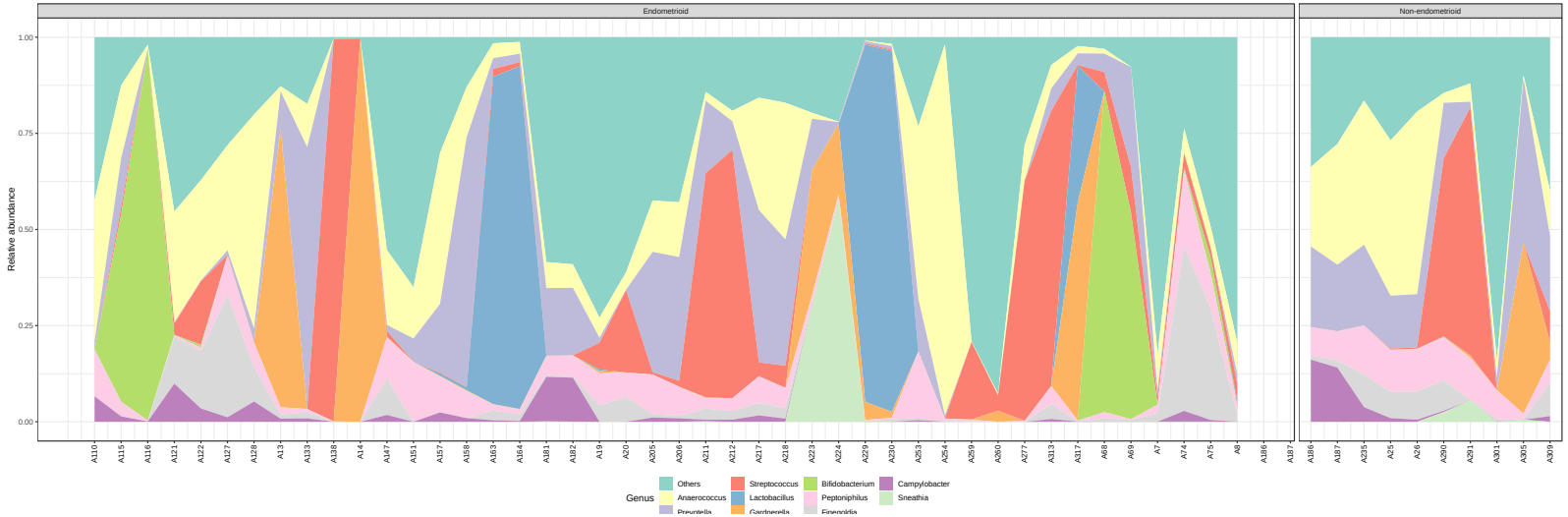
Figure 5.9. Principal component analysis of microbial composition in the lower and upper genital tract of women with endometrial cancer per **A.** histological type and **B.** grade.

A

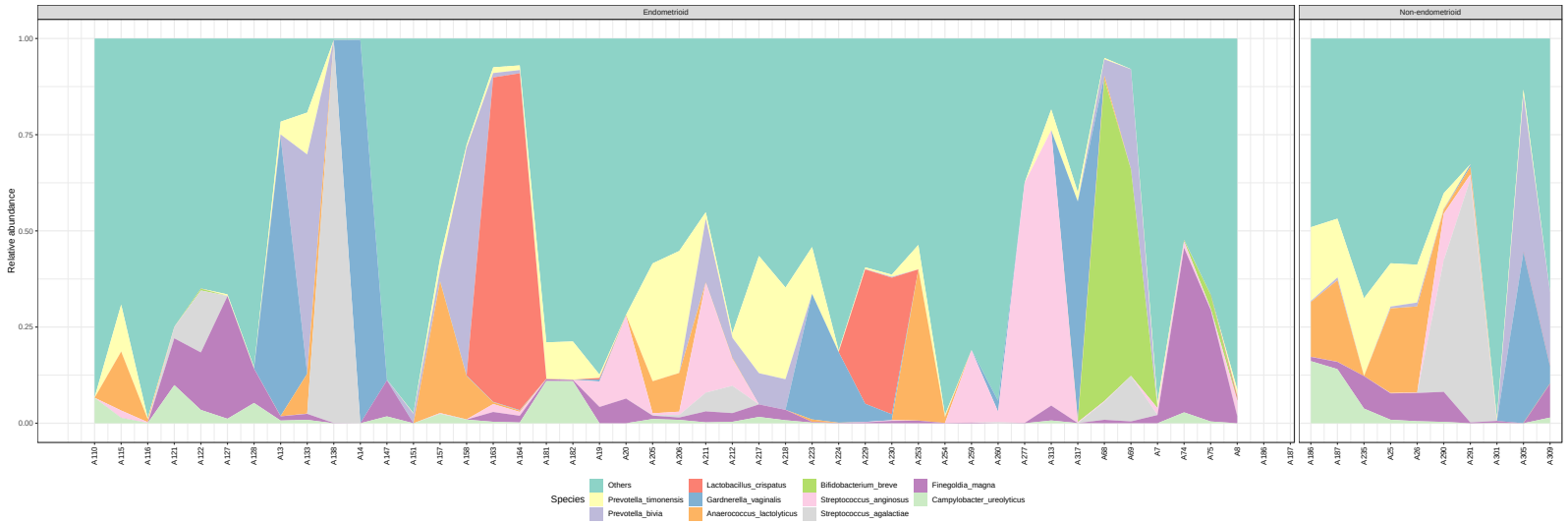
Vagina

Endometrioid | Non-endometrioid

Genera



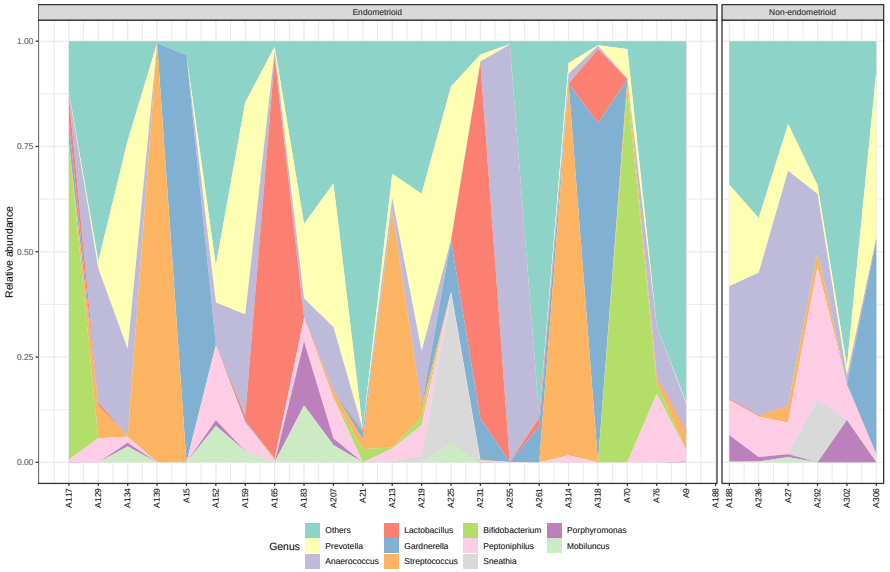
Species



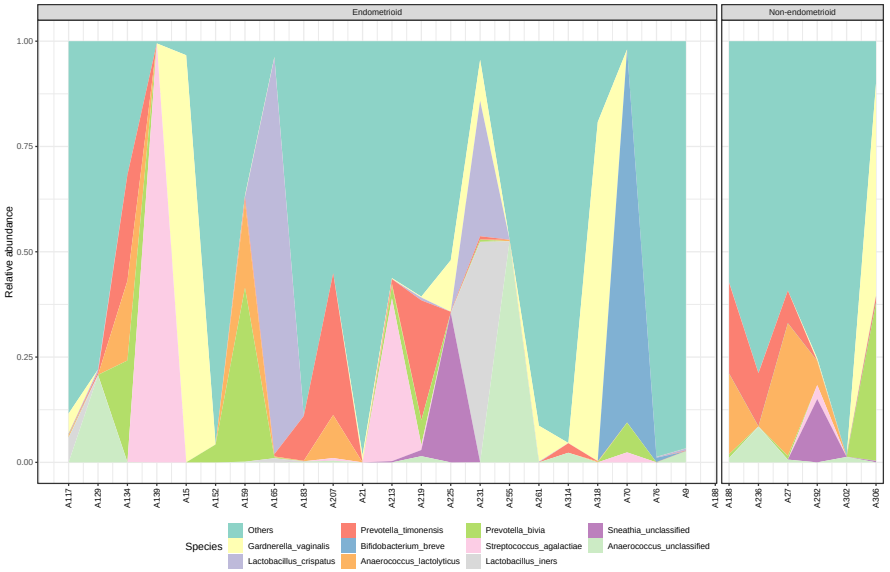
Cervix

Endometrioid | Non-endometrioid

Genera



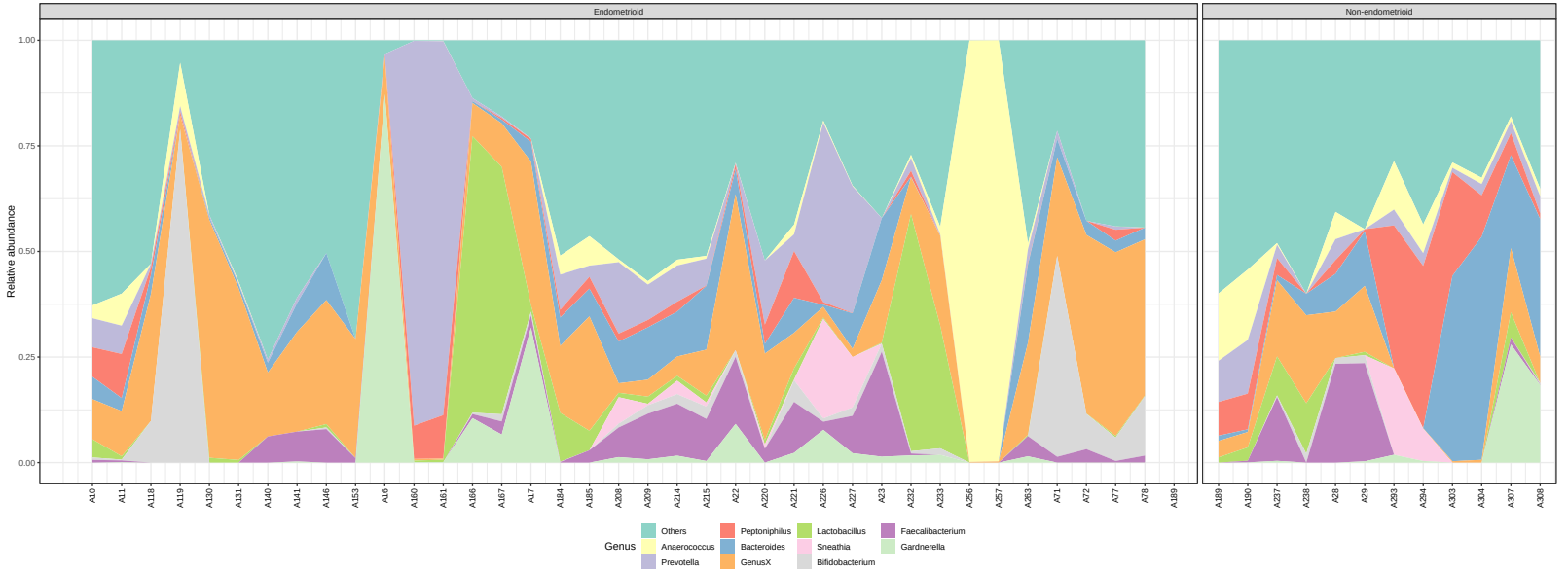
Species



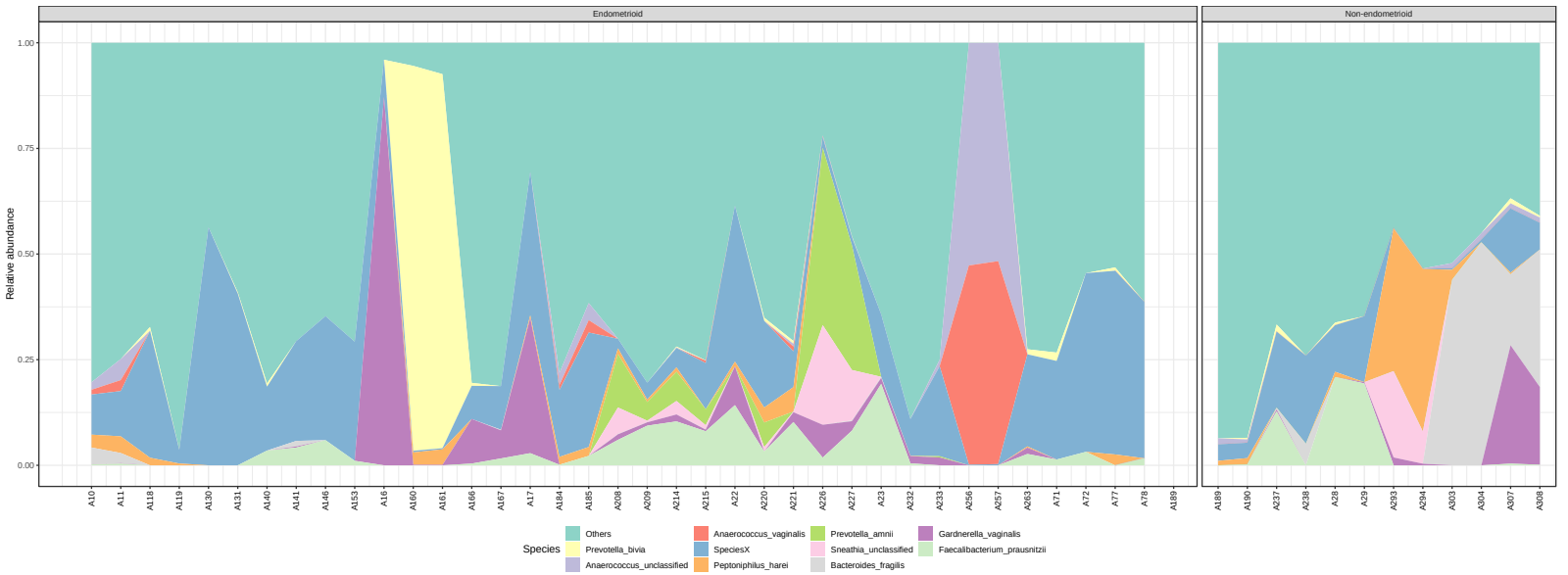
Endometrium

Endometrioid | Non-endometrioid

Genera

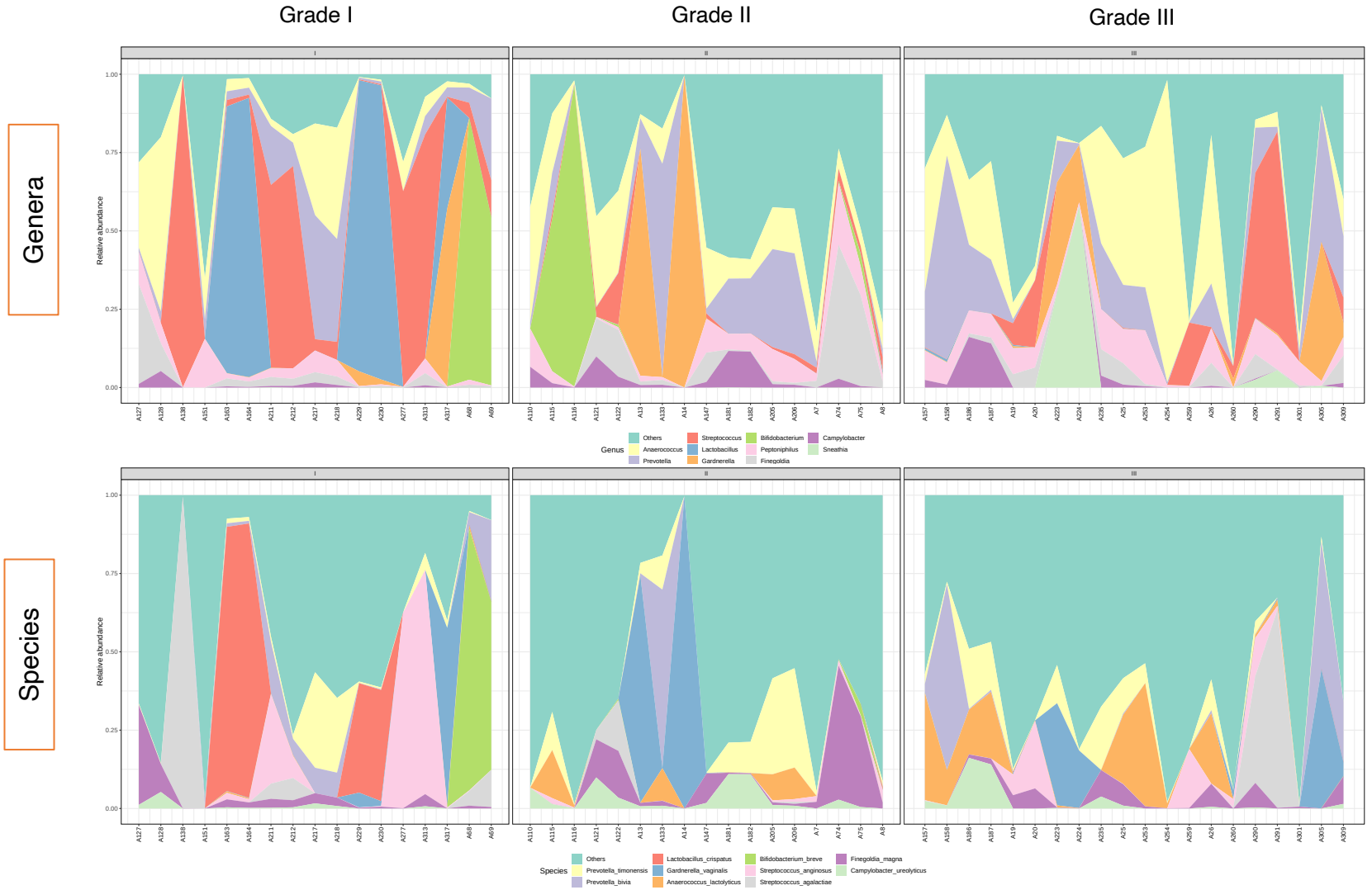


Species



B

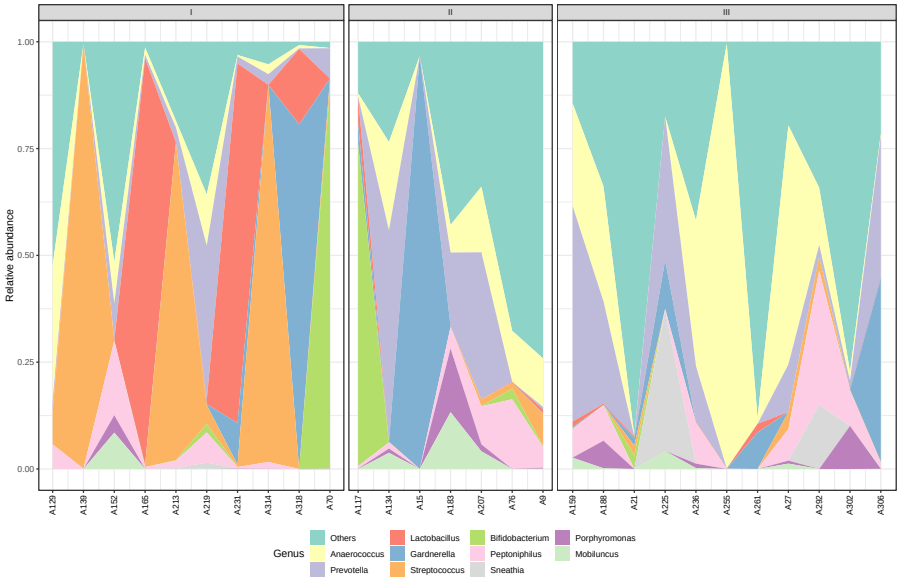
Vagina


Grade I
Grade II
Grade III
Genera
Species
Relative abundance
Genus
Others
Streptococcus
Bifidobacterium
Campylobacter
Anaerococcus
Lactobacillus
Peptoniphilus
Sneathia
Prevotella
Gardnerella
Finegoldia
Species
Others
Lactobacillus_crispatus
Bifidobacterium_breve
Finegoldia_magna
Prevotella_timonensis
Gardnerella_vaginalis
Streptococcus_anginosus
Campylobacter_ureolyticus
Prevotella_bivia
Anaerococcus_lactolyticus
Streptococcus_agalactiae

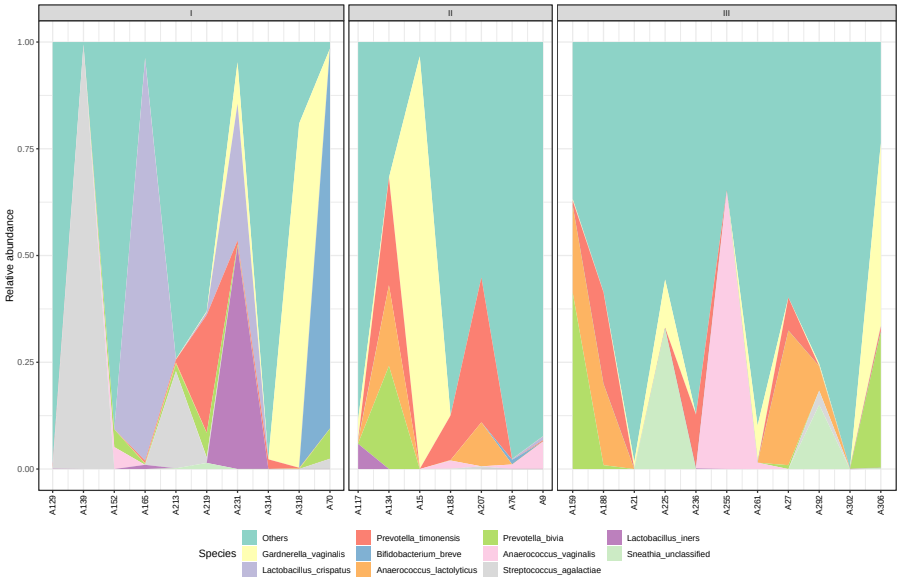
Cervix

Grade I | Grade II | Grade III

Genera



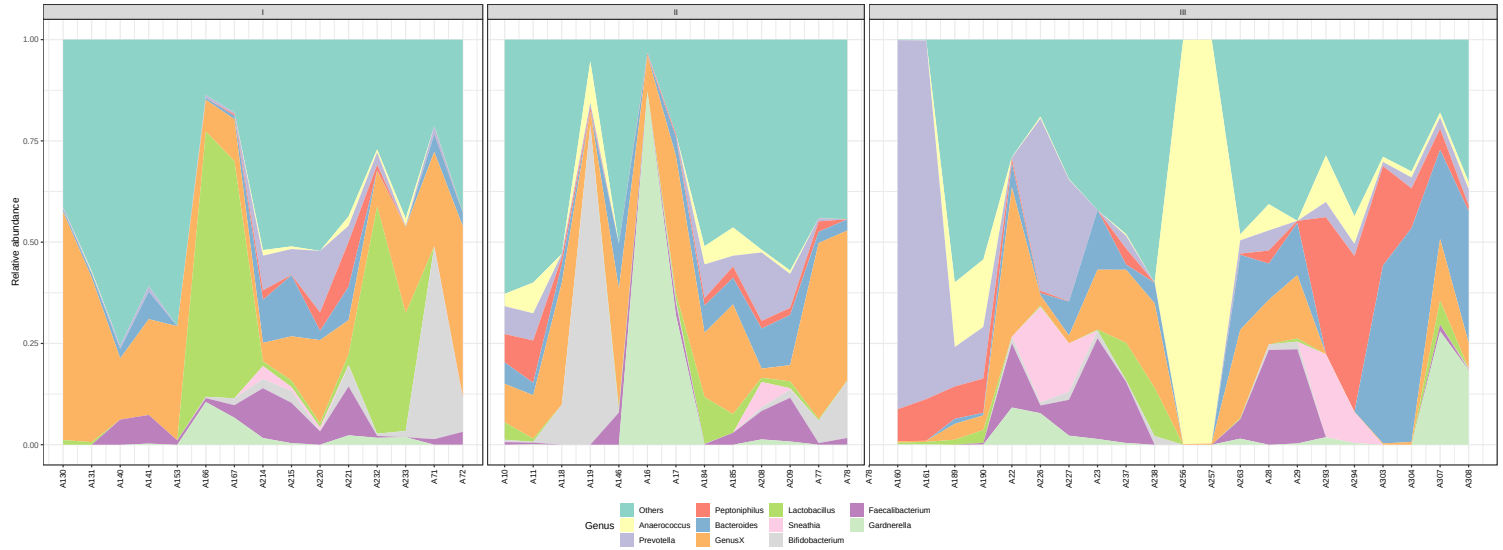
Species



Endometrium

Grade I | Grade II | Grade III

Genera



Species

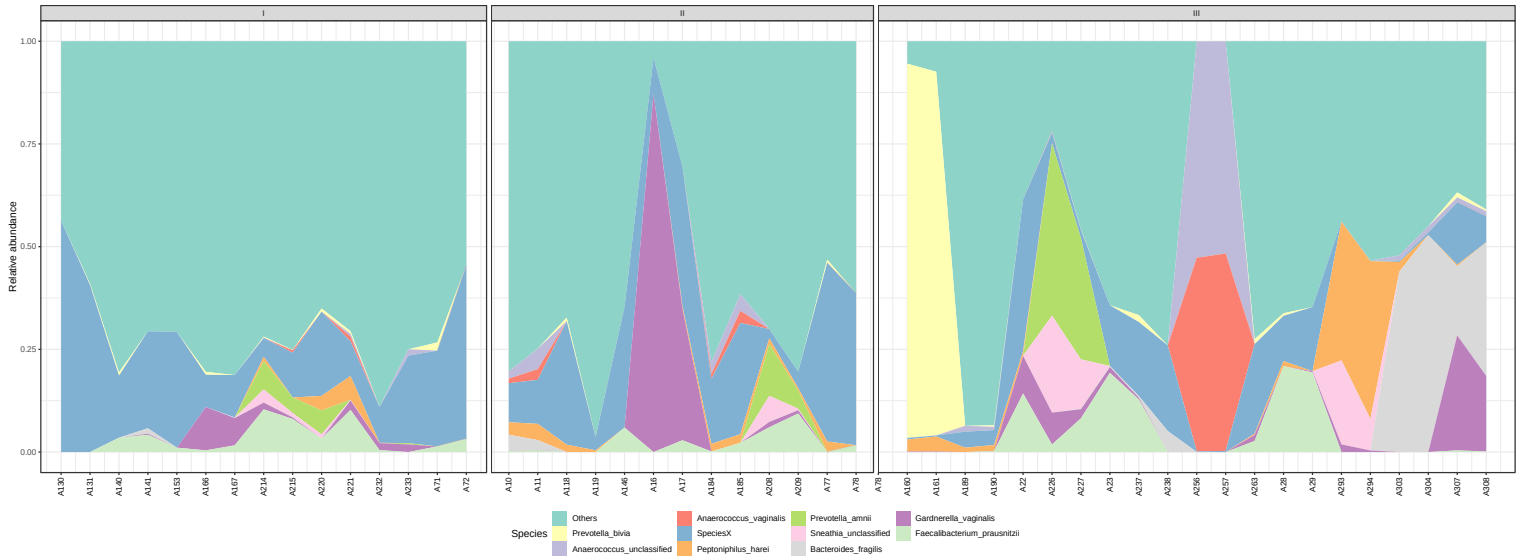
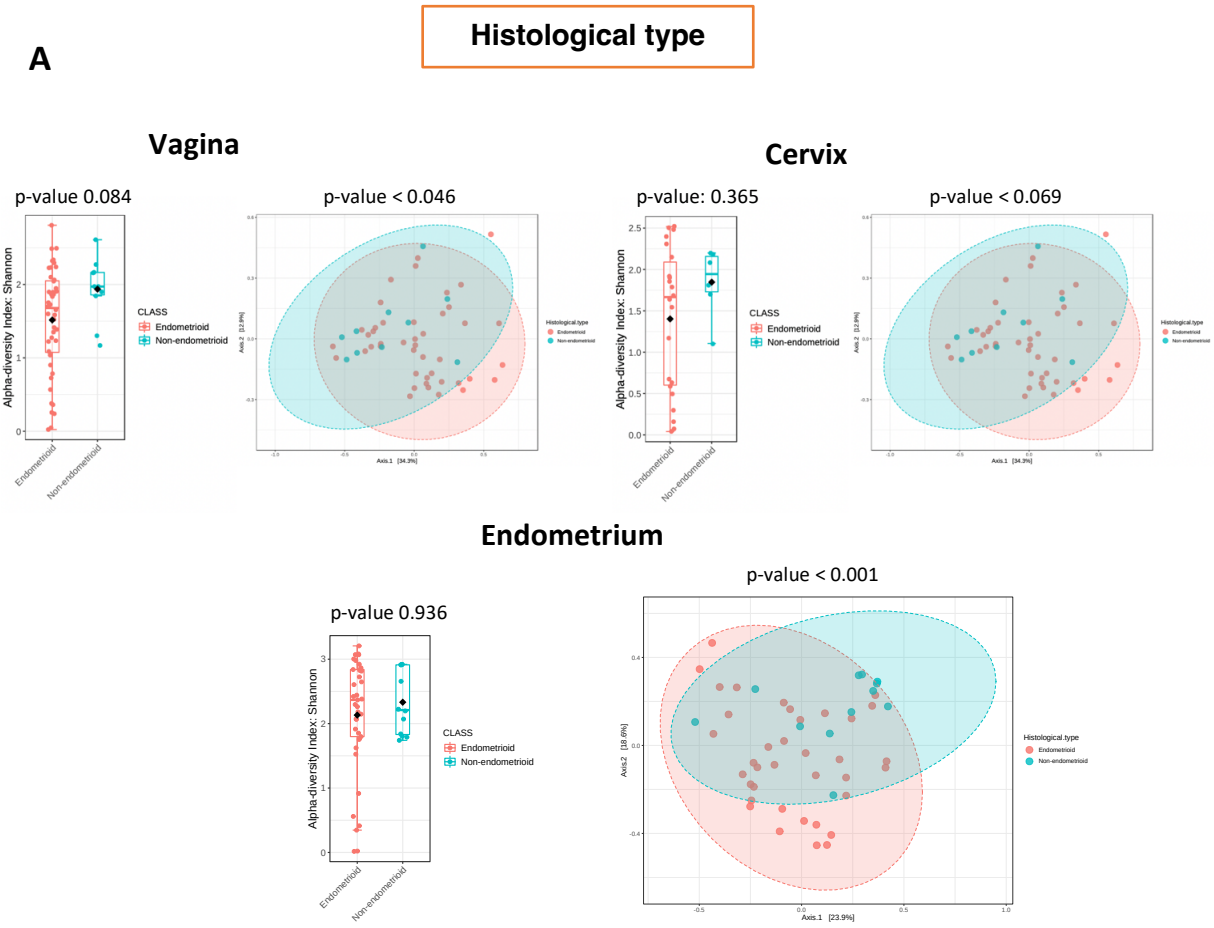


Figure 5.10. Microbial composition of endometrial cancer in the upper and lower female genital tract according to A. histotype and B. grade (genera & species). Stacked area plots showing bacterial abundances in endometrial cancer patients with endometrioid and non-endometrioid, grade I, II and III tumours.



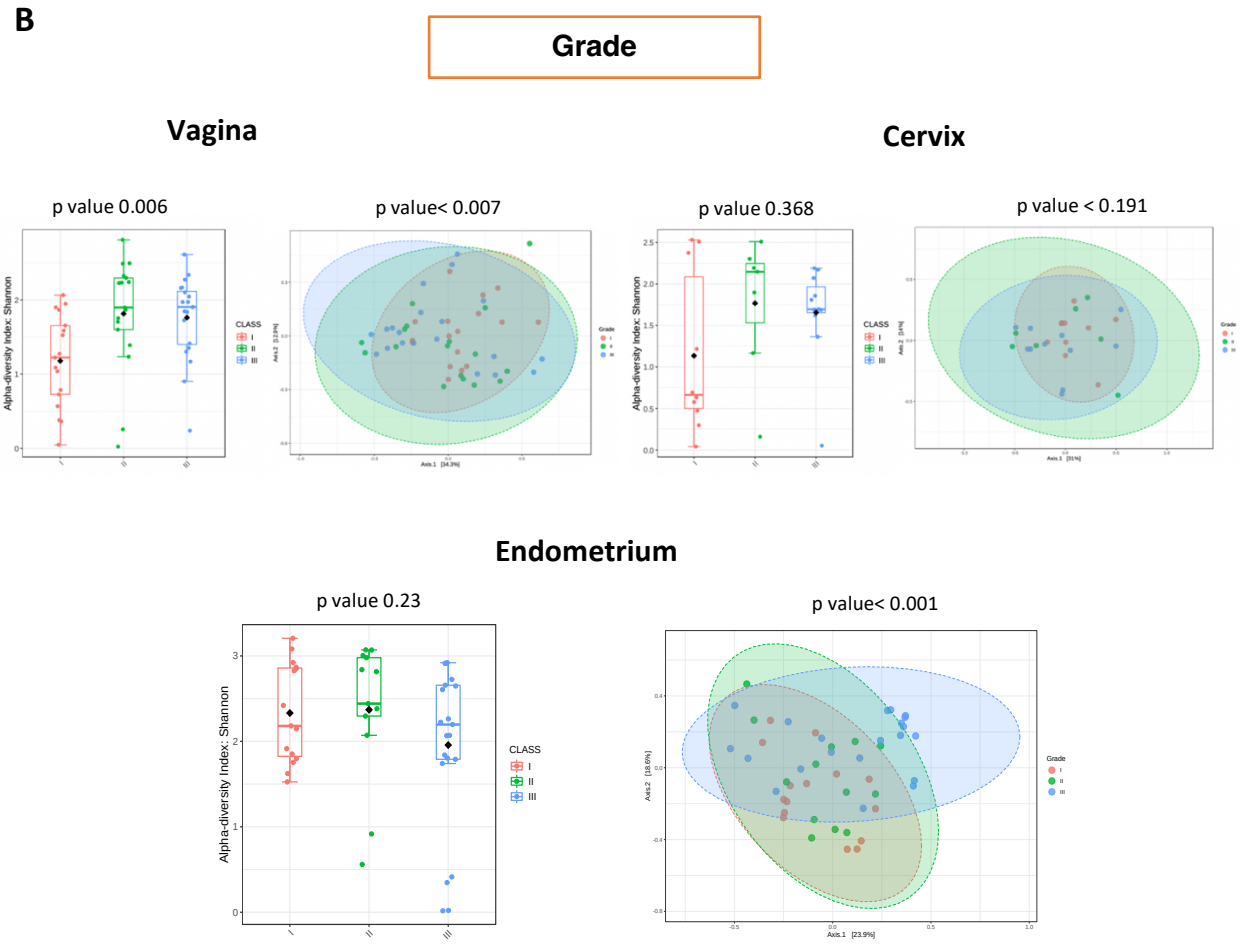


Figure 5.11. Microbiome Shannon α- and β-diversity according to histotype and grade of endometrial cancer per anatomical site (genera). **A.** Microbiome Shannon α-diversity and β-diversity in endometrioid and non-endometrioid endometrial cancer patients. No significant differences were observed in a-diversity at any site examined. β-diversity was different between the two histological types in the vagina and endometrium but not cervix. **B.** Microbiome Shannon α-diversity and β-diversity in different grades of endometrial cancer. α-diversity was lower for Grade I and similar for grades II and III in the vagina, while β-diversity differed significantly among different grades of disease in the vagina and endometrium. β-diversity was calculated using the Bray-Curtis index and PERMANOVA statistical test.

5.4 Discussion

This chapter describes the cross-sectional characterisation and comparison of the female genital tract microbiota of 37 women with and 24 women without endometrial cancer. To the best of my knowledge, this represents the largest such study today to investigate the endometrial cancer microbiome in both the lower and upper genital tract. An unequivocal decrease in *Lactobacillus* spp. relative abundance was observed in the vagina, cervix and endometrium of endometrial cancer patients, which is in accordance with a previous study [308]. This lends support to the more broadly described association between *Lactobacillus* depletion and adverse gynaecological and obstetric outcomes, including cervical cancer and precancerous lesions [310] as well as miscarriages [475], preterm labour [476] and *in vitro* fertilisation failure [144]. Our group has previously shown that cervical intraepithelial neoplasia (CIN) progression is associated with increased vaginal microbiome diversity and *Lactobacillus* depletion [131], which has been since confirmed by several studies and a 2020 systematic review and meta-analysis of longitudinal studies demonstrating a link between a diverse, non-*Lactobacillus* dominant vaginal microbiome and cervical oncogenesis [310]. This microbial signature favours HPV acquisition (overall RR 1.33; RR among young women 1.4; statistical heterogeneity (I^2) 0%) and persistence (RR 1.14; I^2 44.2%), as well as the development of precancerous dysplasia (RR 2.01; I^2 0%) [477].

Ethnicity [126], menopausal status [308 478], obesity [309], use of sex hormones [478], smoking [479], sexual behaviour [480] and vaginal pH [158] have all been recognised as modulators of microbial composition in the female genital tract. Our group has previously demonstrated that obesity drives the vaginal microbiome towards a highly diverse, *Lactobacillus*-depleted composition, characterised by higher levels of *Dialister* (species unclassified) (p=7.47^{e-03}), *Anaerococcus vaginalis* (p=0.02) and *Prevotella timonensis* (p=0.02), as well as lower levels of *Lactobacillus crispatus* (p=0.01), compared to non-obese women [309]. Another study highlighted the importance of menopausal status in FGT composition showing that postmenopausal women exhibit increased diversity and enrichment of *Anaerococcus*, *Peptoniphilus* and

Porphyromonas species [308]. Interestingly, almost half (8/17) of the microbes enriched in postmenopausal women were also associated with endometrial cancer [308]. From a mechanistic standpoint, the interplay between oestrogens and lactobacilli is well-documented in the vaginal mucosa with oestrogens stimulating glycogen storage and promoting metabolism of complex sugars derived from glycogen by *Lactobacillus* species that subsequently produce lactic acid lowering the pH of the vaginal environment [481]. In the present study, no variable showed systematic bias with respect to the two comparison groups (benign vs endometrial cancer) apart from age; endometrial cancer patients were predominantly ≥65 years, while benign patients between 50-64 years of age. Importantly, postmenopausal women were equally distributed between the two cohorts.

Combining these findings with previously published reports, a common FGT microbial pattern was noted in the obese, post-menopausal and endometrial cancer cohorts; that of *Lactobacillus* depletion (predominantly *L. crispatus*) and emergence of *Anaerococcus*, *Porphyromonas, Peptococcus, Prevotella, Peptoniphilus* and *Dialister* species [308 309]. Considering that age, post-menopause and obesity are well-recognised risk factors for the development of endometrial cancer, an attractive theory would be that alterations of the FGT microbial *milieu*, in particular *Lactobacillus* depletion, precedes the development of endometrial cancer and is one of the mechanistic routes that predisposing factors employ to lead to endometrial oncogenesis creating a permissive environment for mutations to occur. This, however, does not preclude exacerbation of *Lactobacillus* depletion secondary to cancer. Even though both our comparison arms were populated primarily by post-menopausal women, who have been shown to display lower vaginal *Lactobacillus* levels compared to premenopausal women [482], I observed a further decrease of *Lactobacillus* abundance in the endometrial cancer cohort. Whether this finding could be attributed to endometrial malignancy or age, which was the only significantly different variable between the two comparison groups, remains an unanswered question. Arguments against endometrial cancer as a cause of *Lactobacillus* depletion are the fact that *Lactobacillus* is a microaerophile and therefore resilient in hypoxic conditions found in cancer

microenvironments. Further, *Lactobacillus* depletion was observed throughout the female genital tract affecting anatomical sites like the cervix and vagina, which are relatively remote from the sites of endometrial cancer development. However, haematogenous transfer of cancer cell metabolites and molecules that could influence microbiota in distant locations remains a possibility. It is also possible that the reduction in *Lactobacillus* species observed in endometrial cancer is partly due to differences in age between the cohorts. The age of the benign cohort ranged between 50-64 years of age and patients were recently menopausal, whilst endometrial cancer patients were ≥65 and post-menopausal for longer than a decade. This could also explain the increased, yet not significant, *Lactobacillus* depletion seen in type II non-endometrioid tumours, which commonly afflict older patients. Longitudinal studies monitoring pre- and post-diagnostic microbial profiles are required to delineate the actual *Lactobacillus* depletion-inducing factors in endometrial cancer.

The degree of taxonomic resolution during analysis is occasionally key to depict the true biological interplay between microbial and host cells since different species of the same genera have diverse functional roles. This is clearly illustrated by the *Lactobacillus* genus and its most prevalent species in the female genital tract *L. crispatus*, *L. gasseri* and *L. iners*. *L. iners* and *L. gasseri* have both been associated with higher odds of high-risk HPV prevalence and cervical neoplasia [310], whilst *L. crispatus* with decreased detection of high-risk HPV (OR 0.49; 95% CI; I^2 10%) and dysplasia (OR 0.50; 95% CI; I^2 0%) [128]. Individual species of *Lactobacillus* do not appear to confer the same level of protection against pathogens and related infections as illustrated by our own and previous findings [483 484] showing that *L. crispatus* is consistently depleted in women with a genital tract microbiome dominated by *Gardnerella*, a known pathogen, while *L. iners* and *L. gasseri* are both able to co-exist with *G. vaginalis*.

The biological significance of *Lactobacillus* communities in the FGT and the impact of *L. crispatus* depletion on carcinogenic pathways remains to be elucidated. To date, a wealth of reports have highlighted the health-promoting effects of *Lactobacillus*

species that are in part mediated by pathogen antagonism. A meta-analysis of 23 studies showed that a *Lactobacillus*-depleted environment as seen in BV is associated with a 60% increase in risk of acquiring HIV-1 [485], while a large longitudinal cohort study found a twofold increase in incident PID risk [486]. Vaginal lactobacilli *in vitro* inhibit the growth of *Neisseria gonorrhoeae* [487] as well as other bacterial pathogens [488]. Lactobacilli function, however, seems to extend beyond their ability to create an unfavourable environment for pathogens by lactic acid, hydrogen peroxide and bacteriocin production. Their ability to influence immune and metabolic pathways as well as epigenetic regulation makes them key constituents of biological systems affecting immunosurveillance and metabolic haemostasis [248 249 489-492]. Anti-cancer effects of *Lactobacillus* species have been reported for *L. casei*, *L. plantarum*, *L. rhamnosus* GG and *L. acidophilus* through natural killer cell activation, dendritic cell maturation or probiotic-derived ferrichrome release [251 493] inducing cancer cell apoptosis *in vitro* [250 494 495] and increasing anti-cancer drug efficacy [249 251 262]. Continuous administration of *L. reuteri* strain ATCC-PTA-6475 to tumour-prone mice for several months reduces the frequency of intestinal pre-cancerous lesions [493], while *L. acidophilus* has been shown to enhance anti-tumour immunity when combined with CTLA-4 blocking immunotherapy in a mouse colon cancer model [262].

An enrichment of *Anaerococcus*, *Porphyromonas*, *Prevotella, Dialister, Fusobacterium, Bacteroides* and *Peptoniphilus* genera was observed in the upper and lower genital tract of endometrial cancer patients, which is in keeping with previous studies [138 158 308]. However, most studies to date only focused on cervicovaginal microbiota and/or on a small number of uterine samples. A 2020 study assessing vaginal swabs from 36 women with endometrial cancer against 69 healthy post-menopausal women found that *Lactobacillus* and *Bifidobacterium* have significantly higher relative abundances in the healthy group, whilst the cancer group was enriched in 16 phylogroups associated with bacterial vaginosis, including *Sneathia*, *Prevotella*, *Peptoniphilus*, *Fusobacterium*, *Anaerococcus*, *Dialister*, *Moryella* and *Peptostreptococcus* [138]. A 2021 study using high-throughput pyrosequencing of barcoded 16S rRNA genes (V3–V4) on bacterial DNA extracted from 30 endometrial

cancer tissue samples reported an enrichment of *Pelomonas, Prevotella, Nocardioides* and *Muribaculum* in the malignant cohort compared to 10 healthy volunteers [496]. Caution should be exercised, however, when interpreting metataxonomic findings because routinely relative abundances are used, which can lead to false interpretation of results and distorted biological relationships. Taxonomic abundance expressed as a percentage inadvertently conceals true biomass; therefore, when examining the effect of an experimental factor on the microbiome of any anatomical site, the depletion of a prevalent species will inevitably lead to an increase of the relative abundance of the least prevalent species. This could be misinterpreted as an enrichment of those taxa, even though their actual abundance has remained the same making the use of quantitative PCR imperative to confirm or refute the enrichment of the taxa in question.

The Shannon α- (within samples) and β- (between samples) diversity using the Bray-Curtis index were also assessed for the microbial composition in patients with and without endometrial cancer. Endometrial cancer was associated with significantly higher α- and β-diversity both at genera and species level in the vagina, cervix and endometrium. These findings are in disagreement with the Walsh study, in which patients with endometrial cancer were not found to have a significantly different α-diversity to patients without endometrial cancer in neither the lower tract (vagina, cervix) nor uterus, while β-diversity was significantly different for the lower genital tract and marginally non-significant (p= 0.07) for the uterus [308]. In the same study, however, postmenopausal patients exhibited increased α-diversity in the lower tract and uterus versus premenopausal, but β-diversity was not significantly different in the uterus that the authors attributed to low sample numbers from this organ. On the other hand, the Lu study showed that endometrial cancer patients have significantly lower alpha diversity measurements compared to the benign uterine pathology group using Observed OTUs (P = .002), Pielou evenness (P = .001), and Shannon index (P<.001) [473]. Similarly, a 2021 study reported decreased bacterial diversity in malignant endometrium relative to healthy controls using the Chao1, observed species and Simpson indexes but no difference using the Shannon index [496]. The discrepancies in

microbial diversity between the present and previously published studies may be attributable to the lower sample size of other studies and use of tissue biopsies instead of swabs to extract microbial DNA that could impact on taxonomic recovery as I showed in Chapter 4 of my thesis.

Lastly, I performed qPCR targeting the 16S rRNA gene to determine and compare the overall bacterial load in endometrial cancer patients versus benign controls at multiple locations. Bacterial copies from the vagina, cervix and rectum of endometrial cancer patients were found to be 1-2 $\log_{10}$ lower than benign patients. No big differences were noted in the endometrium of women with or without malignancy, where bacterial quantities were comparable to those of controls. Species-specific qPCR should be used in future experiments to delineate actual enrichment or depletion of individual taxonomic units.

The overall bacterial quantities in the rectum of chemo- and radio-naïve endometrial cancer patients were significantly reduced compared to benign controls, as confirmed by qPCR, while composition was characterised by *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* depletion. However, α- and β-diversity were not significantly different for the rectum of benign versus endometrial cancer patients. No other studies investigating alterations of gut microbiota in endometrial malignancy were retrieved from literature, except a 1992 study showing significant decrease in the intestinal microbiota of uterine and cervical cancer patients (n=15) after the first radiation exposure with recovery to basal values post-radiotherapy for all bacteria apart from *Enterococcus faecium*, *Lactobacillus* spp. and total anaerobes [497].

A limitation of this study is the use of women with benign pathology as controls instead of healthy individuals that may display differential microbial structure. Women undergoing risk-reducing surgery can provide an ethically acceptable source of healthy FGT surgical specimens in future studies. Longitudinal studies monitoring the bacterial composition intra-individually over time to capture transition from a healthy state to precancerous conditions (complex atypical hyperplasia) and invasive

endometrial carcinoma are required to provide useful information on microbiota dynamics and temporal shifts within each patient. Finally, expansion of the analyses undertaken in this chapter to viral and fungal components of the microbiome using metagenomics approaches could provide further insight into human-microbial and microbial-microbial interactions relevant to endometrial cancer.

Oncobiome signatures are fundamental in understanding how the presence of a "healthy" microbiome may support optimal cell growth and development but if perturbed by antibiotics, pathogen colonisation or other external factors could promote or synergise with mutagenic cues leading to oncogenic sequelae. Future studies could focus on pre- and post-diagnostic, pre- and post-treatment microbial shifts in endometrial cancer to explore potentially causative relationships between altered microbiota with endometrial carcinogenesis as well as potential predictive biomarkers of treatment response.

5.5 Conclusions

In conclusion, the cervicovaginal and rectal bacterial load in endometrial cancer is significantly reduced compared to benign controls, whereas endometrial microbial abundances are similar. *Lactobacillus* depletion and enrichment of *Anaerococcus*, *Porphyromonas*, *Prevotella, Fusobacterium, Bacteroides* and *Peptoniphilus* genera in the lower and upper genital tract are characteristic in endometrial malignancy but whether depletion and/or enrichment of microbes precedes or follows the development of cancer or is exacerbated by malignant transformation is still unclear. High microbial diversity along the genital tract is another prominent bio-feature of endometrial cancer, while rectal biodiversity does not differ significantly in relation to benign controls. Compositionally, the rectum of endometrial cancer patients is depleted in several site-specific commensals. Different histological types and grades of endometrial cancer are not accompanied by significant differences in microbial composition in the upper and lower genital tract, but vaginal diversity is higher in high-grade tumours compared to grade I.

5.6 Statement of Contribution

Processing of 16S rRNA gene sequencing data in Mothur using the MiqSeq SOP pipeline and assignment of OTU taxonomies in Chapters 4 and 5 were performed by Dr Ann Smith.

CHAPTER 6.

Validation of endometrial organoids as tools of gynaecological research

---

Content from this chapter was published as:

Semertzidou A, Brosens JJ, McNeish I, Kyrgiou M (2020). Organoid models in gynaecological oncology research. Cancer Treat Rev 90: 102103.

6.1 Introduction

Cell culture and animal models represent experimental cornerstones for the investigation of tissue, organ and body physiology in the context of gynaecological research. However, their ability to accurately reflect human mechanisms *in vivo* is limited. The development of organoid technologies has begun to address this limitation by providing platforms *ex vivo* that resemble the phenotype and genotype of the multi-cellular tissue from which they were derived more accurately.

Even though primary 2D endometrial cell cultures have been reported [498] [499], their widespread use has been limited because of their short lifespan and poor expansion potential. These limitations have led to the development and widespread use of endometrial cell lines, which are able to undergo indefinite divisions following manipulations that rescues them from cellular senescence [272]. Several endometrial cancer cell lines are currently available, including both type I (AN3, ECC-1, EN, EN-1, EN-11, HEC-1A, HEC-1B, Ishikawa, KLE, MFE-280, MFE-296, MFE-319) and type II (ARK1, ARK2, HEC-155/180, SPEC-2) endometrial tumours [500]. Nevertheless, their major drawback is that, by definition, do not recapitulate normal cell physiology, accumulate mutations and undergo cross-contaminations over time [273] [274], thus necessitating regular authentication of origin [275]. A study that employed DNA profiling analysis on fifty-one ovarian and ten endometrial cell lines demonstrated considerable misidentification and cross-contamination among cell lines [501], while another study compared sixteen endometrial cancer cell lines and observed differential ER-α, ER-β, PR-B, PTEN, hMLH1 and P53 expression and methylation patterns amongst lines [502]. On the other hand, animal models exhibit functional completeness but their use in research involves pain, distress and death of animals making ethics a major concern. Other disadvantages of animal use include the need for skilled manpower, consuming protocols, high cost and decreased scalability.

3D organoid models have recently emerged as an alternative platform to overcome some of the drawbacks of previous models. Human and murine endometrial organoids have been derived from healthy, benign and diseased endometrial tissue and are

expected to shed light on female genital tract organogenesis, physiology and disease pathogenesis. Most importantly, organoids pave the way for personalised medicine since patient-derived cells can be used for drug and radiotherapy screening.

A feature that differentiates 3D organoids from primary cell monolayers is the preservation of a constant stem cell pool that allows long-term propagation in culture. Scientific research has sought to delineate the individual components of media formulations and suitable extracellular matrices that are capable of maintaining stemness features and defer differentiation. The extracellular matrix is a key element in organoid cultures because it constructs a 3D non-cellular microenvironment that surrounds cells but also provides them with biochemical/biomechanical signals that are crucial for their survival [277 278]. ECM is highly tissue-specific and is not static but rather demonstrates dynamic remodelling in response to molecular signals generated by the interaction with resident cells [503 504]. A number of ECM biomaterials have been used in 3D FGT organoid cultures so far, including decellularized tissues (e.g. EHS matrix- Matrigel) [279-286], natural biopolymers (e.g. collagen, hyaluronic acid) [287] and synthetic polymers (e.g. PEG, PGA) [288 289].

Furthermore, culturing media enriched in WNT/β-catenin signalling pathway molecules, such as RSPONDIN-1 and WNT3a, are pivotal determinants of organoid culture thrive and preservation of an artificial stem cell niche *in vitro* [290]. Several growth factors, signalling molecules and hormones, including nicotinamide, TGF-β inhibitor (A83-01), EGF, HGF, IGF-1, FGF, FGF10, p38i and ROCK inhibitor, neuregulin-1, oestradiol and progesterone have been scrutinized in an attempt to increase endometrial organoid proliferation and prolong lifespan.

Organoid culture technology is still in its infancy and its scientific robustness and utility remain to be revealed. Unravelling the morphological and multi-omics traits of benign and malignant endometrial organoids and comparing them to parent tissue is key to validate them as credible research models that accurately recapitulate the tissue characteristics *in vivo* with no or minimal deviations. In this chapter, I aimed to derive organoids from endometrial cancer, expand them in culture and explore their

morphological, mutational and epigenetic similarity to original tissue. I also cultured benign endometrial organoids to spot potential phenotypical disparities in relation to endometrial cancer organoids.

6.2 Aims

- To develop an *in vitro* organoid culture model of human benign and malignant endometrium and compare their biologic characteristics.

- To assess the preservation of morphological/molecular features, genetic mutations and epigenetic signatures in endometrial cancer organoids in relation to progenitor tissue.

6.3 Results

6.3.1 Derivation of human benign and malignant endometrial organoids from pipelle biopsies

Human endometrial glandular organoids were isolated from pipelle biopsies post-operatively from uteri specimens of women undergoing hysterectomy for benign conditions or endometrial cancer (Figure 6.1). The biopsy samples were subjected to enzymatic treatment using collagenase and DNAse. Following digestion, a small amount of digest was frozen at -80°C, while the remaining cells were seeded into Matrigel matrix and submerged in culture medium (see methods for more details). The same medium composition was used for both benign endometrial and endometrial cancer organoids, following the medium previously described for benign organoid generation of 11 components: Nicotinamide, Noggin, Rspondin-1, B27 supplement, N2 supplement, N-acetyl-L-cysteine, A83-01, Y-27632, EGF, FGF-10, HGF (Table 6.1).

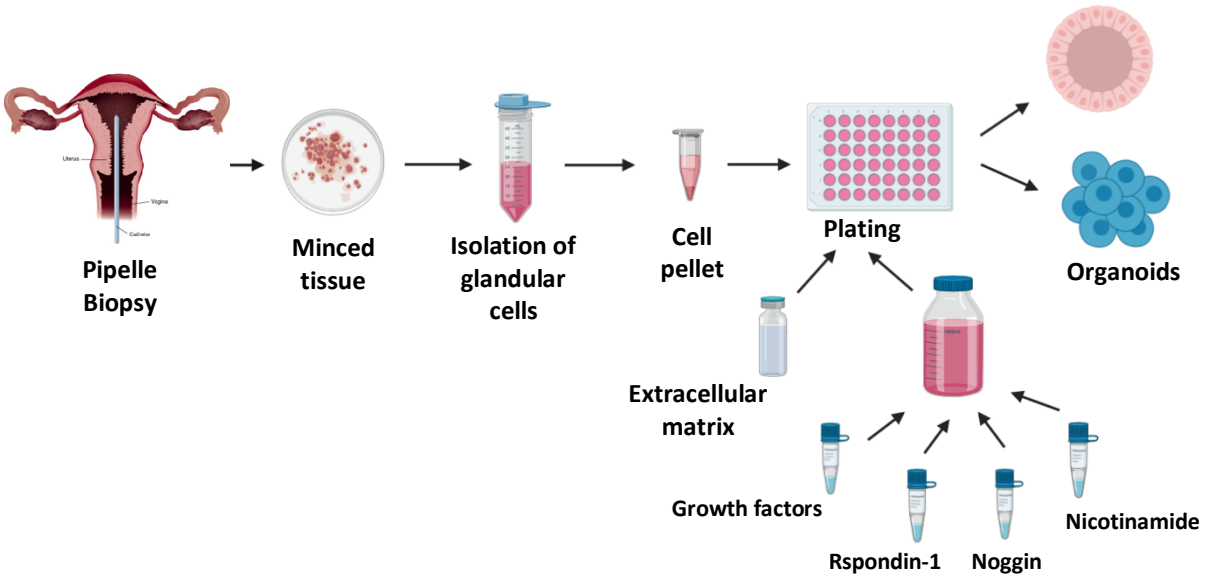


Figure 6.1. Workflow of endometrial organoid culture establishment. An endometrial pipelle biopsy is obtained, minced until puree and endometrial glandular cells are isolated. The generated cell pellet is seeded on a culture plate with Matrigel and medium supplemented with a cocktail of growth factors and signalling molecules, which manipulate developmental pathways and ensure culture prosperity by substituting the endocrine and paracrine signals that cells receive in their natural environment.

Table 6.1. Factors tested for the purpose of endometrial cancer organoid derivation based on previous reports of benign endometrial organoid models.

Factor	Stock	Final concentration	Manufacturer	Catalogue number	Function
N2 supplement 100x	1*	1*	Gibco	17502048	Increases sphere formation efficiency.
B27 supplement 50x	1*	1*	Gibco	12587010	Increases sphere formation efficiency.
N-acetyl-L-cysteine	1*	1.25mM	Sigma	A9165-5G	Increases organoid outgrowth.
A83-01	5.9309mM	500nM	Sigma	SML0788-5MG	Transforming growth factor β (TGF-β) inhibitor.
Nicotinamide	1mM	10mM	Sigma	N0636-100G	Important for long-term organoid maintenance [280 505].
Noggin	100ug/ml	100ng/ml	Peprotech	120-10C	Inhibits differentiation cues from bone morphogenic protein (BMP) signals and generally facilitates expansion of stem cells [291].
Rspondin-1	100ug/ml	500ng/ml	Peprotech	120-38	Canonical Wingless/Int (WNT) signalling pathway potentiator
Y-27632	10mM	10uM	Abcam	ab120129	Increases proliferation and prevents cell death through anoikis.
EGF	100ug/ml	50ng/ml	Peprotech	AF-100-15	Improves long-term maintenance.
FGF-10	100ug/ml	100ng/ml	Peprotech	100-26	Increases organoid outgrowth.
HGF	100ug/ml	50ng/ml	Peprotech	100-39	Increases organoid outgrowth.

A cohort of 71 women was recruited, subdivided into 47 patients with endometrial cancer (38 endometrioid, 6 serous, 1 clear cell, 2 carcinosarcomas) and 24 patients with benign conditions (e.g., fibroids, benign ovarian cysts). The age of patients ranged from 26 to 89 years (Mean age, Benign 46 [26-77]; EC: 65 [50-89]) and organoid derivation efficiency did not seem to be affected by age or menopausal status. Details on patient/disease characteristics, organoid derivation efficiency, expansion potential and use in downstream applications can be found in Table 6.2.

Table 6.2. Endometrial organoid recruitment, derivation efficiency, expansion potential and applications.

Culture ID	Age	Histology	Matched tissue	Organoid derivation	Expansion	Expansion post-cryopreservation	DNA+RNA extraction (organoid+tissue)	Targeted gene sequencing (organoid+tissue)	Methylation profiling (organoid+tissue)
AO1	56	Endometrioid Gr3	-	+	7 days	NA	-	-	-
AO2	70	Serous Gr3	-	+	7 days	NA	-	-	-
AO3	53	Endometrioid Gr3	-	+	5 days	NA	-	-	-
AO4	89	Carcinosarcoma Gr3	-	+	6 days	NA	-	-	-
AO5	56	Benign (leiomyoma)	-	+	35 days	NA	-	-	-
AO6	51	Endometrioid Gr1	-	+	14 days	NA	-	-	-
AO7	62	Endometrioid Gr2	-	-	0 days	NA	-	-	-
AO8	35	Benign	-	+	45 days	NA	-	-	-
AO9	65	Endometrioid Gr2	-	-	0 days	NA	-	-	-
AO10	63	Endometrioid Gr2	-	-	0 days	NA	-	-	-
AO11	64	Endometrioid Gr1	-	+	14 days	NA	-	-	-

AO12	72	Endometrioid Gr1	-	+	10 days	NA	-	-	-
AO13	44	Benign	-	+	60 days	NA	-	-	-
AO14	38	Benign	-	+	45 days	NA	-	-	-
AO15	63	Carcinosarcoma	-	+	8 days	NA	-	-	-
AO16	62	Endometrioid Gr3	-	+	32 days	NA	-	-	-
AO17	57	Endometrioid Gr1	-	+	30 days	NA	-	-	-
AO18	60	Endometrioid Gr1	-	+	25 days	NA	-	-	-
AO19	71	Endometrioid Gr1	-	+	10 days	NA	-	-	-
AO20	34	Benign	-	+	30 days	NA	-	-	-
AO21	48	Benign	-	+	62 days	NA	-	-	-
AO22	75	Endometrioid Gr2	-	+	13 days	Unsuccessful	-	-	-
AO23	69	Endometrioid Gr2	-	+	14 days	NA	-	-	-
AO24	81	Endometrioid Gr2	-	+	7 days	NA	-	-	-
AO25	58	Endometrioid Gr3	+	+	7 days	Unsuccessful	+	+	+
AO26	61	Serous	+	+	5 days	NA	+	+	+
AO27	74	Endometrioid Gr1	+	+	9 days	Unsuccessful	+	+	+

AO28	46	Benign (Ovarian cystadenoma)	-	+	92 days	Unsuccessful	+	-	+
AO29	84	Serous	+	+	2 days	Unsuccessful	+	+	+
AO30	54	Endometrioid Gr1	-	+	14 days	NA	+	-	+
AO31	71	Serous	+	+	14 days	Unsuccessful	+	+	+
AO32	55	Endometrioid Gr3	+	+	7 days	NA	+	+	+
AO33	78	Endometrioid Gr1	+	+	13 days	NA	+	+	+
AO34	44	Benign (leiomyoma)	+	+	25 days	Unsuccessful	+	-	+
AO35	61	Endometrioid Gr1	+	+	12 days	NA	+	+	+
AO36	34	Benign	+	+	36 days	NA	+	-	+
AO37	57	Endometrioid Gr2	+	+	6 days	NA	+	-	+
AO38	62	Clear cell, Gr3	+	+	10 days	NA	+	+	+
AO39	65	Endometrioid, Gr3	+	+	3 days	NA	+	+	+
AO40	84	Endometrioid, Gr1	+	+	5 days	NA	+	+	+
AO41	77	Benign	-	+	29 days	NA	-	-	-

AO42	63	Benign (Borderline ovarian serous)	-	+	37 days	NA	-	-	-
AO43	31	Benign	-	+	41 days	NA	-	-	-
AO44	56	Endometrioid Gr1	-	+	15 days	NA	-	-	-
AO45	80	Endometrioid Gr2	-	-	0 days	NA	-	-	-
AO46	50	Endometrioid Gr1	-	+	4 days	NA	-	-	-
AO47	61	Benign (Ovarian granulosa cell tumour)	-	+	41 days	NA	-	-	-
AO48	52	Endometrioid Gr1	-	+	7 days	NA	-	-	-
AO49	74	Serous Gr3	-	+	16 days	NA	-	-	-
AO50	64	Endometrioid Gr1	-	_	0 days	NA	-	-	-
AO51	72	Mixed endometrioid and serous, Gr3	-	+	9 days	NA	-	-	-
AO52	62	Endometrioid Gr1	-	+	5 days	NA	-	-	-
AO53	26	Benign (immature	-	+	30 days	NA	-	-	-

		ovarian teratoma)							
AO54	55	Endometrioid Gr2	-	+	6 days	NA	-	-	-
AO55	54	Endometrioid Gr1	-	+	9 days	NA	-	-	-
AO56	71	Endometrioid Gr2	-	-	0 days	NA	-	-	-
AO57	57	Endometrioid Gr1	-	+	5 days	NA	-	-	-
AO58	59	Benign	-	+	25 days	NA	-	-	-
AO59	44	Benign	-	+	28 days	NA	-	-	-
AO60	62	Endometrioid Gr2	-	+	12 days	NA	-	-	-
AO61	62	Benign	-	+	35 days	NA	-	-	-
AO62	58	Endometrioid Gr1	-	+	9 days	NA	-	-	-
AO63	75	Benign	-	+	41 days	NA	-	-	-
AO64	83	Endometrioid Gr1	-	+	13 days	NA	-	-	-
AO65	67	Endometrioid Gr2	-	-	0 days	NA	-	-	-
AO66	45	Benign	-	+	31 days	NA	-	-	-
AO67	32	Benign	-	+	14 days (used in experiments after)	Successful	-	-	-

AO68	37	Benign	-	+	14 days (used in experiments after)	Successful	-	-	-
AO69	32	Benign	-	+	14 days (used in experiments after)	Successful	-	-	-
AO70	37	Benign	-	+	14 days (used in experiments after)	Successful	-	-	-
AO71	39	Benign	-	+	14 days (used in experiments after)	Successful	-	-	-

Abbreviations: Gr: Grade; NA: Not attempted

6.3.2 Both benign glandular and endometrial cancer organoids exhibit high formation efficiency, but long-term expansion is only observed in benign endometrial organoids

Organoid formation efficiency was 100% (24/24) from benign endometrial organoids. Following fragmentation, benign endometrial cells were able to self-organise, proliferate and expand in size demonstrating their clonogenic ability. Dense cell aggregates, however, demonstrated higher proliferation and growth capability than single cells. Benign organoids were able to self-organise in 6 hours, increase in size up to 500 μm or fold upon themselves creating tortuous structures when left unpassaged and self-expand for longer than 3 months (Figures 6.2 and 6.3). Proliferation and growth potential of benign uterine organoids was consistent with minimal inter-patient variability.

On the other hand, organoid formation efficiency was 85% (40/47) from malignant endometrial tissue. Even though malignant endometrial organoids self-organised within 12-24 hours after plating, their long-term expansion was severely compromised compared to their benign counterparts, with the most sustainable lines surviving 1-2 weeks (Figures 6.2 and 6.3). Histological type and grade of endometrial cancer did not affect organoid formation ability. Passage frequency was dictated by cell density and was normally undertaken every 7-14 days in 1:2-1:3 ratio.

Dead organoids displayed overall a disrupted, dark morphology. Cell death, confirmed by tryptan blue stain, was manifested with features of necrosis and autophagy, including cell swelling, loss of membrane integrity, translucency of cytoplasm, multiple and large cytoplasmic blebs and karyolysis (Figure 6.4).

Cryopreservation and successful revival following thawing has been reported in literature for endometrial organoids [506]; however, this was not confirmed by my experiments either for tissue or organoids (benign or malignant) stored for 3-6 months.

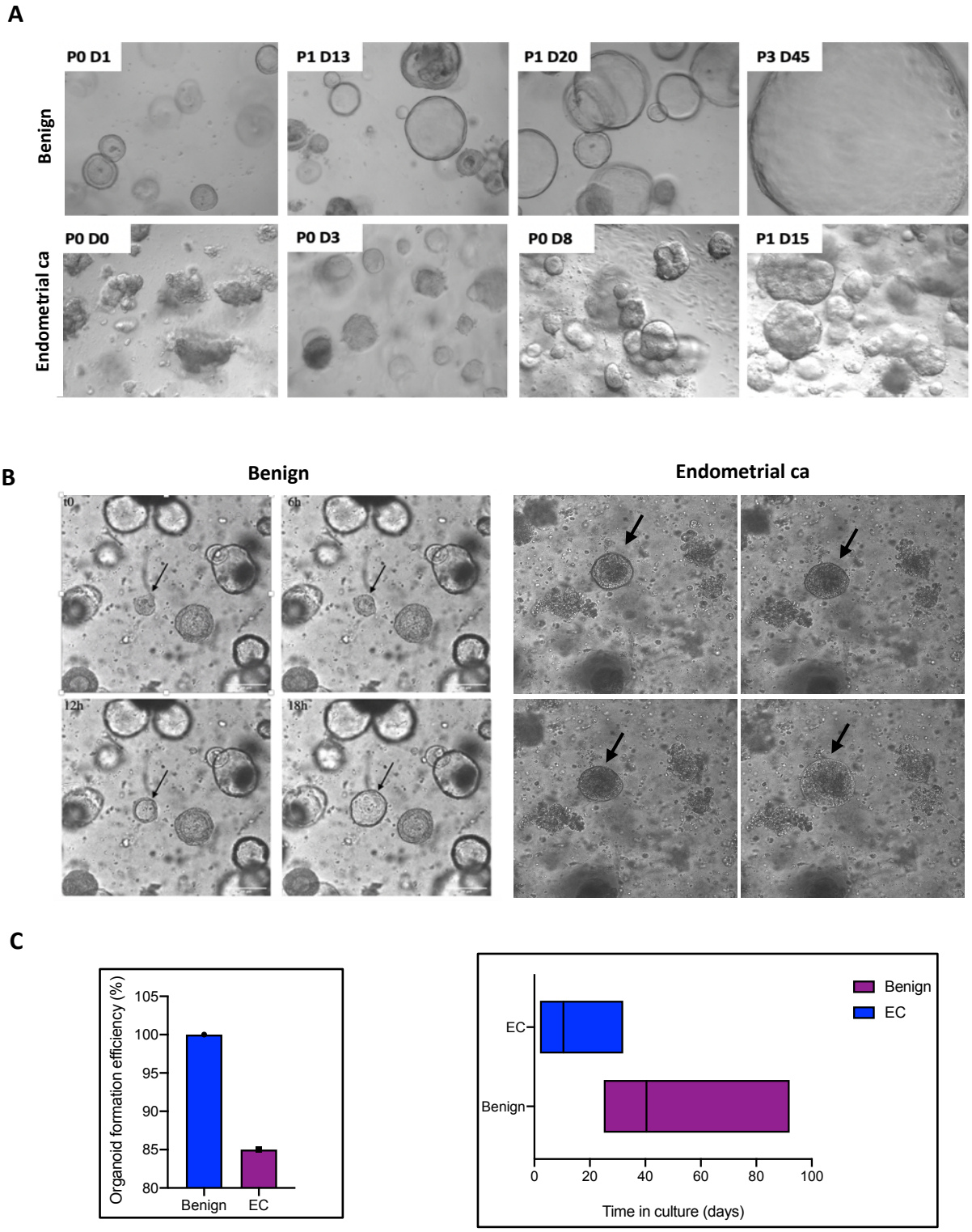


Figure 6.2. Proliferation and growth of human benign and malignant endometrial glandular organoids. A. Bright-field microscopy showing growth and proliferation over time for benign organoids and organoids derived from a G2 endometrioid endometrial tumour. **B.** Live-cell imaging of benign and malignant endometrial organoids showing their expansion at 6-hour intervals. Scale bar 100µm. **C.** Organoid formation efficiency (%) and expansion (days) of benign and malignant endometrial organoids. Line represents mean. EC: Endometrial cancer.

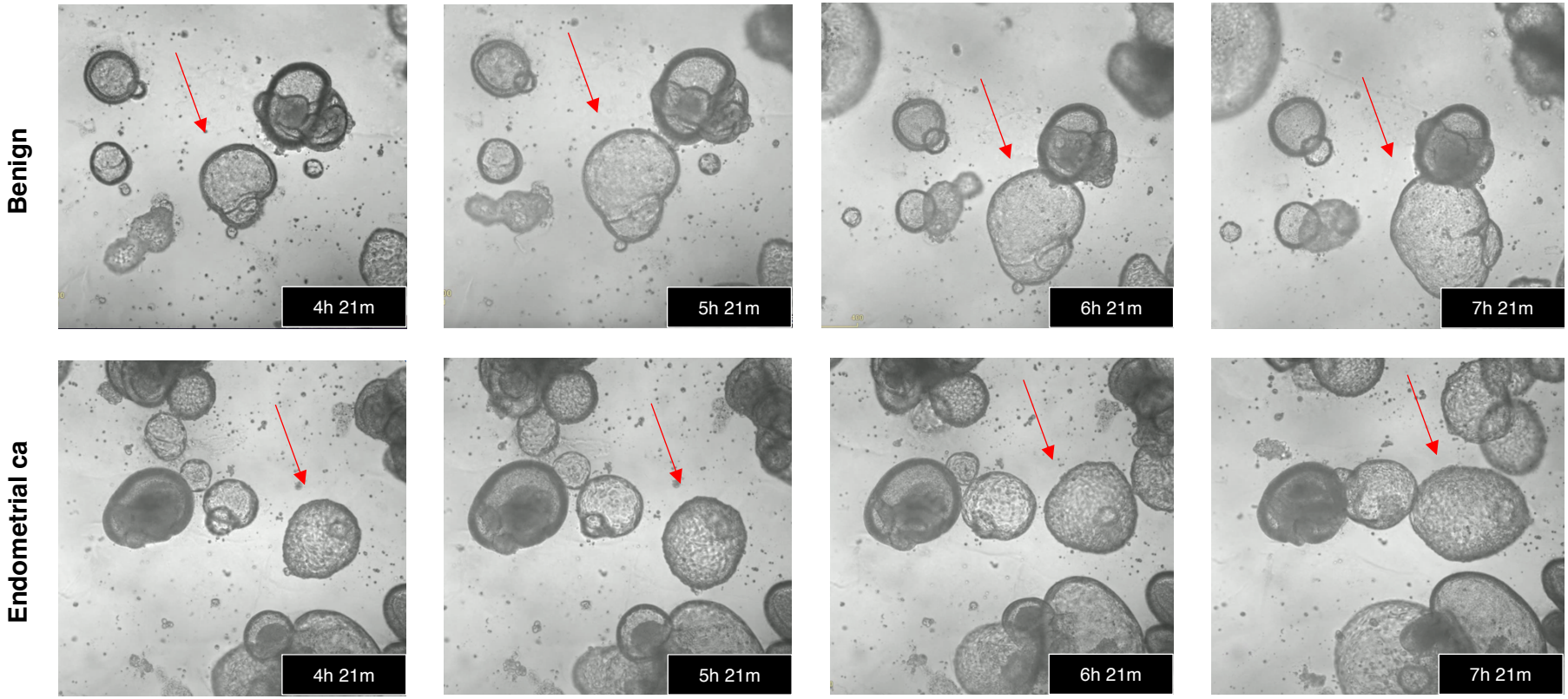


Figure 6.3. IncuCyte S3 live-cell imaging of human benign and malignant endometrial glandular organoids at 3h intervals (total duration: 7 days 21 hours). Benign glandular organoids expand in size and fold upon themselves to form tortuous structures. Endometrial cancer organoids present with either glandular or solid cytoarchitecture. Scale bar 100µm.

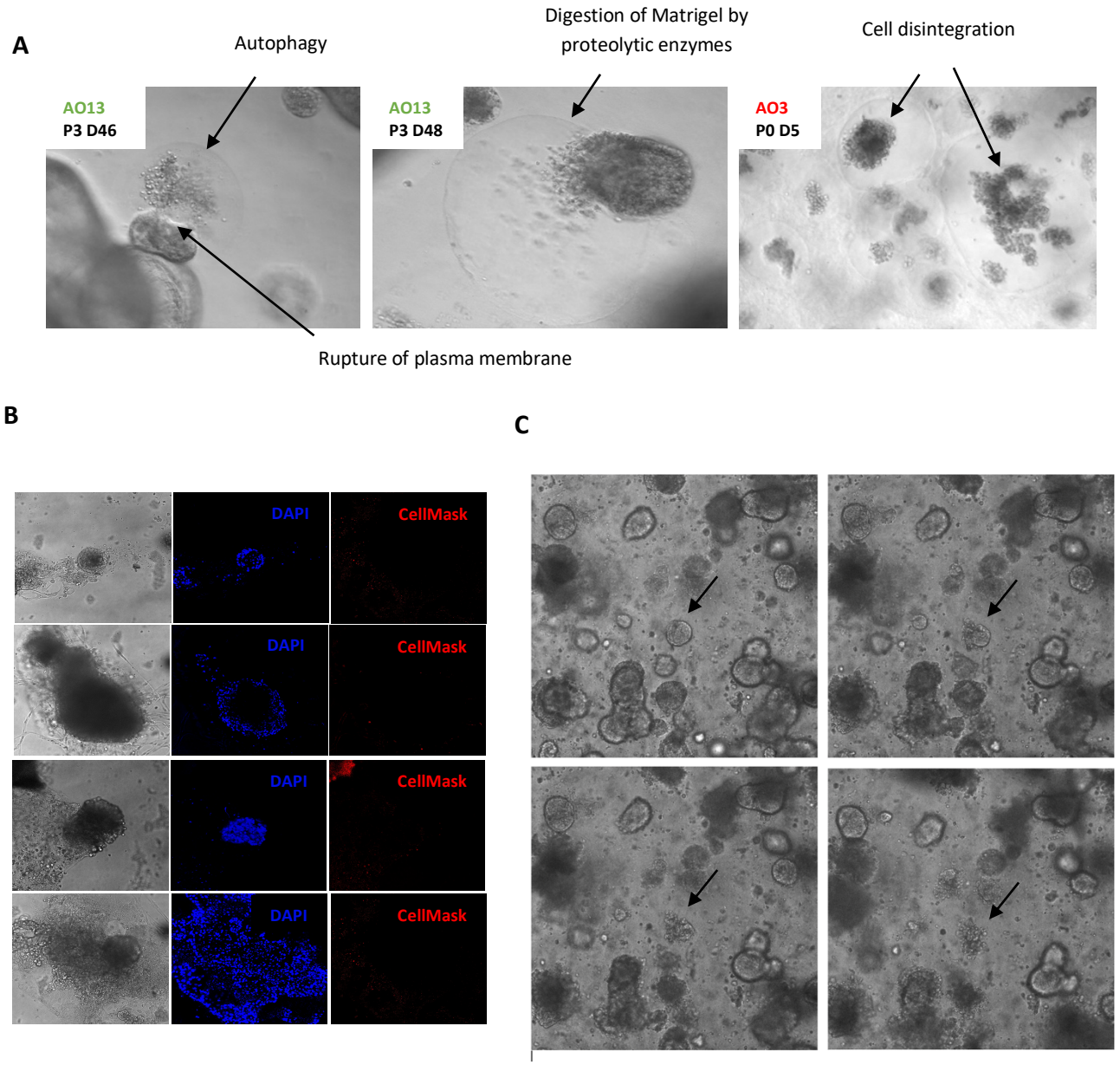


Figure 6.4. Endometrial organoid death. A. Organoid necrosis after one month for a benign line (AO13) and five days for a G3 endometrioid endometrial tumour (AO3), marked by plasma membrane rupture, cell disintegration and cytoplasm/Matrigel digestion. **B.** Non-viable organoids appear dark, with disturbed cytoarchitecture, fragmented nuclei and loss of membrane integrity. DAPI: nuclear stain; CellMask: cell membrane stain. **C.** Live-cell imaging showing endometrial organoid disintegration.

6.3.3 Benign glandular and endometrial cancer organoids recapitulate the phenotypical features of primary tissue

For a cell model to be considered robust and representable of the origin cell or tissue type, morphological and genetic/epigenetic resemblance to parent tissue are fundamental to facilitate reliability of extrapolated research results. Therefore, I explored the cytoarchitecture and molecular markers of human benign and endometrial cancer organoids to establish their kinship to progenitor tissue and concluded that human endometrial organoids capture parent tissue features at morphological level. The glandular architecture of benign endometrial organoids is preserved with a columnar epithelial lining, basal nuclei and microvilli directed towards a central lumen containing cellular debris and a few degenerating viable cells (Figure 6.5). Unlike benign endometrial glandular organoids that display inter-patient homogeneity, endometrial cancer organoids are characterised by morphological heterogeneity. Glandular-like structures reminiscent of benign organoid morphology are encountered primarily in low grade (Grade 1) lesions, whilst dense cellular aggregates (spheroids) or multi-lumen organoids with disturbed cell polarity predominate in organoids derived from high grade (Grade 2-3) lesions, suggesting that the architectural diversity of malignant organoids is reflective of histological grade variations of endometrial cancer. No morphological differences were noted based on histological type (Figure 6.5).

Tissue-specific molecular and cell polarity markers (MUC-1, E-cadherin, cytokeratin 7, Ezrin, cd49f) are maintained in both benign and malignant endometrial organoids along with cellular bi-potency, as illustrated by the presence of both secretory and ciliated cells on transmission electron microscopy (Figures 6.6 and 6.7). MUC1 is normally expressed in the glandular or luminal epithelial cells of uterus; E-cadherin is a 120–130 kDa integral membrane glycoprotein expressed in epithelial cells and is a key component of the adherens junction that serves to connect the lateral plasma membrane of neighbouring epithelial cells, which is also linked to the cytoskeleton via intracellular ligands termed α, β and γ catenins [507-509] forming a complex that is

essential for cell adhesion; Cytokeratin 7 is a low molecular weight cytokeratin distributed in epithelia and their neoplasms [510]; Ezrin is an apical epithelial marker with a critical role in cell polarization by linking membrane protein complexes to actin cytoskeleton [511] and cd49f (integrin a6) represents a basolateral epithelial marker, which is also encountered in many stem cell populations, including cancer stem cells [512].

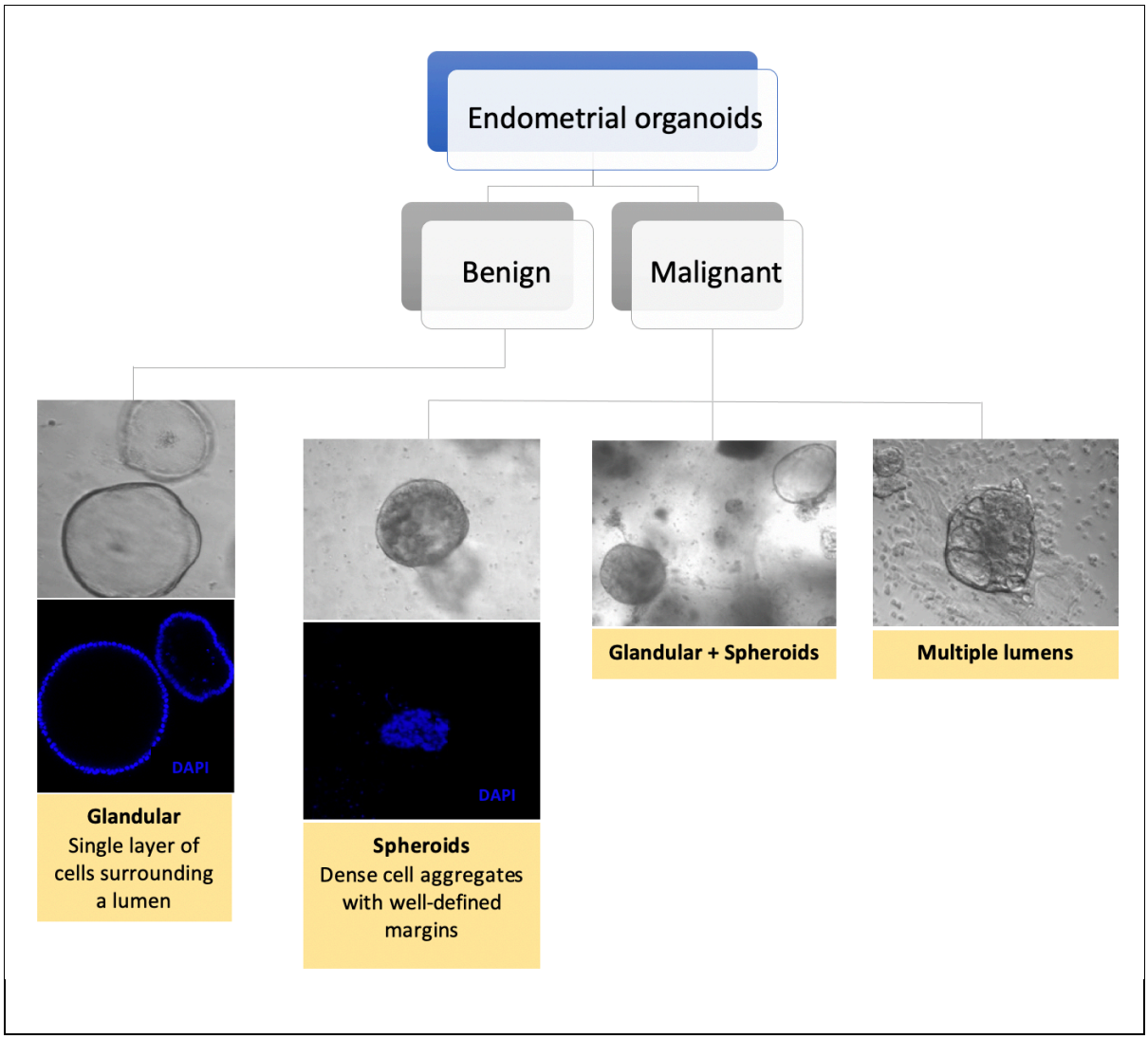


Figure 6.5. Morphological features of human benign and malignant endometrial organoids. Benign organoids exhibit glandular architecture with a central lumen encircled by a single cellular layer, while organoids from endometrial cancer display a solid cellular configuration in combination with or without single- or multi-lumen structures.

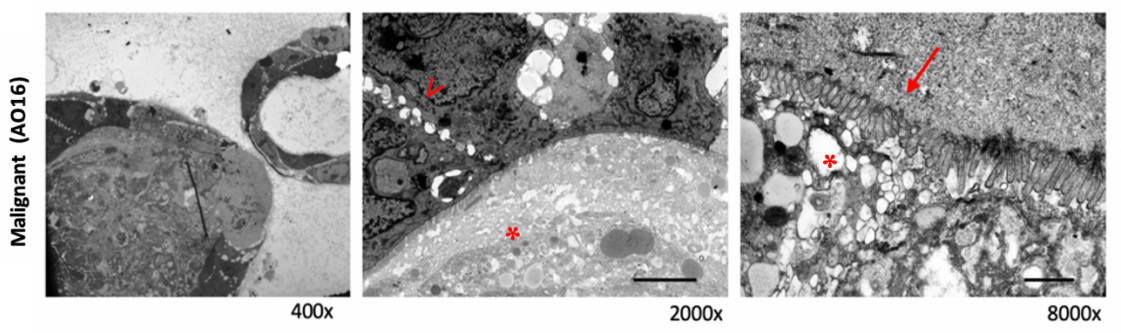


Figure 6.6. Transmission electron microscopy (TEM) images of a malignant organoid derived from G1 endometrioid cancer. Arrowhead (>): tight junctions, asterisk (*): lumen with mucin, arrow (→): apical surface with microvilli. The cytoarchitecture and polarity of cells was revealed with the apical surface harbouring microvilli that face the lumen.

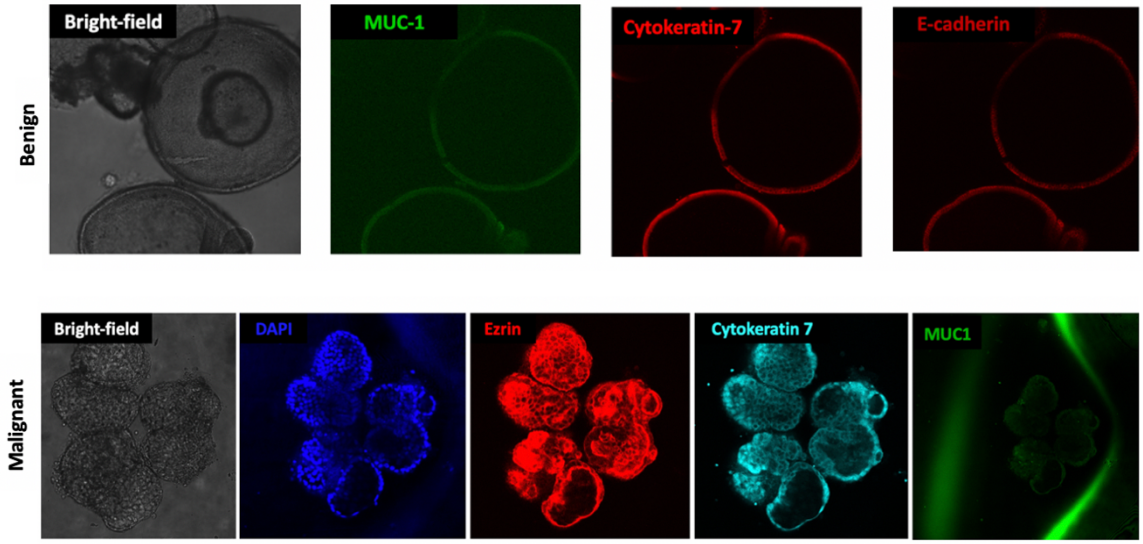
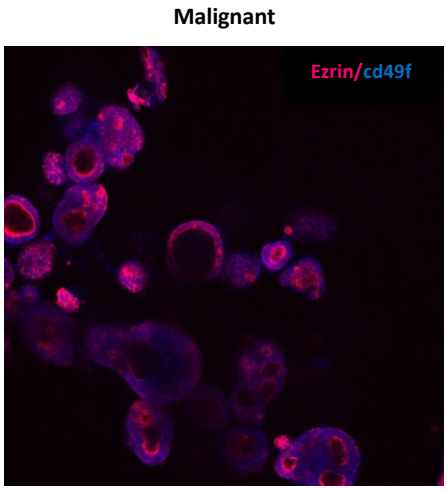


Figure 6.7. Confocal microscopy in benign and malignant endometrial organoids stained for nucleus (DAPI), glandular (MUC1, Cytokeratin-7) and cell polarity (E-cadherin, Ezrin, cd49f) markers. Endometrial organoids retain the glandular molecular characteristics of progenitor tissue. Cell polarity is disturbed in endometrial cancer organoids with the development of multiple mini-luminal structures. Ezrin: apical, E-cadherin: lateral, cd49f: basolateral marker.



6.3.4 Endometrial cancer organoids retain the mutational landscape of parent tumour

Next, I compared the genetic make-up of primary tumours and their corresponding organoids, which were maintained in culture for a maximum of two weeks. A panel of seven genes *(PTEN, ARID1A, PIK3CA, POLE, CTNNB1, KRAS, TP53)*, commonly mutated in endometrial cancer, was interrogated using targeted gene sequencing (NEBNext Direct® Custom Ready Panels, New England BioLabs) in eleven patients with endometrial cancer (7 endometrioid, 3 serous, 1 clear cell) (Table 6.2). The analysis revealed that genetic variations were largely shared by primary tumours and EC-derived organoids, although occasional alterations, mostly synonymous or non-synonymous SNVs (single nucleotide variants), were uniquely present in primary tumours or cultured organoids (Figure 6.8B). Moreover, genetic hits were found to overlap in 7 commonly mutated genes of EC in both organoids and original tumour (Figure 6.8A). In more detail, inactivating mutations were identified in tumour suppressor genes *ARID1A* (P1326R, T1514R, D1633T, F1924S), *PTEN* (C297W, R303Q, W77X, V120fs) and *TP53* (R174X, R141H, R43H, S127F). *PTEN* mutations were present in all endometrioid (AO25, AO27, AO32, AO33, AO35, AO39, AO40) and absent in non-endometrioid tumour-organoid pairs, whereas *TP53* mutations were noted in all non-endometrioid cancer samples (Serous: AO26, AO29, AO31; Clear cell: AO38) but also in one endometrioid grade 1 tumour (AO33). Single-base substitutions were observed in oncogenes *PIK3CA* (E81K, R108H, P539R, M1043I) and *KRAS* (Q61H, G13C, G12V, G12D). *CTNNB1* (G34V) mutations were only found in one endometrioid grade 3 tumour-organoid pair (AO32). *POLE* proofreading mutations, which lead to high numbers of DNA replication errors and high mutation frequency [513], were detected in two endometrioid tumour-organoid pairs (AO25, AO35).

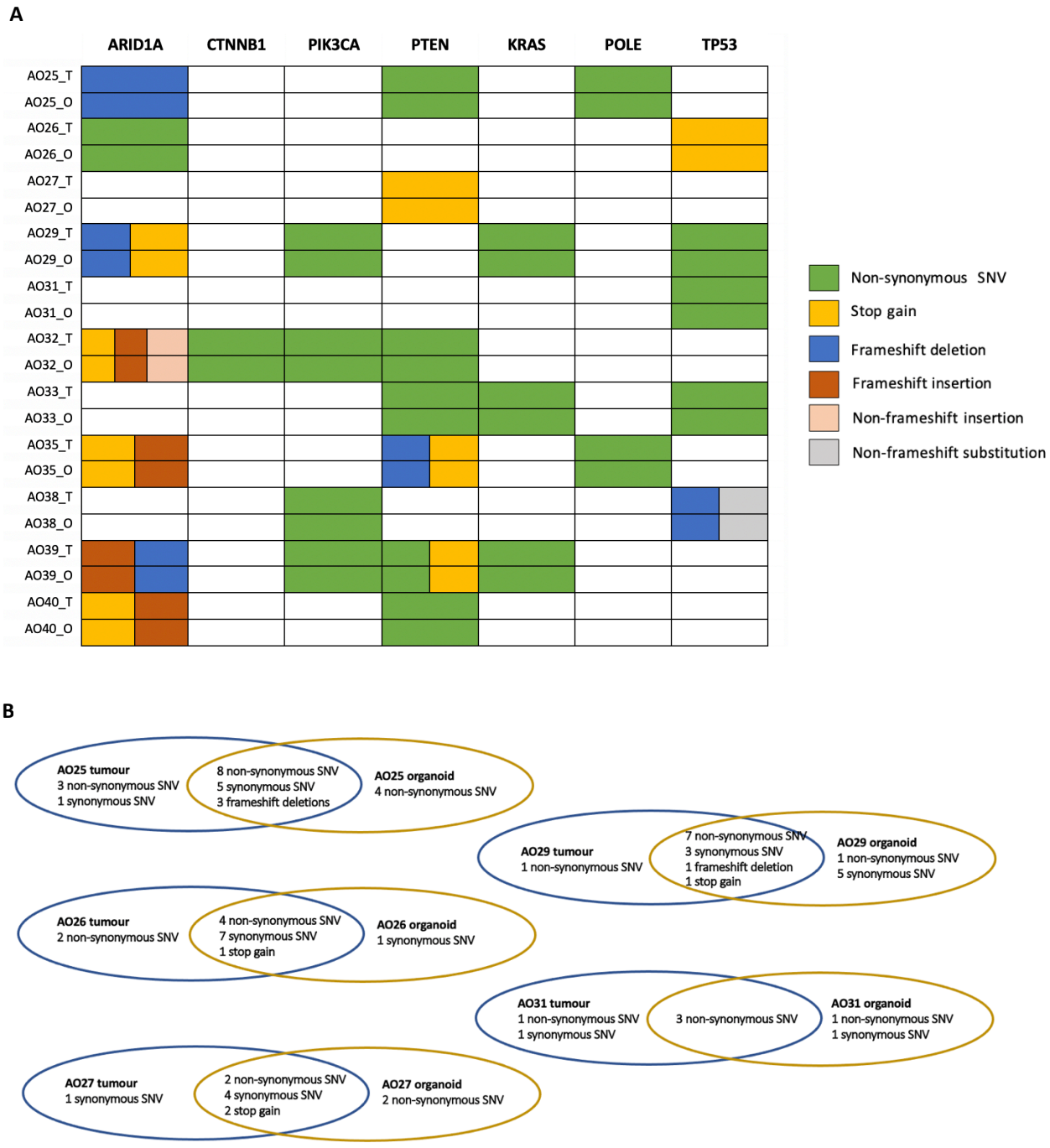


Figure 6.8. EC-derived organoids reflect the mutational landscape of primary tumours. A. A mutation matrix depicting hits in 7 of the most mutated genes in EC as identified by NEBNext targeted gene sequencing in primary EC samples and derived organoids (n=11 pairs). **B.** Genetic variations across the seven genes examined in primary endometrial tumours and corresponding organoids in five patients. SNV: single nucleotide variant.

6.3.5 Benign glandular and endometrial cancer organoids recapitulate the DNA methylation signatures of parent tissue

To assess the extent to which epigenetic signatures are preserved in organoids derived from endometrial tissue, 13 patients (EC n=11; Benign n=3) were recruited and genome-wide DNA methylation (Illumina Infinium HD Methylation Assay, >850,000 CpG islands) was performed in organoid-tissue pairs. The characteristics of these organoid-tissue pairs, including histological type and grade, can be found in Table 6.2. Unsupervised hierarchical clustering revealed close clustering of the organoids with the corresponding tissue (Figure 6.9A), while multidimensional scaling (MDS) provided very similar results (Figure 6.9B). Samples AO30_O and AO37_T represent orphan samples, while samples AO28_O and AO28B_O represent organoid samples harvested 3 months apart. Sixty-eight differentially methylated positions (DMPs) were identified between endometrial cancer organoids and respective tumour tissue. Organoid hypermethylation was observed in 66 regions, while hypomethylation in 2 regions in comparison to parent tumour. A list of DMPs is provided in Table 6.3.

The tree-based dendrogram also showed a distinct separation of benign (AO28, AO34, AO36) and endometrial cancer samples and retention of cancer histotype-specific epigenetic characteristics with endometrioid cancer types forming two clear arms, including samples AO39, AO37, AO25 on one arm and AO35, AO40, AO30, AO32, AO27 on the other (Figure 6.9A). On the other hand, non-endometrioid tumours formed a separate arm, including serous (AO26, AO29 and AO31) and clear cell (AO38) samples. It is worth mentioning that endometrioid sample AO33 grouped more closely with benign samples than any other group, probably suggesting normal tissue contamination. Multidimensional scaling yielded again identical results (Figure 6.9B), confirming distinct epigenetic profiles among benign, endometrioid and non-endometrioid cancer samples.

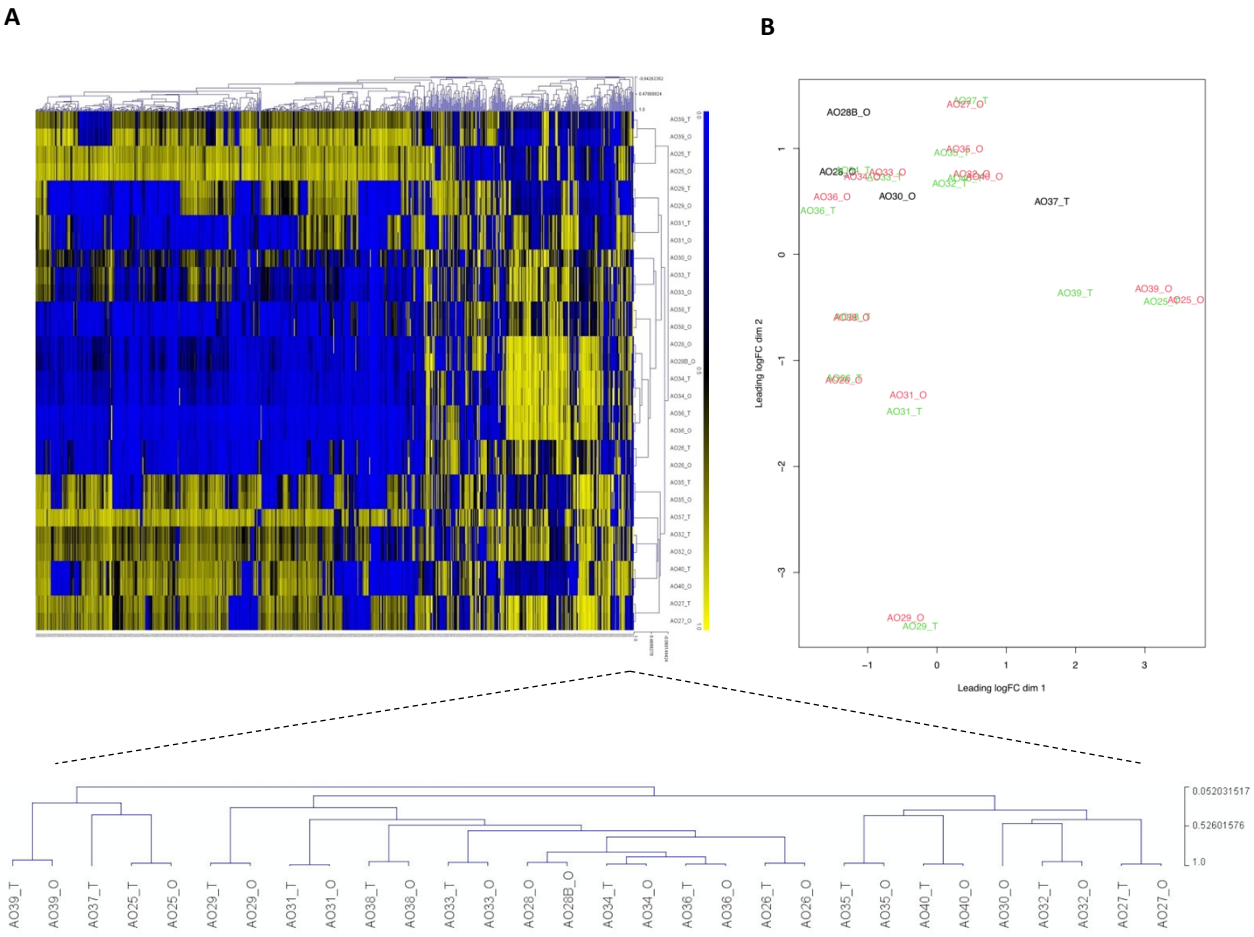


Figure 6.9. DNA methylation landscapes of benign and malignant endometrial organoids retain the original tumour epigenetic profile. A. Heatmaps demonstrating unsupervised hierarchical clustering for organoid-tissue samples. Yellow represents methylated CpGs and blue unmethylated CpGs (SWAN-normalised beta values, Pearson correlation, average linkage clustering). **B.** MDS plot further highlights matched organoid (red)-tissue (green) clustering based on their DNA methylation characteristics. Samples AO30_O and AO37_T represent orphan samples, while samples AO28_O and AO28B_O represent organoid samples harvested 3 months apart. T: Tissue, O: Organoid.

Table 6.3. Top 20 differentially methylated regions (DMRs) between endometrial cancer tissue and derived organoids.

Probe ID	Chromosome	Gene	Start of DMR (bp)	End of DMR (bp)	Log fold change Tissue/Organoid	adjPVal
cg21465162	9	UBQLN1	86322017	86323215	-2.8401721	0.022830545
cg01877318	17	CDC27	45266251	45266974	-2.51061861	0.001096811
cg15801789	9	HSPA5	128002945	128003972	-2.29768868	0.009288536
cg17749261	3	TIPARP	156391811	156393607	-2.26884815	0.030874994
cg11192793	1	ID3	23885682	23886212	-2.22178508	0.001214784
cg27041724	9	MLLT3	20620728	20621862	-2.17993595	0.003225464
cg19079372	5	DHX29/SKIV2L2	54603209	54603904	-2.10352048	0.041811314
cg19375796	3	GLYCTK	52321783	52322054	-2.04578254	0.030874994
cg00542493	10	NUDT5/CDC123	12237832	12238377	-1.82924238	0.006226334
cg00678630	12	SCAF11	46383561	46384756	-1.77119933	0.01134537
cg10532262	3	NCEH1	172428416	172428950	-1.70481941	0.045640335
cg04921814	1	PIAS3	145575130	145576219	-1.64029813	0.047365475
cg19233923	11	OTUB1	63753414	63754454	-1.59738758	0.007510546
cg06561932	8	LOC389641/TNFRSF10A	23081956	23082975	-1.51767409	0.024810257
cg22082682	1	SAMD13	84764108	84764906	-1.51629821	0.043209243
cg04609859	17	HOXB4	46654053	46654369	-1.50535649	0.016263487
cg10191855	2	IKZF2	214016357	214017105	-1.49788403	0.033260114
cg12538421	8	LRRC6	133687573	133687998	-1.47036934	0.037559533
cg21352612	8	HAS2	122651665	122652389	-1.45930498	0.01134537
cg14470647	9	ABCA1	107689847	107690798	-1.35895256	0.025937643

6.4 Discussion

Suitable cell culture models for microbiome mechanistic experiments are critical for further understanding host-microbe interactions. To date, most work in this area has been performed using cell monolayers, both primary and established cell line cultures, which have inherent limitations because they lack the fundamental component of spatial organization and cell polarity that is critical in achieving structural and functional tissue fidelity. Organoids, on the other hand, are increasingly being used as complex, multi-dimensional, multi-cell structures resembling entire organs and have now been derived from a variety of tissues, including endometrial [279 280 514]. Representing the next generation of cancer cell models, organoids necessitate validation to confirm their morphological and (epi)genetic resemblance to primary tissue, which was the aim of this chapter.

This is the first report of endometrial organoid generation from minimally invasive pipelle biopsies and of large-scale establishment of organoids from endometrial cancer in 47 patients, including different histological types and grades. Successful formation and long-term maintenance of organoid models relies on the starting cell population and exogenous cues, like the extracellular matrix and culture medium. Organoid media components aim to meet nutritional requirements of cells ensuring their proliferation and growth but also harness their stem cell properties allowing for long-term expansion. Following separation from stromal cells, endometrial glandular cells were seeded in Matrigel and were successfully grown in medium supplemented with a cocktail of growth factors and Wnt/ BMP (Rspondin-1, Noggin) signalling molecules that have been previously described for generation of benign uterine and other type organoids. Clevers and Sato were the first to provide significant insight in WNT requirements in small intestine and colon organoid models showing that "mini-guts" can be formed from single Leucine-rich repeat containing G-protein-coupled receptor 5 (Lgr5) stem cells, localized in crypts, when Rspondin-1, EGF (epidermal growth factor), Noggin (BMP inhibitor), nicotinamide and p38 inhibitor were concurrently supplemented to the medium [291]. In a similar context, Boretto and colleagues demonstrated that in murine endometrial organoid models Rspondin-1 and Wnt3a are not required at early stages but are indispensable for further passages [279], while another study showed that the need for exogenous Wnt3a supplementation is significantly reduced owing to the fact that Lgr^- epithelial cells supply their Lgr^+ counterparts with Wnt ligands 4/7b endogenously [297]. Human benign endometrial and endometriotic organoids were observed to thrive in Rspondin-1 and Noggin supplemented media, while WNT3a supplementation was not required for long-term expansion [279]. Further analysis revealed that endometrial organoids do not express themselves the Rspo ligands 1, 2, and 3 but strongly express the Rspo receptors LGR4 and 5 [279].

Benign uterine organoids have been derived from a variety of sources displaying high formation rates, including non-pregnant secretory endometrium (100%) and decidual tissue (96-100%) when cultured over 7-14 days [280] [281], while considerably lower formation efficiency has been observed in organoids generated from endometriotic

lesions [282]. Of note, organoid formation ability has been shown to be unaffected by menstrual cycle, menopausal status and benign/premalignant pathology, as indicated by successful derivation from proliferative, atrophic, ectopic and hyperplastic endometrium [279] [280] [282], while the most sustainable benign endometrial organoid cultures have been reported to exceed 3 years [281]. The current study corroborated above findings showing that all cultures derived from benign endometrium, whether proliferative, secretory or atrophic, lead to organoid formation.

Histological type and grade of endometrial cancer have not been reported to affect organoid formation ability; reported efficiency rates, however, do vary among studies (20-100%) [282] [298-300], which is in accordance with the low formation rates reported for malignant organoids derived from prostate and esophageal cancer [301] [302]. Malignant endometrial organoids form within 12h-20d, but unlike their healthy/benign counterparts their longevity is compromised with only a subset (20-62%) expanding long-term (>2 weeks) reaching a size of >100µm [282] [298] [299]. These observations are in agreement with the present study which showed a lower formation efficiency for endometrial cancer organoids compared to benign, which could be partly attributed to the fact that malignant endometrial organoid cultures are normally more overcrowded at seeding owing to the cellular abundance from endometrial cancer pipelle biopsies compared to the endometrial sampling of women with benign uterine or non-uterine pathology, which could lead to growth arrest by contact inhibition or antagonism for nutrients. In addition to this, long-term expansion was severely compromised in malignant endometrial organoid cultures with the most resilient lines surviving 1-2 weeks.

The shortened longevity of cancer organoids remains a paradox since malignant cells are considered to escape the Hayflick limit and maintain limitless replicative potential [515] [516]. A plausible explanation could be the differential biochemical needs of cancer stem cells and need for media optimisation. Studies have shown that both endometrial and high-grade ovarian cancer organoids demonstrate longevity in Rspondin-1, Wnt3a and Noggin-depleted media, indicating that Wnt-free environments and increased

BMP signalling preserve cancer stemness potential [298] [517]. Interestingly, triple knockdown (KD) (p53, PTEN, Rb) fallopian tube organoids also prosper in Wnt-independent niches, while Wnt inhibitors were found to be upregulated in HGSOC and KD FT organoids promoting preservation and expansion of the cancer stem cell cargo [518]. Other groups, however, showed that inhibition of the Wnt pathway disrupts ovarian cancer organoid formation [519] [520]. Whether some malignant organoid cultures may benefit from supplementation with selective Wnt family members warrants further investigation.

Even though successful revival post-cryopreservation has been described for endometrial organoids [281] [514], this was not confirmed by the current study. Failure to revive frozen tissue and organoids was probably due to the fact that cells were frozen at -80^{o}C instead of liquid nitrogen, which was the case in reports of successful cryopreservation. Organoid formation and expansion following freezing of digested endometrial glands was accomplished with five benign organoid lines, kindly provided by Prof Jan Brosen's lab at Warwick University, following a protocol of gradual freezing at -80^{o}C for a day, which was succeeded by storage in liquid nitrogen for two years before thawing and plating. The cryoprotectant used did not differ from the one used in the current project.

Morphologically, human endometrial organoids capture parent tissue features as demonstrated by previous reports. In benign endometrial organoids, the glandular architecture is preserved with a single layer of epithelial cells and a central lumen containing hyaline, cellular debris and degenerating cells [279-281]. Tissue-specific molecular and cell polarity markers are also maintained [279-281]. Conversely, malignant endometrial organoids encompass heterogenous organoid populations that mirror the histological type and grade variations of gynaecological cancer. Low-stage and -grade endometrial cancer organoids exhibit a highly discernible lumen with surrounding cells resembling a gland, whereas high-grade and -stage endometrial lesions generate dense cell clusters and cribriform structures lacking a lumen [282] [300]. Endometrial serous adenocarcinoma organoids, on the other hand, demonstrate small buddings that

mirror the native tissue papillary structure [299]. My study corroborated the above-mentioned divergent phenotypes based on grade but no differences were noted based on histotype. The most common appearance for endometrial cancer organoids was that of cell clusters forming a spheroid with no lumen or multiple mini lumens, while glandular structures were also present in a number of cultures, especially in organoids derived from low-grade tumours. Grade is linearly related to the extent of solid areas found in a tumour, therefore high-grade lesions harbour significant solid components as opposed to grade 1 lesions that exhibit less than 5% solid areas and preservation of well-formed glands with atypic nuclei [521]. Therefore, the glandular structures observed in a subset of malignant endometrial organoid cultures either represent mildly disrupted cancerous cells that have retained a glandular configuration or contamination by adjacent healthy tissue given that pipelle biopsies blindly sample the uterine cavity.

Disruption of cell polarity is one of the hallmarks of epithelial cancer which is important for tumour initiation and progression [522]. Endometrial cancer-derived organoids presented with a disordered structure and developed multiple mini-luminal structures with some inter-patient variation in their degree of complexity and the extent of disruption of polarity.

A reliable cell platform for disease modelling also mandates proof of functional relevance to parent tissue. In this regard, the (epi)genetic features, receptors, signalling transduction pathways and gene expression patterns of the precursor tissue have to be interrogated. The Human Cancer Models Initiative (HCMI), constituted by the National Cancer Institute (NCI), Cancer Research UK (CRUK), Hubrecht Organoid Technology (HUB), and Wellcome Sanger Institute (WSI), has committed its efforts to derive hundreds of organoid cell models from colon, oesophageal, pancreatic, lung, breast and ovarian cancers, genomically characterize them and perform drug screens. With regards to endometrial organoids, evidence suggests maintenance of the parental gland genetic make-up in benign organoids and of chromosomal stability from early (P2-4) until late passage (P8-15) [280]. In organoids generated from hyperplastic endometrial tissue of Lynch syndrome patients, the *MSH2* and *MSH6* mutations are

also preserved [282], while a recent study has showed that endometrial cancer organoids recapitulate the mutations of the primary tumour and exhibit disease-specific gene expression [279]. Similarly, in the present study a panel of seven genes *(PTEN, ARID1A, PIK3CA, POLE, CTNNB1, KRAS, TP53)*, commonly mutated in endometrial cancer, was interrogated in paired tissue-organoid samples from eleven patients with endometrial cancer. My results confirmed previous findings that endometrial cancer-derived organoids retain the mutational signature of original lesions [282].

A vital key layer of biological information rests in DNA methylation but a well-concerted effort to epigenetically characterise organoids is still lacking. For the first time in this study, whole-genome DNA methylation profiling using a microarray that interrogated more than 850,000 CpG sites, was performed to evaluate the epigenetic signature of three benign and eleven malignant endometrial organoids in relation to corresponding tissue. My findings showed preservation of the DNA methylation fingerprint in cultured organoids, which remained unaltered over three months of culture in a benign line. Moreover, I observed that endometrial cancer organoid DNA methylation profiles are distinct to benign endometrial organoids and further demonstrate histotype-specific epigenetic traits. Similar independent studies on intestinal, oesophageal, lung, pancreatic and stomach cancer organoids demonstrated stable epigenetic signatures when compared to matching primary tissue [523 524].

6.5 Conclusions

3D endometrial organoids can successfully be derived from pipelle biopsies for most women with an atrophic endometrium or endometrium harbouring a malignant tumour. Organoid formation efficiency is high from benign and malignant endometrial tissue but long-term expansion was only demonstrated for benign organoids with most malignant organoids not surviving beyond second week. Endometrial organoids can reliably be used as research tools of endometrial disease as they closely recapitulate the phenotype, genotype and DNA methylation patterns of primary tissue. This

observation renders 3D endometrial organoids the most physiologically relevant cell system creating extraordinary expectations in disease modelling research.

In the following chapter, endometrial organoids were used to begin to explore the functional role of genital tract microbiota in endometrial cancer with a view this knowledge to be used for microbiome-based therapeutics as adjuncts to existing treatment modalities.

6.6 Statement of Contribution

Bioinformatics analysis of targeted gene sequencing and genome-wide DNA methylation profiling data was conducted by bioinformaticians, Dr Richard Williams and Dr Nadia Fernandes.

CHAPTER 7.

Role of female genital tract microbiota in endometrial organoid proliferation and inflammation

---

Content from this chapter is currently under preparation as:

Semertzidou A, Smith A, Brosens J, Marchesi J, Bennett P, MacIntyre D and Kyrgiou M. The role of genital tract microbiota continuum in endometrial cancer. *(Under preparation)*

7.1 Introduction

The route from observational to functional studies is pivotal to robustly establish causal relationships and disclose ways of intervention that could help improve clinical outcomes. Even though there is currently a solid body of evidence on eubiotic and dysbiotic microenvironments in human cancer, mechanistic studies exploring cause and effect are still lacking.

Microbiota actions mediated by bacterial cell wall components (e.g., LPS, LTA), metabolites (e.g., SCFAs) and signalling molecules encoded by the bacterial genome could exert either a beneficial or harmful effect on the host. Pathogen antagonism, bacteriocin production and pH regulation are the most comprehensively characterised actions of the genital microbiota but emerging evidence of microbial ability to alter host genome expression and exert a tumour-stimulative or suppressive role is rapidly gaining ground. Host function is modulated by micro-organisms through multiple mechanisms, such as by modifying epigenetic, transcriptomic, metabolomic, immune and cell survival processes [525].

In the wider gynaecological oncology scene, functional studies have cast light on the ways microbes might employ to influence carcinogenic pathways. A study demonstrated that *Lactobacillus* products, L- and D-lactate, enhance DNA repair and modulate the resistance of cervical carcinoma cells to anticancer drugs via histone deacetylase inhibition and hydroxycarboxylic acid receptor 1 activation [249]. Łaniewski reported that local immune checkpoint proteins in cervicovaginal lavages of cervical cancer patients are associated with *Lactobacillus* dominance with programmed cell death ligand 1 (PD-L1) and lymphocyte activation gene 3 (LAG-3) levels showing a negative, whereas Toll-like receptor 2 (TLR2) a positive correlation [526]. Metabolomic studies have also linked specific metabolites, such as anti-inflammatory nucleotides, with *Lactobacillus* dominance independently of the severity of the cervical neoplasm [527]. In ovarian cancer, functional inferences based on microbiome data revealed forty-six significantly different KEGG pathways in the ovarian bacteria of the cancer group

compared to the controls [472]. In endometrial cancer microbiome research, transcriptomic analysis has identified 8 robust associations between *Prevotella* and fibrin degradation-related genes expressed within endometrial tumours as well as serum D-dimer (DD) and fibrin degradation products (FDPs) [496].

The microbiota-associated local cytokine profiles in gynaecological malignancy have been the subject of intense scrutiny. Dysbiotic, non-*Lactobacillus* dominance has been associated with several proinflammatory (IL-36γ), chemotactic (IFNγ-induced protein 10 (IP10), macrophage-inflammatory protein 1β (MIP1β) and RANTES, haematopoietic (FLT3 ligand) and adaptive immune response cytokines (IL-2, IL-4, soluble CD40 ligand) in women with cervical cancer or dysplasia [528]. Cancer biomarkers, including growth and angiogenic factors (e.g., basic fibroblast growth factor (FGF2), stem cell factor (SCF), apoptosis-related proteins (soluble Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL)), hormones (prolactin), proinflammatory cytokines and chemokines (macrophage migration inhibitory factor (MIF) and TNF) and osteopontin have also been positively correlated with non-*Lactobacillus* dominance [529]. Furthermore, *Fusobacterium* spp. have been associated with elevated cervical expression of anti-inflammatory IL-4 and transforming growth factor β1 (TGFβ1) promoting an immunosuppressive microenvironment [530]. In a study using endometrial adenocarcinoma cell lines, *Lactobacillus vaginalis* was found to induce the production of IL-8, whereas *Atopobium vaginae* and *Porphyromonas somerae* induced the production of IL-1α, IL-1β, IL-17α, and TNFα, but not IL-8. Collectively, these data highlight the multi-faceted interaction of microbes with immune host functions laying the groundwork for the systematic pursuit of underlying mechanisms.

Endometrial cancer is thought to arise in an environment of chronic, low-grade inflammation induced by free fatty acids and increased LPS circulating levels secondary to increased gut permeability in obese patients. Following establishment of endometrial cancer, cytokines are secreted by cancer cells, the tumour microenvironment and infiltrating immune cells but their role in oncogenic processes

remains enigmatic. In this chapter, I describe a preliminary study designed to begin to test the hypothesis that *L. crispatus*, which often dominates the benign vagina, cervix and in some women, the endometrium, and was found to be depleted in respective locations of endometrial cancer patients, has an anti-inflammatory, anti-mitogenic effect on endometrial organoids.

7.2 Aim

- To investigate the mechanistic interplay between endometrial microbiota and endometrial carcinogenesis by evaluating the impact of *L. crispatus* on endometrial organoid proliferation and inflammation.

7.3 Results

7.3.1 Patient and clinical characteristics

Glandular organoids were derived from the endometrium of five benign and six endometrial cancer patients. The benign samples involved patients attending the miscarriage clinic, either for recurrent miscarriage or recurrent *in vitro* fertilisation failure (Table 7.1).

Table 7.1. Recruits for the investigation of interactions between endometrial microbiota and endometrial organoids.

ID	Age	Biopsy	Benign/Malignant	Pathology
AO51	72	Endometrial	Malignant	Mixed endometrioid + serous endometrial ca, high grade
AO54	55	Endometrial	Malignant	EEC, Grade 2
AO55	54	Endometrial	Malignant	EEC, Grade 1
AO57	57	Endometrial	Malignant	EEC, Grade 2
AO62	58	Endometrial	Malignant	EEC, Grade 1
AO64	83	Endometrial	Malignant	EEC, Grade 1
AO67	32	Endometrial	Benign	RM
AO68	37	Endometrial	Benign	RIVF
AO69	32	Endometrial	Benign	RM
AO70	37	Endometrial	Benign	RM
AO71	39	Endometrial	Benign	RIVF

EEC: Endometrioid endometrial cancer, **RM:** Recurrent miscarriage, **RIVF:** Recurrent In Vitro Fertilisation failure

7.3.2 Effect of *L. crispatus* on endometrial organoid proliferation

L. crispatus supernatant was added to organoid culturing medium and the viability of endometrial organoids was assessed at increasing concentrations of *L. crispatus*-conditioned (LCC) media (10%, 20%, 30%, 50% v/v). Concentrations of 50% v/v LCC was lethal across all endometrial organoid lines within two days (data not shown), and therefore 10% v/v, 20% v/v and 30% v/v concentrations were used for further experiments. Benign endometrial organoids demonstrated a trend towards increased proliferation at 10% v/v LCC and decreased proliferation dynamics at 20% and 30% v/v concentrations but this did not reach significance (Figure 7.1A). In malignant organoids, proliferation showed an inverse relationship with increasing LCC concentrations at 48h, irrespective of *L. crispatus* origin (*L. crispatus* vaginal isolate from a recruited patient or commercial isolate), but this trend reached statistical significance only for the 30% v/v concentration (vaginal isolate: 0.0009; commercial isolate: 0.0003) (Figure 7.1D).

I next constructed an inflammation model using 1µg/mL *E.coli*- derived LPS (O111:B4) to treat benign endometrial organoids for 24h to bio-mimic the chronic inflammation that precedes endometrial cancer development in obese patients. Firstly, I showed that benign and malignant organoids express TLR-4 (Toll-like receptor 4), the receptor for LPS (Figure 7.1D). Following treatment of benign endometrial organoids with LPS and co-incubation with LCC for 24h, I observed that organoid proliferation was significantly reduced in co-incubation of LPS and 30% v/v LCC (p= 0.0097) but unaltered when LPS alone was used or combined with other LCC concentrations (10%, 20% v/v) (Figure 7.1B). To delineate whether the impact on cell proliferation was driven by the *L. crispatus* metabolites themselves or the MRS broth in which they were secreted, I performed the same experiment incubating benign organoids with MRS broth alone, which also revealed significantly decreased proliferation in the 30% v/v concentration (p= 0.027) (Figure 7.1C).

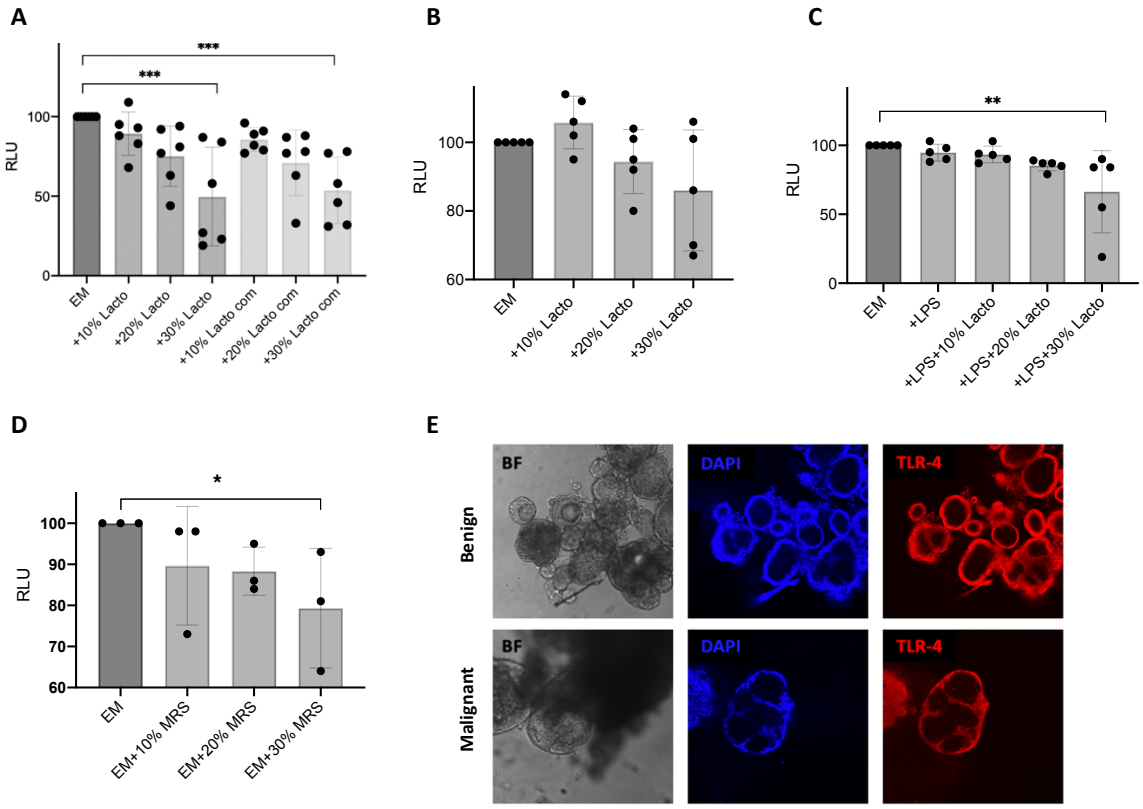


Figure 7.1. Endometrial organoid viability in response to various treatments. A. Endometrial cancer organoids treated with increasing concentrations of *L. crispatus*-conditioned media for 48h (n=6). **B.** Benign endometrial organoids treated with increasing concentrations of *L. crispatus*-conditioned media for 48h (n=5). **C.** Benign endometrial organoids treated with LPS with or without increasing concentrations of *L. crispatus*-conditioned media for 24h (n=5). **D.** Benign endometrial organoids treated with increasing concentrations of MRS broth for 48h (n=3). **E.** Confocal microscopy in benign and malignant endometrial organoids stained for TLR-4 expression. RLU: Relative light units, EM: Expansion medium, Lacto: *L. crispatus* isolated from patient vaginal swab, Lacto com: commercially available *L. crispatus*. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001; adjusted p values calculated by Dunn's multiple comparisons test.

7.3.3 Effect of *L. crispatus* on endometrial organoid inflammation

I next investigated the impact of LCC media on the constitutive and LPS-induced cytokine secretion of endometrial organoids. Organoids were grown in expansion medium supplemented with 10%, 20% and 30% v/v LCC medium and secreted cytokines were detected in culture supernatant after 48h. Basal cytokine secretion of

IL-1β, IL-6, IL-8, TNF-α by malignant organoids was higher than benign organoids but only IFN-γ reached statistical significance (p= 0.0286) (Figure 7.2). For endometrial cancer organoids, the addition of *L. crispatus* metabolites at any concentration did not significantly alter the basal secretion of IL-6, IL-8, TNF-α, IFN-γ, IL-1ra, IL-2, G-CSF, GM-CSF, VEGF and chemokine CCL5/RANTES. A significant increase of chemokine CCL4/MIP1beta was noted in response to 10% v/v LCC medium (p= 0.0105), of CCL4/MIP1beta (p= 0.0105) and IL-1β (p= 0.0048) in response to 20% v/v LCC medium and a significant decrease of GM-CSF was observed in the 30% vs 10% v/v concentration (p= 0.0437) (Figure 7.3A). When endometrial cancer organoids were treated with supernatant of commercially available *L. crispatus*, none of the cytokines/chemokines was significantly affected (Figure 7.3B). Similarly, for benign endometrial organoids the secretion of IL-6, IL-8, IL-10, TNF-α, IFN-γ remained unaffected following 10% and 20% v/v LLC medium supplementation. IL-1β, on the other hand, increased significantly in 20% versus 10% v/v LCC media (p= 0.037), while 30% v/v LCC significantly increased TNF-α (p= 0.037) and decreased IL-10 secretion (p= 0.0155) (Figure 7.3C). I next constructed an inflammation model using 1μg/mL *E. coli*- derived LPS (O111:B4) to treat benign endometrial organoids for 24h to mimic the chronic inflammation that precedes endometrial cancer development. Organoids were co-incubated with increasing LCC media concentrations but no significant changes were noted in the inducible secretion of IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ (Figure 7.3D).

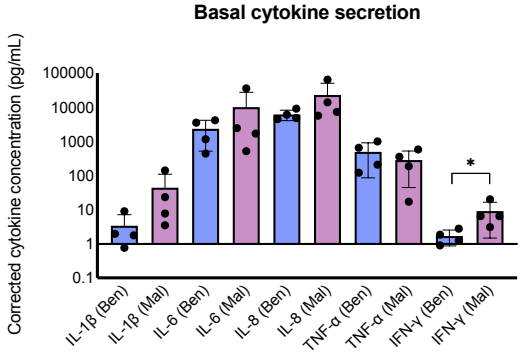


Figure 7.2. Comparison of basal cytokine secretion by benign and endometrial cancer organoids after 48h of culture. *Ben: Benign, Mal: Malignant*. * p-value < 0.05.

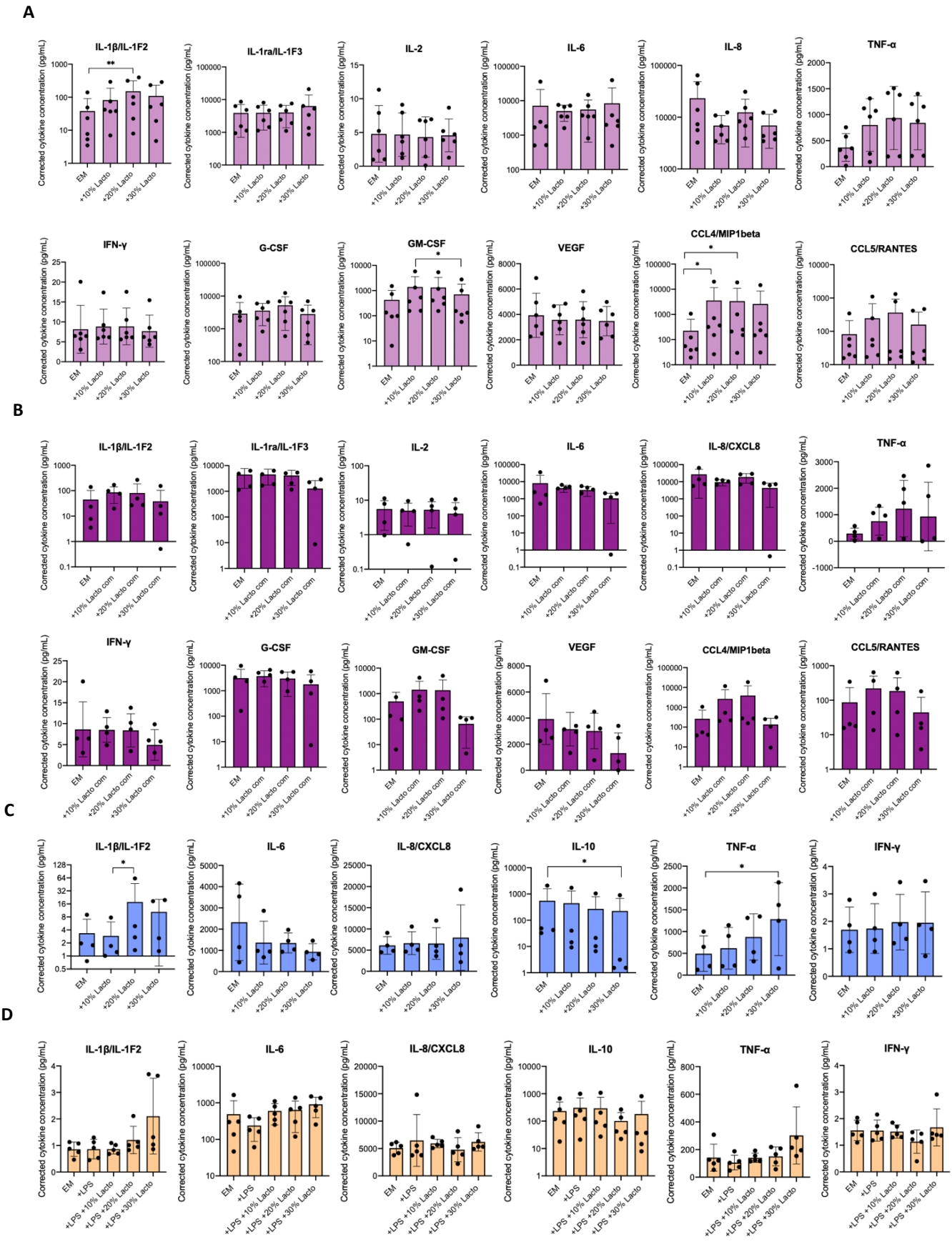

A
IL-1β/IL-1F2
IL-1ra/IL-1F3
IL-2
IL-6
IL-8
TNF-α
IFN-γ
G-CSF
GM-CSF
VEGF
CCL4/MIP1beta
CCL5/RANTES
Corrected cytokine concentration (pg/mL)
EM
+10% Lacto
+20% Lacto
+30% Lacto
B
IL-1β/IL-1F2
IL-1ra/IL-1F3
IL-2
IL-6
IL-8/CXCL8
TNF-α
IFN-γ
G-CSF
GM-CSF
VEGF
CCL4/MIP1beta
CCL5/RANTES
EM
+10% Lacto com
+20% Lacto com
+30% Lacto com
C
IL-1β/IL-1F2
IL-6
IL-8/CXCL8
IL-10
TNF-α
IFN-γ
EM
+10% Lacto
+20% Lacto
+30% Lacto
D
IL-1β/IL-1F2
IL-6
IL-8/CXCL8
IL-10
TNF-α
IFN-γ
EM
+LPS
+LPS +10% Lacto
+LPS +20% Lacto
+LPS +30% Lacto

Figure 7.3. Cytokine/Chemokine secretion in culture supernatant by endometrial organoids in response to treatments. A. Endometrial cancer organoids grown in increasing concentrations of *L. crispatus*-conditioned media for 48h (n=6). *L. crispatus* was isolated from patient vaginal swab. **B.** Endometrial cancer organoids grown in increasing concentrations of *L. crispatus*-conditioned media for 48h (n=4). Commercially available *L. crispatus* used. **C.** Benign endometrial organoids grown in increasing concentrations of *L. crispatus*-conditioned media for 48h (n=4). **D.** Benign endometrial organoids treated with LPS and increasing concentrations of *L. crispatus*-conditioned media for 24h (n=5). EM: Expansion medium, Lacto: *L. crispatus* isolated from patient vaginal swab, Lacto com: commercially available *L. crispatus*. * p-value < 0.05, ** p-value < 0.005; adjusted p values calculated by Dunn's multiple comparisons test.

7.4 Discussion

Endometrial cancer is thought to arise in an environment of chronic, low-grade inflammation characterised by an increase of pre-diagnostic circulating levels of CRP, IL-6, TNF-α, sTNFR1 and sTNFR2 and IL-1Ra (EPIC cohort) [75 531], which have also been linked to high BMI [311 532], highlighting the interrelationship of the obesity-inflammation-endometrial carcinogenesis axis. Following establishment of endometrial cancer, cytokines are secreted by cancer cells, the tumour microenvironment and infiltrating immune cells but their role in oncogenic processes remains ambiguous. Cytokine involvement in cancer progression manifested as local growth or distant metastasis has been documented in the literature [533] alongside the ability of non-steroidal anti-inflammatory agents to reduce tumour formation in several mouse models and in patients at high risk for colon carcinoma [534 535].

To investigate the potential role of altered microbiota in endometrial carcinogenesis, *L. crispatus* was selected as a candidate health-promoting microbe, given that it is significantly depleted along the genital tract (vagina, cervix, endometrium) of endometrial cancer versus benign patients and substantial evidence suggests that commensal *Lactobacillus* species have anti-inflammatory and anti-cancer properties [248-251 489-495 536-540]. *L. crispatus* culture supernatant was supplemented to the medium used for benign and malignant endometrial organoid cultures.

Exposure of malignant endometrial organoids to *L. crispatus*-conditioned media led to reduced viability, which was not observed in benign endometrial organoids to the same extent. Viability of benign endometrial organoids was compromised when high concentrations of LCC media were combined with LPS treatment. Reduced viability at increased LCC media concentrations could be partly attributed to the MRS broth contained in LCC media, which was used as a control. It is unclear whether MRS broth is poorly tolerated by endometrial organoids or it is the dilution of expansion medium ingredients at 30% v/v concentrations that restricts proliferation and/or induces lethality. Similarly, Shiroda *et al.* evaluated the effect of different alive *Lactobacillus* species on telomerase-immortalized human endometrial stromal cells (T-HESC) and showed that *L. crispatus* attached to host cells but did not increase host cell death. Conversely, the anti-proliferative and pro-apoptotic effects of *Lactobacillus* species and their metabolites on colon cancer cells have also been documented in literature [250 494 495].

The potential intersection of *L. crispatus* with inflammatory pathways as reflected by cytokine secretion was next interrogated. A set of ten cytokines (IL-1β, IL-1ra, IL-2, IL-6, IL-8, G- CSF, GM- CSF, IFN-γ, TNF-α) and two chemokines (CCL4/MIP1beta, CCL5/RANTES), known to be secreted by endometrial tumours [541], were measured in endometrial cancer organoids and six cytokines (IL-1β, IL-6, IL-8, IL-10, IFN-γ, TNF-α), commonly secreted in response to inflammatory stimuli, were selected for benign organoids, which were left unstimulated or treated with LPS. I found that IL-1ra, IL-2, IL-6, IL-8, G- CSF, GM- CSF, IFN-γ and CCL5/RANTES levels in malignant organoids remained unchanged in response to LCC-supplemented medium irrespective of *L. crispatus* origin (own patient-derived or commercial), while an increase was noted for IL-1β and CCL4/MIP1beta. A study using *Lactobacillus vaginalis* on a human endometrial adenocarcinoma cell line noticed over-production of only IL-8 from a number of cytokines tested [542].

In benign endometrial organoids, the constitutive secretion of IL-1β, IL-6, IL-8 and IFN-γ in response to LCC medium remained unaltered, while a significant decrease was

observed for IL-10, implying that *L. crispatus* metabolites do not exert a pro- or anti-inflammatory action on endometrial cells. Other studies have also investigated the immunomodulatory effect of *Lactobacillus* species on endometrial cells. The Shiroda study concluded that in human endometrial stromal cell lines, total levels of p38, a key Mitogen Activated Protein Kinase (MAPK) for the production of TNF-α and COX-2, were reduced in the presence of *Lactobacillus* species, *L. crispatus*, *L. gasseri*, *L. reuteri*, while phosphorylated p38 demonstrated a reduced trend without reaching statistical significance. A study on bovine endometrial cells showed that *Lactobacillus ruminis* and *Lactobacillus amylovorus* are able to induce increased IL-1A, IL-6, IL-8 and prostaglandin-endoperoxide synthase 2 mRNA levels, while *Lactobacillus buchneri* did not significantly influence cytokine expression [543]. Taken together this evidence, it can be argued that the immunomodulatory effects of *Lactobacillus* might be species- or even strain-specific.

TNF-α was the only cytokine consistently increased in response to *L. crispatus* conditioned media, in both cancer and benign organoids reaching, however, statistical significance only for the latter. Schirmer *et al.* found that the gut microbiome accounts for 10% of inter-individual systemic cytokine variability and TNF-α appears to be the cytokine more strongly influenced by the gut microbial structure [544]. In accordance with the current study, intrauterine administration of *Lactobacillus buchneri* in cows showed higher TNF levels and increased infiltration with immune cells versus placebo after one week of administration [545].

Treatment of organoids with LPS to mimic the inflammatory cues that benign endometrial cells receive prior to cancerous conversion failed to induce a significant inflammatory reaction, possibly due to low LPS concentration or organoids being refractory to the LPS type used.

The impact of other bacterial taxa on endometrial cytokine expression has also been explored in literature. Caselli *et al.* tested the effect of *Atopobium vaginae* and *Porphyromonas somerae* on the expression of pro-inflammatory

cytokines, determined by ELISA, in HEC-1A cells (human endometrial adenocarcinoma cells) [542]. The authors showed that both bacteria, when alive, were able to induce production of IL-1α, IL-1β, IL-17α and TNF-α after 24h with no further increase at later timepoints, while killed bacteria had no effect on cytokine release. Real-time PCR confirmed the transcriptional upregulation of above cytokines as well as chemokines CCL13, CCL8, CXCL2, IL22, and IL9. In another study, Lu et al. found that IL-6, IL-8 and IL-17 endometrial mRNA levels were significantly higher in endometrial cancer versus benign controls and that the relative abundances of *Micrococcus* were positively correlated with IL-6, and IL-17 mRNA levels [473].

Collectively, this evidence suggests that inflammatory responses to bacterial stimuli might be species- and cytokine-specific, even though data assessment from different studies should take into account the cell type used (e.g., glandular vs stromal endometrial, primary vs cell lines, benign vs malignant), cell source (e.g., human vs animal), bacterial species and strains, alive/killed bacteria vs bacterial metabolites used as stimuli and differential experimental conditions (e.g., monolayers vs 3D organoids, controls used). For example, bacterial viability/non-viability is recognised by the host through distinct immune pathways [546], while the mode of bacterial killing, whether heat-or antibiotic-killed, incites divergent inflammatory responses [547]. Moreover, cellular dimensionality determines inflammatory responses, as illustrated by a study showing that the 3D culture environment upregulates both sensitivity and inflammatory response to TNF-α in human umbilical vein endothelial cells (HUVECs) compared to 2D cultures [548].

7.5 Conclusions

L. crispatus-conditioned medium significantly decreases malignant endometrial organoid viability at high concentrations and benign endometrial organoid viability when combined with LPS, even though to a lesser extent. LCC medium does not significantly affect cytokine production in neither benign nor malignant endometrial organoids, implying that even when lactobacilli reside in the endometrium, they have

minimal or no role to play in inflammatory signalling. However, this observation does not preclude that *L. crispatus* or other bacterial species could influence other signalling pathways, e.g., metabolic/oestrogen, locally or could have an impact on infiltrating immune cell cytokine secretion, which could subsequently exert an action on endometrium. Transitioning from reductionist cell models to more comprehensive, physiologically relevant cell systems that capture the cellular diversity of organs, including stromal, mesenchymal and immune cells, is mandatory to reliably assess the functional role of resident microbiota and devise ways of manipulation that could restore tissue homeostasis.

CHAPTER 8. General Discussion

8.1 Summary of hypotheses

Endometrial cancer is the most common cancer of the female genital tract in higher-income countries [549] [550] but its aetiology remains a puzzle with many missing pieces. Obesity and diabetes have long been acknowledged as metabolic drivers, while risk factors, such as unopposed oestrogen exposure, tamoxifen use, nulliparity, infertility and late menopause highlight the hormonal elements of disease pathogenesis. Genetic predisposition has also been recognised in Lynch and Cowden syndrome patients, whereas more recent work has uncovered cancer susceptibility candidate genes [551] as well as several genomic loci harbouring common low-risk variants [552]. The role of the genital tract microbiome in endometrial cancer has only recently been suggested and intensive microbiome research on a variety of diseases over the last decade has brought the depth and breadth of microbe-host interactions within our conceptual and observational reach.

The overarching hypothesis of this work was that endometrial cancer pathogenesis is influenced by metabolic, microbial and inflammatory cues. Therefore, the first aim was to assess the strength of evidence available in literature suggesting a link between diabetes or anti-diabetic interventions and endometrial cancer. Despite an abundance of epidemiological studies interrogating the relationship between diabetes and incidence/mortality of endometrial cancer, robust statistical methods are required to evaluate bias and validity of reported outcomes. The umbrella review conducted was expanded to also include other gynaecological and obstetric outcomes. The next step was to explore the presence of a genuine microbial signature in the upper female genital tract above background contamination, which inevitably impacts on findings from low bacterial biomass sites. The microbiome of endometrium, fallopian tubes and ovaries of patients with benign uterine or extra-uterine pathology was investigated alongside the endometrium of endometrial cancer patients. Subsequently, a potential continuum in bacterial colonisation along the length of female genital tract and correlations between vaginal-rectal microbiota were sought to uncover bacterial seeding among sites. The next aim was to characterise and compare the genital tract

and rectal microbiome of endometrial cancer patients and benign controls and identify microbial signatures based on histological type and grade of cancer. To investigate the functional role of microbiota in endometrial oncogenesis, endometrial cancer organoids were developed as a tool for disease modelling. The morphological, genetic and epigenetic traits of matched organoid-tissue pairs were compared to interrogate the physiological relevance of the model. Finally, the last aim was to examine whether genital tract resident bacteria can impact on proliferation and inflammatory pathways of benign and endometrial cancer organoids thus intersecting with oncogenic processes.

8.2 Summary of methods

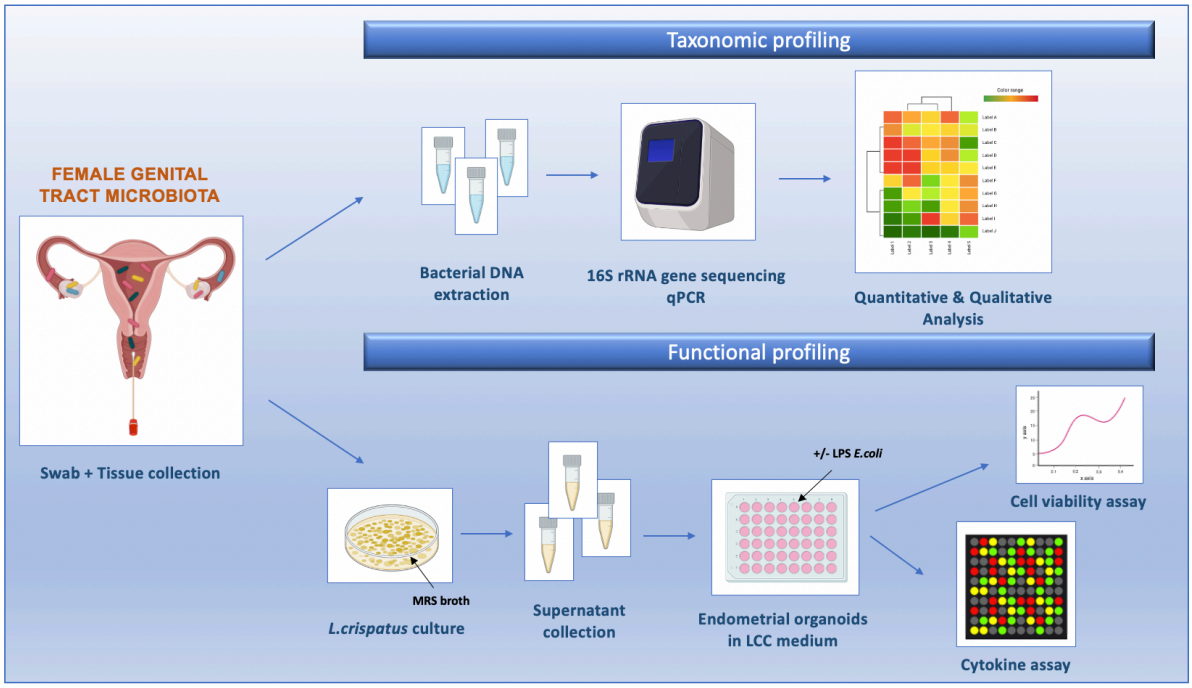


Figure 8.1. Overview of the experimental workflow for the taxonomic and functional profiling of female genital tract microbiota. Microbiome swab and tissue samples were collected from the female genital tract and rectum of endometrial cancer patients and benign controls. Bacterial DNA was extracted and used for qPCR and 16S rRNA gene sequencing to determine the absolute bacterial quantities and composition of microbial communities. A patient-derived *L. crispatus* isolate was cultured and the supernatant was used to supplement the culture medium of benign and malignant endometrial organoid cultures, with or without LPS co-treatment. Organoid viability and cytokine secretion were measured to determine the downstream effects of *L. crispatus*-conditioned medium on endometrial organoids. LCC: *L. crispatus* conditioned.

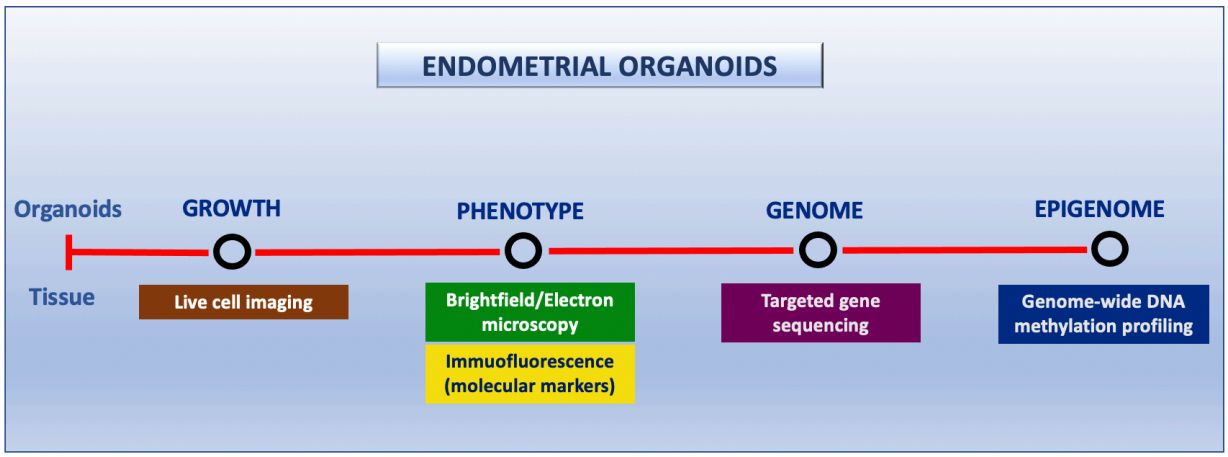


Figure 8.2. Endometrial organoid project workflow. Experiments performed for the characterisation of benign and malignant endometrial organoids and the investigation of their morphological and (epi)genetic resemblance to parent tissue.

8.3 Summary of findings

8.3.1 Diabetes and anti-diabetic interventions and the risk of gynaecological and obstetric morbidity: an umbrella review of the literature

Key findings

- Diabetes increases the risk of endometrial cancer (suggestive association).
- Metformin improves endometrial cancer survival compared to other anti-diabetic drugs (suggestive association).
- Diabetes increases endometrial cancer mortality (weak association).
- Diabetes increases the risk of pre-malignant/malignant endometrial polyps (weak association).

This umbrella review evaluated 117 meta-analyses of observational studies and 200 meta-analyses of randomised clinical trials on the association between diabetes/anti-diabetic interventions and gynaecological or obstetric morbidity. Strong correlations i.e., strongly statistically significant results and no evidence of bias, were only revealed for obstetric outcomes (CS, LGA) and gestational diabetes. The association between diabetes and increased incidence of endometrial cancer was supported by suggestive evidence, which was also the case for the association between metformin and better endometrial cancer survival. On the other hand, the evidence on the connection between diabetes and increased endometrial cancer mortality was classified as weak. Similarly, the association of diabetes with premalignant/malignant endometrial polyps was only supported by weak evidence.

The underlying mechanisms of the diabetes-endometrial cancer interplay remain largely obscure. The current hypothesis suggests that insulin resistance causes hyperinsulinaemia that activates the insulin-like growth factor-1 (IGF-1) signal transduction pathway, which in turn activates the PI3K/mTOR pathway stimulating cell proliferation and inhibiting apoptosis [90-94 96 105]. Additionally, insulin may impact

on endometrial cancer development by reducing blood concentrations of sex-hormone-binding globulin (SHBG), thus increasing oestrogen bioavailability [553].

8.3.2 Female genital tract microbiota: genuine signatures in low biomass sites, continuum and correlation with rectal microbiota

Key findings

- A subset of patients, whether benign or endometrial cancer, harbours microbiota in the upper genital tract that are quantitatively and compositionally distinguishable from background contaminants.
- Laparoscopic hysterectomy practices introduce cross-site contamination in one quarter to one third of cases.
- Epithelial surfaces are more heavily colonised with microbes compared to underlying tissue.
- The most abundant species of the lower genital tract also colonise the upper genital tract in benign patients but continuum is less cohesive in endometrial cancer patients.
- Vaginal microbiota display poor correlation with rectal microbiota.

Following integration of technical controls for environmental contaminants and stringent decontamination analyses, 15% of total sequence data was discarded as background DNA contamination. The presence of a low-abundance microbiome was confirmed in the endometrium, fallopian tubes and ovaries comparable to the bacterial biomass of controls, which was one-four orders of magnitude lower than the heavily colonised vagina, cervix and rectum. A bacterial load above that of controls was observed in 62% of benign endometrium, 50% of malignant endometrium, 85% of benign fallopian tube and 95% of benign ovary. Compositionally, the malignant endometrium displayed a bigger bacterial overlap with controls, hinting that endometrial cancer patients may not harbour a true microbiome in their uterus. Cross-site contamination during laparoscopic hysterectomies, involving the insertion of a uterine manipulator and vaginal retrieval of specimen, was observed in one quarter to

one third of cases and needs to be considered when analysing taxonomic data from low-biomass sites. Bacterial quantities in tissue biopsies were lower in relation to swabs that sampled the mucosa, suggesting that few microbes reside in the underlying tissue, which were also compositionally dissimilar to swabs in two thirds of cases.

A microbial continuum along the length of the female genital tract from vagina to ovary was noted in 75% of patients with benign conditions for the most abundant vaginal species. Conversely, in endometrial cancer patients only 7.2% of the most abundant vaginal and cervical bacterial species were also shared by the endometrium. Intra-individually, microbial composition is highly correlated between lower and higher vagina but less correlated between lower and fundal endometrium, highlighting the importance of researchers clearly stating the sampling site in taxonomic studies. Lastly, vaginal microbiota of benign patients were poorly correlated with matched rectal microbiota, while endometrial cancer patients demonstrated stronger, yet still limited, phylogenetic affiliations. It is therefore unlikely that the vagina is seeded, at least in its entirety, by the rectal microbiome.

8.3.3 Comparison of female genital tract and rectal microbiota in endometrial cancer patients and benign controls

Key findings

- Cervicovaginal and rectal bacterial load is reduced in endometrial cancer, but endometrial bacterial load does not differ to benign controls.
- *Lactobacillus*-depletion, high microbial diversity and enrichment of *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus* in the lower genital tract and endometrium are characteristics of endometrial cancer patients.
- No compositional differences are observed in cervicovaginal and endometrial microbiota according to histotype and grade of endometrial cancer.
- Rectal depletion of *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* is noted in endometrial cancer.

Bacterial copies, as confirmed by qPCR, from the vagina (both lower 2/3 and higher 1/3), cervix and rectum of endometrial cancer patients were 1-2 log_{10} lower than benign patients with the strongest differences observed in the higher vagina and cervix. No significant differences were noted in the endometrium, fundal or lower, of women with or without malignancy, where bacterial quantities were comparable to those of environmental controls. Hierarchical clustering analysis (HCA) at genera level revealed *Lactobacillus*-dominance (≥75% relative abundance) and *Gardnerella/Lactobacillus*-dominance (≥50%/40%) among benign patients in the vagina, cervix and endometrium, while High diversity & Other (defined as high α-diversity or predominance of other genera) was the most prevalent cluster among endometrial cancer patients, which was also accompanied by *Lactobacillus* depletion and enrichment of *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus*. At species level, *Lactobacillus crispatus* accounted for the majority of *Lactobacillus* abundance at all sites (vagina, cervix, endometrium), while *Gardnerella vaginalis* was the second most abundant bacterial species across all samples. α- and β-diversity in endometrial cancer patients were significantly increased to benign controls in the lower and upper genital tract at genera and species level.

Greater microbial resemblance was observed between benign samples and endometrioid/ grade I endometrial tumours in the vagina and cervix than non-endometrioid/ high-grade lesions. Even though there was a trend towards *Lactobacillus* (*L. crispatus*) and *Bifidobacterium* (*B. breve*) depletion and *Anaerococcus* (*A. lactolyticus*) enrichment in the non-endometrioid/ high-grade samples in relation to endometrioid/ grade I samples in vagina, cervix and endometrium, this observation did not reach statistical significance. A bigger sample size might be required to reveal true relationships. Finally, the rectum of endometrial cancer patients was significantly depleted of *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* versus benign controls.

The only confounder that was significantly different between the two comparison arms was age, with the majority of benign patients falling into the 50-64 age range, while

patients with endometrial malignancy were predominantly ≥65. Whether alterations in microbial quantities and composition are secondary to age or whether they precede or follow the development of endometrial cancer is still debatable and mandates further longitudinal studies to record intra-patient colonisation patterns over time.

8.3.4 Validation of endometrial organoids as tools in gynaecological research

Key findings

- Human benign and malignant endometrial organoid cultures can be successfully derived from pipelle biopsies.
- Organoid derivation efficiency is not affected by age, menopausal status, histological type/ grade of endometrial cancer.
- Both benign glandular and endometrial cancer organoids exhibit high formation efficiency, but the latter do not usually survive beyond the second week.
- Benign glandular and endometrial cancer organoids recapitulate the phenotypical features and DNA methylation signature of parent tissue.
- Endometrial cancer organoids retain the mutational landscape of progenitor tissue.

Human endometrial glandular organoids were successfully generated from pipelle biopsies collected post-operatively from uteri specimens of women undergoing hysterectomy for benign conditions or endometrial cancer. Organoids were grown in Matrigel and culture medium containing a cocktail of factors, such as Nicotinamide, Noggin, Rspondin-1, B27 supplement, N2 supplement, N-acetyl-L-cysteine, A83-01, Y-27632, EGF, FGF-10 and HGF. Organoid derivation efficiency was unaffected by age and menopausal status and organoid generation was feasible from all histological types and grades of endometrial cancer. Organoid formation efficiency was 100% from benign endometrial organoids, organoids were able to self-organise in 6 hours, proliferate and self-expand for longer than 3 months. On the other hand, organoid formation efficiency was 85% from malignant endometrial tissue, tumoroids self-organised within 12-24 hours after plating and their long-term expansion was severely

compromised with the most sustainable lines surviving 1-2 weeks. Positivity for glandular markers confirmed the epithelial origin of cultured organoids, while cell polarity markers demonstrated disturbed polarization in endometrial cancer organoids.

Morphologically, organoids derived from benign endometrium displayed a glandular morphology with a discernible central lumen and a single layer of epithelial cells surrounding the lumen. Glandular-like structures reminiscent of benign organoid morphology were encountered primarily in low grade (Grade 1) lesions, whilst dense cellular aggregates (spheroids) or multi-lumen organoids predominated in organoids derived from high grade (Grade 2-3) lesions, suggesting that the morphological heterogeneity of malignant organoids mirrors the histological grade variations of gynaecological cancer. Genomically, endometrial cancer-derived organoids retained the mutations of the *PTEN, ARID1A, PIK3CA, POLE, CTNNNB1, KRAS,* TP53 genes when compared to matched tissue. DNA methylation of benign and malignant endometrial organoids also closely recapitulated that of original tissue with very few differentially methylated genomic regions. Overall, cytoarchitecture, mutations and DNA methylation signatures were largely maintained in endometrial organoids, thus building confidence to the model and laying the groundwork for the multi-omic characterisation of endometrial organoids.

8.3.5 Role of female genital tract microbiota in endometrial organoid proliferation and inflammation

Key findings

- *L. crispatus*-conditioned (LCC) medium significantly decreases malignant endometrial organoid viability at high concentrations.
- In endometrial cancer organoids, the basal secretion of IL-6, IL-8, TNF-α, IFN-γ, IL-1ra, IL-2, G-CSF, GM-CSF, VEGF and CCL5/RANTES remains unchanged in response to LCC medium, while an increase of CCL4/MIP1beta and IL-1β is noted.

- In benign organoids, the basal secretion of IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-γ remains unaffected following LLC medium supplementation, while high LCC medium concentrations significantly increase TNF-α and decrease IL-10 secretion.

L. crispatus was selected as a candidate health-promoting microbe considering that it was significantly depleted in the vagina, cervix and endometrium of endometrial cancer patients. The supernatant of *L. crispatus* cultures, containing MRS broth with the metabolites and small molecules produced by *L. crispatus*, was added to organoid cultures at increasing concentrations. Toxic effects were observed at ≥50% v/v LCC supplementation, which could also be ascribed to the dilution effect on culture medium. The viability of endometrial cancer-derived organoids was significantly reduced in response to high LCC medium concentrations (30% v/v), which was lower compared to benign organoid viability and controls (plain MRS broth), suggesting that *L. crispatus* might have an anti-proliferative or cytotoxic effect against endometrial cancer cells.

Given that endometrial cancer is considered to develop in a chronic, low-grade inflammatory context, the effect of *L. crispatus* on basal and LPS-induced cytokine production by benign and malignant endometrial organoids was inquired. In endometrial cancer organoids, the basal secretion of IL-6, IL-8, TNF-α, IFN-γ, IL-1ra, IL-2, G-CSF, GM-CSF, VEGF and CCL5/RANTES remained unchanged in response to LCC medium, while an increase of CCL4/MIP1beta and IL-1β was noted. In benign organoids, the basal secretion of IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-γ remained unaffected following LLC medium supplementation, while high LCC medium concentrations significantly increased TNF-α and decreased IL-10 secretion. A pro-inflammatory model was devised by using LPS to mimic the inflammatory stress that endometrial cells undergo before malignant conversion. Additionally, benign endometrial organoids were co-treated with LCC medium to monitor the potential of *L. crispatus* to mitigate inflammation and preserve homeostasis. No cytokine response was observed in LPS-treated organoids, suggesting that a low LPS concentration was used or organoids were refractory to the type of LPS used. Collectively, these data

show that *L. crispatus*, a female genital tract commensal, either does not intersect with inflammatory pathways in endometrial cells or has a selective pro-inflammatory action impacting on specific cytokines and/or chemokines. Alternatively, the model might not reflect the conditions *in vivo* and refinement of the host cell and bacterial microenvironment is necessary to illustrate interactions.

8.4 Proposed mechanism of microbiome-inflammation-metabolic dysregulation-endometrial carcinogenesis interplay

Endometrial cancer has been associated with a systemic, chronic, low-grade inflammatory state induced by obesity, diabetes, physical inactivity and intestinal dysbiosis. In patients with metabolic dysregulation presented as obesity and/or diabetes, excess circulating free fatty acids (FFA), cytokines and chemokines secreted by the visceral adipose tissue and microbial products released by dysbiotic gut microbiota synergistically promote a pro-inflammatory environment impacting on signalling pathways in end organs. Moreover, genomic mutations in key components of signalling pathways (e.g., PTEN, K-ras, PIK3CA, p53) in endometrial cells dysregulate homeostasis leading to uncontrollable cell proliferation and invasiveness. In this context, a local eubiotic or dysbiotic microenvironment in the lower female genital tract and endometrium could potentially intersect with inflammatory and carcinogenic pathways either by suppressing or activating them. In this model, protective commensal microbes promote immune surveillance and tumour suppressor gene expression, thus empowering the host immune system and antagonizing the deleterious effects of carcinogenic stimuli. Conversely, disruption of the microbial equilibrium and predominance of harmful pathogens might induce immune dysregulation promoting cancer development and progression (Figure 8.1).

TLRs are a family of pattern-recognition receptors that play a critical role in the innate immune system by activating proinflammatory signalling pathways in response to microbial pathogens. TLR4, the best characterized TLR, binds to LPS of Gram-negative bacterial cell walls, while TLR2 is activated by lipoteichoic acid (LTA), a major immunostimulatory constituent of Gram-positive bacteria. FFA, on the other hand, can be recognised by both TLR2 and 4 [554]. In more detail, LPS/LTA/FFA bind and activate TLR4 and/or TLR2, which dimerize, and recruit downstream adaptor molecules such as myeloid differentiation protein 88 (MyD88)/MyD88 adapter-like protein (MAL) to mount an inflammatory response [84]. The activated MyD88/MAL then activates IL-1 receptor associated kinase (IRAK) [85 555], TNF receptor-associated factor (TRAF6) [86],

transforming growth factor B-associated kinase 1 (TAK1) [87], and JNK [556] and IKK complexes [89]. The activated IKK complex phosphorylates IκBa leading to its polyubiquitination and ultimately proteasomal degradation and NF-κB release. NF-κB translocates to the nucleus thereafter, activating pro-inflammatory molecule expression [89]. The activation of JNK and IKK can also induce IRS (Insulin Receptor Substrate) serine phosphorylation, which is pivotal to establish insulin resistance [557].

The IRS family of proteins, which encompasses 4 members, IRS-1 through IRS-4, is the substrate of the insulin and IGF-1 receptors [112]. Under normal conditions, tyrosine-phosphorylated IRS acts as a scaffold to organise and mediate signalling complexes. The signal is then propagated via two main branches: the PI3K-Akt-mTOR, responsible for glucose uptake, metabolism and cell growth and the Ras-ERK pathway that controls cell proliferation and differentiation [113] [114]. Apart from the tyrosine phosphorylation of IRS, which is an integral part of insulin signal transduction, multi-site phosphorylation of Serine/Threonine of IRS-1 can regulate insulin signalling both positively and negatively. Increased IR serine phosphorylation has been observed in insulin-resistant states, both in rodents and in humans [113] [114]. This post-translational modification of IRS-1, which is considered an insulin resistance marker, could represent a connecting bond between the microbiome and metabolic dysregulation [558].

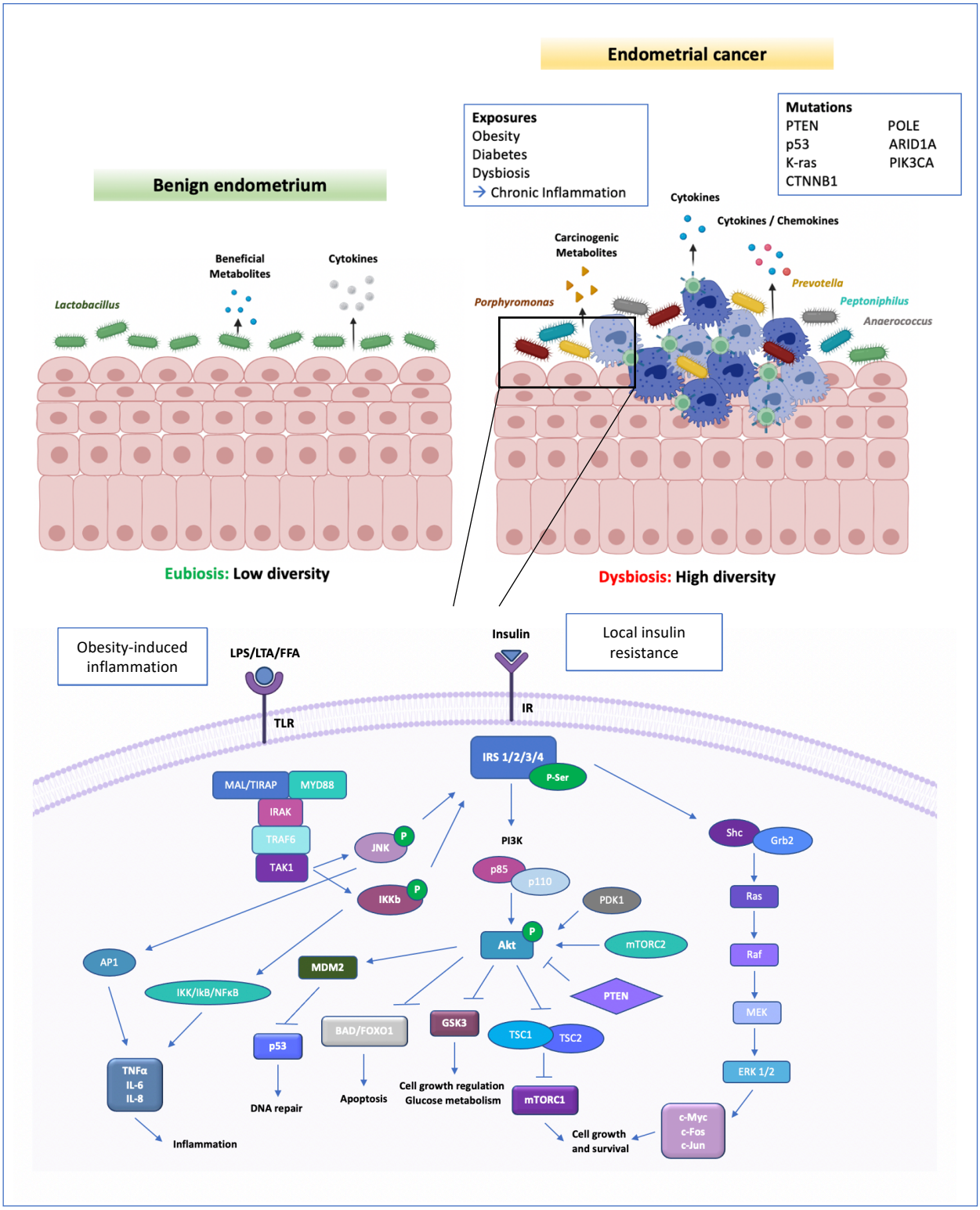


Figure 8.3. Proposed model of bacterial pro- and anti-tumorigenic effects through modulation of inflammatory, metabolic and cell survival pathways. Various sources of chronic inflammation (obesity, diabetes, gut dysbiosis) and genetic mutations initiate endometrial malignant transformation. Protective resident microbiota are able to preserve tissue homeostasis, whereas their depletion with concurrent predominance of potential pathobionts permits and/or amplifies pro-inflammatory, carcinogenic effects. Bacteria or their components, for example LPS and LTA, bind and activate Toll-

like receptors, which dimerize, and recruit downstream molecules such as MyD88/MAL, which in turn activate IRAK, TRAF6, TAK1 and JNK/ IKK complexes. JNK binds to activator protein 1 (AP1) leading to transcription of pro-inflammatory molecules such as TNFa, IL-6 and IL-8. A similar effect has the IKK complex, which induces NF-κB release by IκBa allowing its translocation into the nucleus. IRS, an insulin receptor substrate, is phosphorylated by JNK and IKK complexes on serine/threonine residues, which is important to establish insulin resistance. The signal is then propagated via two main branches: the PI3K-Akt-mTOR and Ras-MAPK-ERK pathways. PI3K consists of two subunits p85 and p110, which phosphorylate PIP2 to PIP3. PIP3 recruits Akt which is then phosphorylated and activated by PDK1 and mTORC2. Downstream effects of Akt are upregulation of mTORC1 through TSC complex inactivation and downregulation of GSK3, FOXO1, BAD and p53; thus, impairing apoptotic and DNA repair mechanisms. The MAPK pathway involves docking of Grb2, Shc and SOS proteins and sequential activation of Ras, Raf, MEK, ERK1/2 proteins, which upregulate expression of proto-oncogenes c-Myc, c-Fos and c-Jun.

8.5 Translational value

The work carried out in this thesis has revealed quantitative and qualitative alterations in the female genital tract and rectal microbiota of endometrial cancer patients, which might constitute causal or complicit factors in the initiation and/or progression of endometrial cancer. Additionally, the present study has captured the phenotypic, genetic and epigenetic kinship of endometrial cancer organoids to parent tissue and established their utility as a model system for studying cancer. In the context of endometrial cancer, the knowledge gained in this project could serve as a stepping stone to a) cancer prevention and early diagnosis, b) microbiome-based therapeutics and c) use of endometrial organoids for disease modelling and drug screening.

The identification of a distinct microbial signature in endometrial cancer and precancer patients backed up by mechanistic studies that shed light to the pathways affected by microbes could be used for risk stratification that would aid *disease prevention*. Microbial profiling to identify patients that are predisposed to endometrial cancer could lead to increased surveillance and prophylactic surgery for patients at high risk. Furthermore, prevention could be achieved by reversing perturbed microbiomes to healthy eco-structures through pre-/ pro- and post-biotics and microbiota

transplantation of health-promoting bacteria or use of antibiotics against potentially carcinogenic bacteria.

In established endometrial cancer, microbial profiling could help *early diagnosis*, predict response to treatment, side effects and disease prognosis. The diagnostic value of microbiota has been showcased by Poore et al. in a *Nature* publication that demonstrated that microbiome analysis of blood and tissues could be used to discriminate within and between most types of cancer [559]. Prediction of treatment response, toxicity and disease prognosis based on gut colonisation has been supported by a number of studies, which associated increased diversity and/or distinct microbial signatures with a favourable response to chemoradiation in cervical cancer [191], favourable prognosis in early breast cancer and fewer neurological and enteropathy side effects post-chemotherapy [560 561].

Microbiome-based therapeutics could be used as adjuncts to other anti-cancer treatments, i.e., radio-, chemo-, immunotherapy, to boost therapeutic efficacy and mitigate side effects of treatment. Endometrial cancer is typically treated by surgery, radiation and chemotherapy, while immunotherapy represents an exciting new addition to the anti-cancer armamentarium. Immune checkpoint inhibitors for endometrial cancer are currently used in advanced and recurrent cases as mono- or combination therapy with chemotherapeutics or PARP/kinase inhibitors. The highest rates observed so far were in MMR tumours with MMR deficiency/MSI (microsatellite instability) and PD-L1 (programmed death-ligand 1) positivity [268]. Interestingly, studies have demonstrated that checkpoint blockade of T cells is influenced by the intestinal microbiome [258]. A seminal study in 2019 reported a correlation between decreased efficacy of immunotherapy and survival in patients with melanoma and non-small lung cancer who had received antibiotic treatment before immunotherapy, implying that gut microbiota eradication via antibiotic treatment directly impacts on therapeutic responses [267]. In addition to this, *L. acidophilus* was shown to enhance anti-tumour immunity when combined with CTLA-4 blocking immunotherapy in a mouse colon cancer model [262]. Biotherapeutics could also be used to improve the safety profile of

existing treatments. Various studies have shown that several probiotics, can ameliorate chemotherapy-induced mucositis, through suppressing inflammation [562-564], restoring gut barrier integrity [565] and inhibiting intrinsic apoptosis [564].

Microbiome-based therapeutics encompass pre-, pro-, postbiotics, antibiotics and microbiota transplantation. The overarching goal of microbiota interventions is the normalisation of body-specific perturbed microbiota and even though these interventions have not yet been yet tested in endometrial cancer, insights from other cancer types or benign gynaecology could allow biological inferences and inform the future endometrial research agenda.

Prebiotics are defined as nondigestible food ingredients, such as carbohydrates and fibres, that selectively stimulate the growth and beneficial activities of specific bacteria [566]. Potential anti-cancer properties of prebiotics have mainly been investigated for colorectal cancer (CRC) [567], where several *in vitro* and mouse studies have showed a preventive effect in CRC progression, while the few human-based studies available have yielded contradictory results [568] [569]. Interestingly, prebiotics demonstrate interpatient variability of efficacy and even have adverse effects in some patients. Prebiotics could have an indirect effect on endometrial pathology through modulation of the gut oestrobolome, which could subsequently regulate circulating oestrogens and female genital tract bacterial communities.

Probiotics were defined in 2001 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organisation (WHO) as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. An expert panel convened in 2013 and concluded that the term was still relevant and sufficiently accommodating for current and anticipated applications [570]. Evidence of a health benefit is required for a probiotic and must be safe for the intended use. Dead microbes, microbial products and microbial components do not come under the probiotic classification.

In the realm of gynaecology, vaginal probiotic lactobacilli, such as *L. crispatus* strain CTV-05 (as a vaginal suppository; LACTIN-V), have been tested in clinical trials, mainly for the treatment of BV [571] [572] and urinary tract infection (UTI) [573] with encouraging results achieving *L. crispatus* colonisation after 28 days of treatment, depletion of BV-associated bacteria and prevention of recurrent UTIs. Cervical cancer and HPV clearance represent the second most researched area of probiotic use in gynaecology. Sungur *et al.* reported that human vagina-isolated *L. gasseri* strains, G10 and H15, inhibit HeLa cell proliferation [574]. Mechanisms of action include direct cytotoxic effects with Bax and Caspase3 upregulation together with anti-inflammatory effects reflected by TNF-α reduction and IL-10 increase. Regarding HPV clearance, *L. crispatus* 2743 has been shown to have an inhibitory effect on mRNA expression of E6 and E7, whereas *L. gasseri* 3396 has a smaller inhibitory impact on the E6 gene without any significant effect on the E7 gene [575]**.** In the first interventional human study, daily consumption of a probiotic drink (Yakult) containing *Lactobacillus casei* Shirota (LcS) by 54 LSIL patients showed greater clearance of HPV infections (29% vs 19%), and cytologic abnormalities (60% vs 31%) in 6 months compared to the untreated cohort [576]. A more recent randomised controlled trial in 117 women with bacterial vaginosis or vaginitis and concomitant HPV infections, reported that a 6-month use of vaginal *L. rhamnosus* BMX 54 **(**NORMOGIN ®) was associated with greater regression of cytologic abnormalities **(**79.4% vs 37.5%, p= 0.041) and negative HPV test (31.2% vs 11.6%, p = 0.044) compared to a 3-month use [577].

Postbiotics refer to the soluble by-products and metabolites (e.g., SCFA) secreted by microbiota modulating biological activities of the host. They can be found in conditioned medium or culture supernatants. Wang et al. [578] showed that *Lactobacillus* supernatants from *L. crispatus*, *L. jensenii*, and *L. gasseri*, inhibit the proliferation of Caski cells, while certain postbiotics, including lactate dehydrogenase, from *Lactobacillus* species, have been shown to induce apoptosis or inhibit invasion in CRC cell lines [579] [580]. Validation of postbiotics in *in vivo* studies is still lacking. Given the enormous diversity of metabolites and molecules secreted by microbes, exploring their functionality and safety profile remains a challenging task.

Antibiotics have been reported to confer protection against cancer development and progression. Doxycycline [581 582] and mefloquine [583] have been shown to exert a pro-apoptotic, anti-proliferative effect in cervical cancer cells. Meanwhile, depletion of deleterious bacteria, such as *Bacteroides fragilis* [584] and *Fusobacterium* [585], has been linked with reduced CRC growth and proliferation in preclinical models.

Microbiota transplantation is a new, controversial investigational approach for disease treatment. A 2019 pilot study (*n* = 5) demonstrated that vaginal microbiota transplantation resulted in long-term remission and reconstitution of a *Lactobacillus*-dominant microbiome in women with recurrent BV [586]. Two relevant clinical trials in the USA and Israel are currently underway to assess the efficacy and safety of this biotherapeutic in BV (ClinicalTrials.gov). Increased scepticism and uncertainty still remains regarding the safety profile of the intervention since experimental evidence has reported transmission of unrecognised pathogens and disease-causing host genes between individuals [587-592].

Lastly, the current study validated patient-derived endometrial organoids as reliable platforms to model disease as well as screen drugs and microbiota-based therapeutics paving the way for exciting advances in microbiome and cancer research. Organoids that morphologically and functionally reflect tissues *in vivo* could be used to delineate host-microbe interactions and carcinogenic pathways with a view to reveal biologic vulnerabilities that could be exploited for novel drug development. Furthermore, patient-derived organoids could be used to predict chemo- and radioresistance prior to treatment enhancing precision medicine by addressing the individual characteristics of each patient's disease and guiding treatment strategies. Last but not least, establishing organoids as an alternative research tool to animal models not only renders extrapolation of findings physiologically more relevant but also helps to overcome the skepticism related to ethical considerations of animal use for scientific purposes.

8.6 Future Directions

8.6.1 Future directions of female genital tract microbiome research

The thorough characterisation of the female genital tract microbiota in health and disease can lead to clinical inferences and modulation of commensal and pathogenic micro-organisms for patient benefit. In this direction, longitudinal cohort studies are paramount to capture intra-individual microbial stability and shifts over time, especially in cases of gynaecological precancer where early aberrations could provide insights in disease pathogenesis. In addition to this, future epidemiological studies should encompass women of different races, ethnicities and socio-economic backgrounds to account for demographic microbial disparities and collect behavioural, diet and genetic data to identify potential confounders.

Absolute quantification of enriched or depleted taxa by quantitative real-time PCR should be performed given that the 16S rRNA gene sequencing technique is not reliable in this regard. Compositionally, 16S rRNA gene sequencing, which targets the highly conserved 16S rRNA gene is more accurate at identifying genera and less precise at classifying bacterial species. Nevertheless, evidence suggests that different species confer divergent health benefits to the host, suggesting that the remaining genomic differences impact on their functionality [593-595]. Shotgun metagenomic sequencing, which scans the entire genomic DNA in a sample instead of a specific DNA region, can provide strain-level taxonomic resolution of the taxa and is the way forward in microbiome studies despite the higher cost. More recently, metagenomic sequencing of tens of thousands of samples was carried out in large scale projects studying the role of microbiome in early-onset type 1 diabetes (T1D) [596 597], IBD [598], pre-diabetes [599], and colorectal cancer [600 601] generating massive databases of microbial reference genomes [602 603].

Integrative multi-omics studies, including metagenomics, metatranscriptomics, metaproteomics, and metabolomics could help explore the functional activities of microbial communities and complex interplay with the host, facilitating microbiome-

targeted drug discovery. However, this integration represents a laborious task given that multi-omics analysis is increasingly reliant on sophisticated bioinformatic tools and advanced statistical methods, such as multivariate statistics and machine-learning approaches (readers are directed to the following representative reviews for more details [604-609]). Furthermore, bacterial viability assays or cultivation of microbes isolated from fresh samples should be used to determine populations of alive, biologically active bacteria in tissues and differentiate them from dead bacteria and extracellular DNA found in biofilms. Expanding microbiome studies to all types of microorganisms (including archaea, fungi, and viruses) would complement our knowledge about symbiotic and dysbiotic relationships with the human host [593-595 610].

A move beyond descriptive characterisation of microbial community structures to the investigation of the function and influences of microbiomes should be attempted using *in vivo* and 3-dimensional models *in vitro*. In this study, *L. crispatus* was not found to significantly affect cytokine secretion in neither benign nor malignant endometrial organoids. Further studies should interrogate different signalling pathways (metabolic/hormonal), different taxa and make use of multi-cellular models that also include stromal and immune cells. Finally, future translational microbiome research should aim to uncover drug-microbiome interactions, which according to recent reports are important contributors to the disparate patient responses to anti-cancer treatment.

8.6.2 Future directions of endometrial organoid research

In this project, endometrial organoids were derived from human benign and malignant endometrial tissue using pipelle biopsies. Future research should enhance the robustness of the model and prolong the lifespan of endometrial cancer organoids in culture. Optimisation should involve (i) cell components, (ii) extracellular matrix and (iii) culture media (Figure 8.2).

The uterus represents a collection of different cell types with diverse functions that are in perpetual crosstalk modulating intracellular signalling and governing gene expression. It is composed of epithelial glandular cells surrounded by stromal cells

that accommodate arterial, venous, lymph vasculature and neural networks overlying a layer of smooth muscle cells of mesenchymal origin. A serous membrane of epithelial cells and connective tissue covers the uterus externally, while red blood and immune cells are continuously transferred via bloodstream and lymph. Bacterial cell populations also colonize the endometrium, as shown in present and previous studies [141 142 448]. It has been shown in 2D models that endometrial stromal cells regulate growth, differentiation and hormonal responsiveness of endometrial epithelium and co-cultures alter gene expression, involved in immune function and tissue repair, as well as cytokine secretion of epithelial cells [611]. Another study has demonstrated that Ishikawa cells, a widely used endometrial epithelial cell line, acquire a differentiated secretory phenotype resembling normal epithelium when co-cultured with stromal cells [612]. Therefore, it can be easily inferred that isolating a cell population in a dish is a one-dimensional approach to recapitulate physiology *in vivo* that may distort experimental findings. A shift towards co-culture systems is the way forward for endometrial organoid models as already reported for other cell types, e.g., co-culture of peripheral immune cells with gastric [613], colorectal [614] and pancreatic [615] cancer organoids, dendritic cells with gastric organoids [616], mesenchymal stem cells with lung organoids [617], bacterial cells with various organoid types [286 618-622].

The extracellular matrix is another vital component of endometrial organoid cultures that mandates optimisation. ECM composition is organ-specific and undergoes alterations in disease states as explicitly depicted in endometrial, ovarian [623] and cervical carcinogenesis [624 625]. ECM changes are associated with tumour stiffness and impact on local invasion and distant migration [623]. In endometrial cancer, evidence suggests that ECM-derived TGF-β signalling promotes metastatic potential [626]. As a consequence, synthetic customized hydrogels, tailored to cell type and experimental needs, are needed to generate sophisticated cell models and enhance physiological relevance.

Medium optimisation to address cell type-specific nutritional and growth needs is key to achieve long-term cell prosperity and replicate signalling *in vivo*. Wnt [627], Notch [628]

and BMP [629] signalling pathways seem to be pivotal regulators of intracellular processes and as a result their components are typically part of media formulations for organoids [282 295 630]. Combinations of different growth factors and signalling molecules in conjunction with or without hormones (e.g., oestradiol, progesterone, insulin) should be sequentially tested on endometrial cancer organoids to determine optimal conditions of growth. Other experimental variables, fundamentally important for cell culture health and fidelity *in vivo*, include spatiotemporal oxygen gradients [631], acid-base equilibrium and pH maintenance [632], venous and organ-specific lymph vasculature [633] and should be taken into account in future experiments.

Besides refining culture conditions to recreate tissue replicas and ensure long-term expansion, the multi-omics characterization of endometrial organoids also warrants further investigation. The only published study to date has demonstrated that endometrial cancer organoids capture disease heterogeneity by preserving the genetic and transcriptomic traits of original tissue using array comparative genomic hybridization (aCGH) or low-coverage whole-genome sequencing and RNA sequencing respectively [282]. The current study has confirmed retention of the mutational landscape in endometrial cancer-derived organoids along with epigenetic characteristics as demonstrated by genome-wide DNA methylation profiling. Further studies should focus on the (epi)genetic and transcriptomic stability of endometrial cancer organoids over time and additionally explore their proteomic and metabolomic features in relation to parent tissue.

Lastly, beyond the attempts of reconstructing a faithful copy of the *in vivo* conditions, emphasis should also be placed on optimising experimental assays and imaging techniques for 3D structures so that scalability is enhanced, interpretation of results is accurate and reliable conclusions can be drawn.

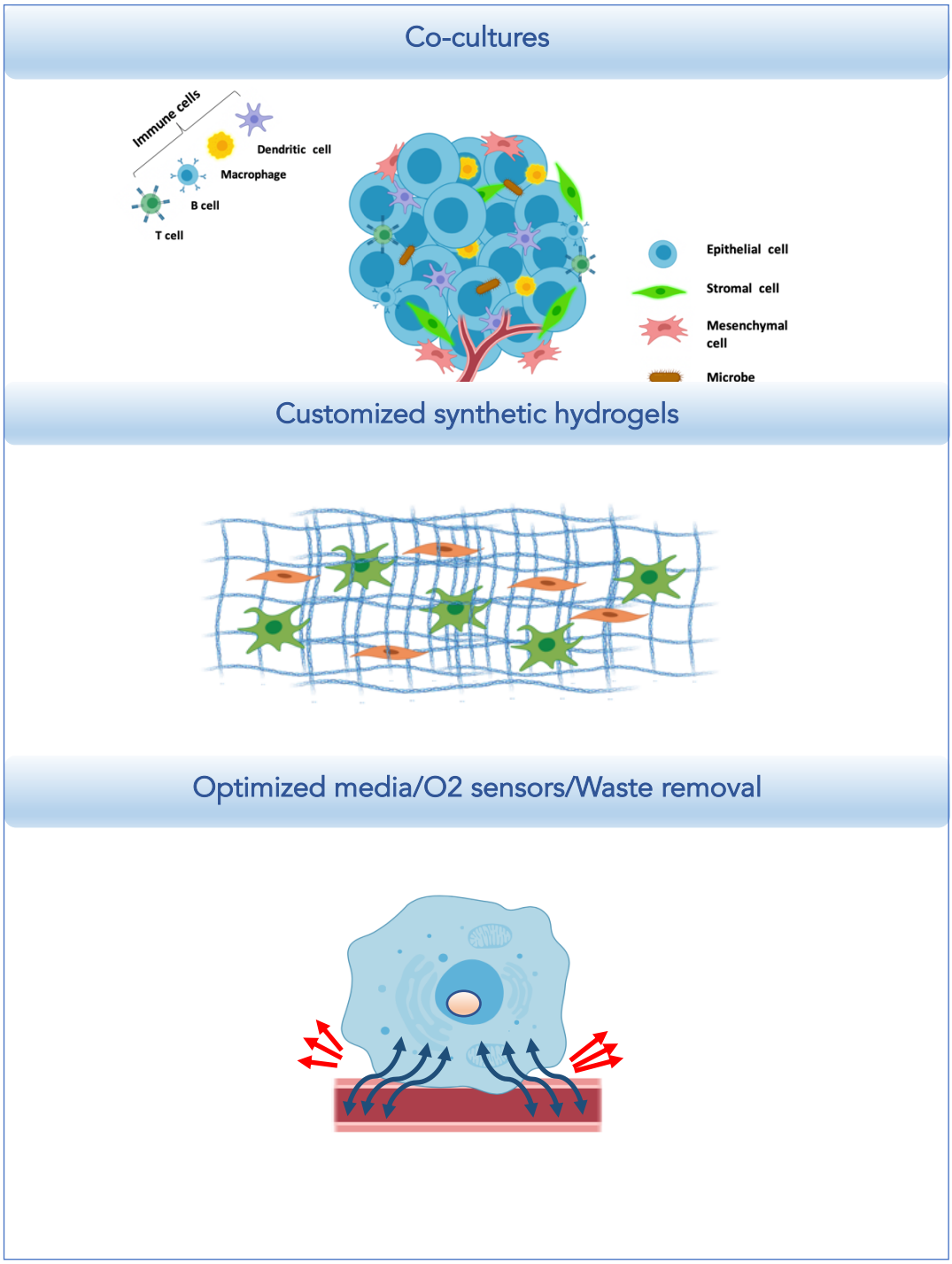


Figure 8.4. Next-generation female genital tract organoid cultures. Refinement of organoid models requires co-cultures of tissue resident cells, immune cells, microbial populations and vascular networks, customized synthetic hydrogels to mimic the extracellular matrix *in vivo* and optimization of media, oxygen sensors and waste removal systems to enable oxygen and nutrients to be efficiently delivered to organoids and toxic waste products to be removed maintaining pH (adapted from Semertzidou *et al.* [304], permission to reproduce not required as the author of this article).

8.7 Summary Conclusion

The work presented in this thesis has shed light on fundamental questions of bacterial colonisation in the upper genital tract of women with benign or malignant uterine pathology. It was shown that the upper genital tract is not sterile with a subset of benign and endometrial cancer patients harbouring microbiota that are quantitatively and compositionally distinguishable from background contaminants. Epithelial surfaces were found to be more heavily colonised compared to underlying tissues. A microbial continuum along the genital tract was demonstrated for the majority of benign patients but continuum was less cohesive in endometrial cancer patients. On the other hand, vaginal microbiota displayed poor correlation with rectal microbiota in both cohorts.

This work also reiterated that endometrial cancer is a multifactorial disease with metabolic and putative microbial components. Following extensive review of the literature and application of stringent statistical criteria, it was concluded that valid evidence associates diabetes with an increased risk of endometrial cancer and metformin with reduced endometrial cancer mortality rates. Microbiome analysis, on the other hand, highlighted the differences between endometrial cancer and benign patients in the lower and upper genital tract as well as the rectum. Endometrial cancer was associated with reduced cervicovaginal and rectal bacterial load together with *Lactobacillus*-depletion, high microbial diversity and enrichment of *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus* in the lower genital tract and endometrium. No significant differences were observed according to histotype and grade of endometrial cancer.

Finally, mechanistic experiments involving 3D endometrial organoid models were performed to delineate the microbiome-host interactions. Endometrial organoids were derived from human, benign and malignant endometrial tissue and were found to morphologically and (epi)genetically recapitulate the progenitor tissue. *L. crispatus*, which was significantly depleted along the genital tract of endometrial cancer patients, was selected as a potential beneficial microbe. *L. crispatus*-conditioned medium

appeared to significantly decrease endometrial cancer organoid viability at high concentrations, which was not observed in benign organoids to the same extent. Furthermore, LCC medium significantly increased CCL4/MIP1beta and IL-1β secretion by endometrial cancer organoids, while an increase of TNF-α and decrease of IL-10 secretion was noted in benign organoids. The secretion of the majority of cytokines and chemokines interrogated remained unaffected. Whether commensal microbiota selectively affect inflammatory pathways warrants further investigation. Unravelling the molecular mechanisms underlying the host-microbiome crosstalk is key to uncover the clinical relevance and utility of microbiota-based interventions.

References

1. Evans T, Sany O, Pearmain P, et al. Differential trends in the rising incidence of endometrial cancer by type: data from a UK population-based registry from 1994 to 2006. *Br J Cancer* 2011;104(9):1505-10. doi: 10.1038/bjc.2011.68 [published Online First: 2011/04/28]
2. Duncan ME, Seagroatt V, Goldacre MJ. Cancer of the body of the uterus: trends in mortality and incidence in England, 1985-2008. *BJOG* 2012;119(3):333-9. doi: 10.1111/j.1471-0528.2011.03201.x [published Online First: 2011/11/16]
3. Lu KH, Broaddus RR. Endometrial Cancer. *N Engl J Med* 2020;383(21):2053-64. doi: 10.1056/NEJMra1514010 [published Online First: 2020/11/19]
4. Sheikh MA, Althouse AD, Freese KE, et al. USA endometrial cancer projections to 2030: should we be concerned? *Future Oncol* 2014;10(16):2561-8. doi: 10.2217/fon.14.192 [published Online First: 2014/12/23]
5. Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol* 1983;15(1):10-7. doi: 10.1016/0090-8258(83)90111-7 [published Online First: 1983/02/01]
6. Lax SF, Kurman RJ. A dualistic model for endometrial carcinogenesis based on immunohistochemical and molecular genetic analyses. *Verh Dtsch Ges Pathol* 1997;81:228-32. [published Online First: 1997/01/01]
7. Sherman ME. Theories of endometrial carcinogenesis: a multidisciplinary approach. *Mod Pathol* 2000;13(3):295-308. doi: 10.1038/modpathol.3880051 [published Online First: 2000/04/11]
8. Lax SF. Pathology of Endometrial Carcinoma. *Adv Exp Med Biol* 2017;943:75-96. doi: 10.1007/978-3-319-43139-0_3 [published Online First: 2016/12/03]
9. Suarez AA, Felix AS, Cohn DE. Bokhman Redux: Endometrial cancer "types" in the 21st century. *Gynecol Oncol* 2017;144(2):243-49. doi: 10.1016/j.ygyno.2016.12.010 [published Online First: 2016/12/21]
10. Murali R, Soslow RA, Weigelt B. Classification of endometrial carcinoma: more than two types. *Lancet Oncol* 2014;15(7):e268-78. doi: 10.1016/S1470-2045(13)70591-6 [published Online First: 2014/05/30]
11. Talhouk A, McConechy MK, Leung S, et al. A clinically applicable molecular-based classification for endometrial cancers. *Br J Cancer* 2015;113(2):299-310. doi: 10.1038/bjc.2015.190 [published Online First: 2015/07/15]
12. Cancer Genome Atlas Research N, Kandoth C, Schultz N, et al. Integrated genomic characterization of endometrial carcinoma. *Nature* 2013;497(7447):67-73. doi: 10.1038/nature12113 [published Online First: 2013/05/03]
13. Urick ME, Bell DW. Clinical actionability of molecular targets in endometrial cancer. *Nat Rev Cancer* 2019;19(9):510-21. doi: 10.1038/s41568-019-0177-x [published Online First: 2019/08/08]
14. McConechy MK, Talhouk A, Leung S, et al. Endometrial Carcinomas with POLE Exonuclease Domain Mutations Have a Favorable Prognosis. *Clin Cancer Res* 2016;22(12):2865-73. doi: 10.1158/1078-0432.CCR-15-2233 [published Online First: 2016/01/15]

15. Clarke BA, Gilks CB. Endometrial carcinoma: controversies in histopathological assessment of grade and tumour cell type. *J Clin Pathol* 2010;63(5):410-5. doi: 10.1136/jcp.2009.071225 [published Online First: 2010/04/27]
16. McConechy MK, Ding J, Cheang MC, et al. Use of mutation profiles to refine the classification of endometrial carcinomas. *J Pathol* 2012;228(1):20-30. doi: 10.1002/path.4056 [published Online First: 2012/06/02]
17. Cho KR, Cooper K, Croce S, et al. International Society of Gynecological Pathologists (ISGyP) Endometrial Cancer Project: Guidelines From the Special Techniques and Ancillary Studies Group. *Int J Gynecol Pathol* 2019;38 Suppl 1:S114-S22. doi: 10.1097/PGP.0000000000000496 [published Online First: 2018/03/10]
18. Society AC. Endometrial cancer risk factors 2019 [accessed November 2021.
19. Raglan O, Kalliala I, Markozannes G, et al. Risk factors for endometrial cancer: An umbrella review of the literature. *Int J Cancer* 2019;145(7):1719-30. doi: 10.1002/ijc.31961 [published Online First: 20190220]
20. Liao C, Zhang D, Mungo C, et al. Is diabetes mellitus associated with increased incidence and disease-specific mortality in endometrial cancer? A systematic review and meta-analysis of cohort studies. *Gynecol Oncol* 2014;135(1):163-71. doi: 10.1016/j.ygyno.2014.07.095 [published Online First: 2014/07/30]
21. Friberg E, Orsini N, Mantzoros CS, et al. Diabetes mellitus and risk of endometrial cancer: a meta-analysis. *Diabetologia* 2007;50(7):1365-74. doi: 10.1007/s00125-007-0681-5 [published Online First: 2007/05/04]
22. Zhang ZH, Su PY, Hao JH, et al. The role of preexisting diabetes mellitus on incidence and mortality of endometrial cancer: a meta-analysis of prospective cohort studies. *Int J Gynecol Cancer* 2013;23(2):294-303. doi: 10.1097/IGC.0b013e31827b8430 [published Online First: 2013/01/05]
23. Gunter MJ, Hoover DR, Yu H, et al. A prospective evaluation of insulin and insulin-like growth factor-I as risk factors for endometrial cancer. *Cancer Epidemiol Biomarkers Prev* 2008;17(4):921-9. doi: 10.1158/1055-9965.EPI-07-2686 [published Online First: 2008/04/10]
24. Yang TK, Chung CJ, Chung SD, et al. Risk of Endometrial Cancer in Women With Pelvic Inflammatory Disease: A Nationwide Population-Based Retrospective Cohort Study. *Medicine (Baltimore)* 2015;94(34):e1278. doi: 10.1097/MD.0000000000001278 [published Online First: 2015/08/28]
25. Setiawan VW, Yang HP, Pike MC, et al. Type I and II endometrial cancers: have they different risk factors? *J Clin Oncol* 2013;31(20):2607-18. doi: 10.1200/JCO.2012.48.2596 [published Online First: 2013/06/05]
26. Brinton LA, Felix AS, McMeekin DS, et al. Etiologic heterogeneity in endometrial cancer: evidence from a Gynecologic Oncology Group trial. *Gynecol Oncol* 2013;129(2):277-84. doi: 10.1016/j.ygyno.2013.02.023 [published Online First: 2013/03/15]
27. Felix AS, Weissfeld JL, Stone RA, et al. Factors associated with Type I and Type II endometrial cancer. *Cancer Causes Control* 2010;21(11):1851-6. doi: 10.1007/s10552-010-9612-8 [published Online First: 2010/07/16]
28. Sherman ME, Sturgeon S, Brinton LA, et al. Risk factors and hormone levels in patients with serous and endometrioid uterine carcinomas. *Mod Pathol* 1997;10(10):963-8. [published Online First: 1997/11/05]

29. Yang HP, Wentzensen N, Trabert B, et al. Endometrial cancer risk factors by 2 main histologic subtypes: the NIH-AARP Diet and Health Study. *Am J Epidemiol* 2013;177(2):142-51. doi: 10.1093/aje/kws200 [published Online First: 2012/11/23]
30. Kurman RJ, Kaminski PF, Norris HJ. The behavior of endometrial hyperplasia. A long-term study of "untreated" hyperplasia in 170 patients. *Cancer* 1985;56(2):403-12. doi: 10.1002/1097-0142(19850715)56:2<403::aid-cncr2820560233>3.0.co;2-x [published Online First: 1985/07/15]
31. Hayes MP, Wang H, Espinal-Witter R, et al. PIK3CA and PTEN mutations in uterine endometrioid carcinoma and complex atypical hyperplasia. *Clin Cancer Res* 2006;12(20 Pt 1):5932-5. doi: 10.1158/1078-0432.CCR-06-1375 [published Online First: 2006/10/26]
32. Mutter GL, Lin MC, Fitzgerald JT, et al. Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *J Natl Cancer Inst* 2000;92(11):924-30. doi: 10.1093/jnci/92.11.924 [published Online First: 2000/06/08]
33. Joshi A, Miller C, Jr., Baker SJ, et al. Activated mutant p110alpha causes endometrial carcinoma in the setting of biallelic Pten deletion. *Am J Pathol* 2015;185(4):1104-13. doi: 10.1016/j.ajpath.2014.12.019 [published Online First: 2015/02/24]
34. Liaw D, Marsh DJ, Li J, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* 1997;16(1):64-7. doi: 10.1038/ng0597-64 [published Online First: 1997/05/01]
35. Blumenthal GM, Dennis PA. PTEN hamartoma tumor syndromes. *Eur J Hum Genet* 2008;16(11):1289-300. doi: 10.1038/ejhg.2008.162 [published Online First: 2008/09/11]
36. Nelen MR, van Staveren WC, Peeters EA, et al. Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. *Hum Mol Genet* 1997;6(8):1383-7. doi: 10.1093/hmg/6.8.1383 [published Online First: 1997/08/01]
37. Djordjevic B, Barkoh BA, Luthra R, et al. Relationship between PTEN, DNA mismatch repair, and tumor histotype in endometrial carcinoma: retained positive expression of PTEN preferentially identifies sporadic non-endometrioid carcinomas. *Mod Pathol* 2013;26(10):1401-12. doi: 10.1038/modpathol.2013.67 [published Online First: 2013/04/20]
38. Rudd ML, Price JC, Fogoros S, et al. A unique spectrum of somatic PIK3CA (p110alpha) mutations within primary endometrial carcinomas. *Clin Cancer Res* 2011;17(6):1331-40. doi: 10.1158/1078-0432.CCR-10-0540 [published Online First: 2011/01/27]
39. Oda K, Stokoe D, Taketani Y, et al. High frequency of coexistent mutations of PIK3CA and PTEN genes in endometrial carcinoma. *Cancer Res* 2005;65(23):10669-73. doi: 10.1158/0008-5472.CAN-05-2620 [published Online First: 2005/12/03]
40. Urick ME, Rudd ML, Godwin AK, et al. PIK3R1 (p85alpha) is somatically mutated at high frequency in primary endometrial cancer. *Cancer Res* 2011;71(12):4061-7. doi: 10.1158/0008-5472.CAN-11-0549 [published Online First: 2011/04/12]

41. Cheung LW, Hennessy BT, Li J, et al. High frequency of PIK3R1 and PIK3R2 mutations in endometrial cancer elucidates a novel mechanism for regulation of PTEN protein stability. *Cancer Discov* 2011;1(2):170-85. doi: 10.1158/2159-8290.CD-11-0039 [published Online First: 2011/10/11]
42. Lax SF, Kendall B, Tashiro H, et al. The frequency of p53, K-ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct molecular genetic pathways. *Cancer* 2000;88(4):814-24. [published Online First: 2000/02/19]
43. Terakawa J, Serna VA, Taketo MM, et al. Ovarian insufficiency and CTNNB1 mutations drive malignant transformation of endometrial hyperplasia with altered PTEN/PI3K activities. *Proc Natl Acad Sci U S A* 2019;116(10):4528-37. doi: 10.1073/pnas.1814506116 [published Online First: 2019/02/21]
44. Byron SA, Gartside M, Powell MA, et al. FGFR2 point mutations in 466 endometrioid endometrial tumors: relationship with MSI, KRAS, PIK3CA, CTNNB1 mutations and clinicopathological features. *PLoS One* 2012;7(2):e30801. doi: 10.1371/journal.pone.0030801 [published Online First: 2012/03/03]
45. Wang H, Douglas W, Lia M, et al. DNA mismatch repair deficiency accelerates endometrial tumorigenesis in Pten heterozygous mice. *Am J Pathol* 2002;160(4):1481-6. doi: 10.1016/S0002-9440(10)62573-4 [published Online First: 2002/04/12]
46. Ayhan A, Mao TL, Suryo Rahmanto Y, et al. Increased proliferation in atypical hyperplasia/endometrioid intraepithelial neoplasia of the endometrium with concurrent inactivation of ARID1A and PTEN tumour suppressors. *J Pathol Clin Res* 2015;1(3):186-93. doi: 10.1002/cjp2.22 [published Online First: 2016/08/09]
47. Matias-Guiu X, Prat J. Molecular pathology of endometrial carcinoma. *Histopathology* 2013;62(1):111-23. doi: 10.1111/his.12053 [published Online First: 2012/12/18]
48. Sherman ME, Bur ME, Kurman RJ. p53 in endometrial cancer and its putative precursors: evidence for diverse pathways of tumorigenesis. *Hum Pathol* 1995;26(11):1268-74. doi: 10.1016/0046-8177(95)90204-x [published Online First: 1995/11/01]
49. Le Gallo M, Rudd ML, Urick ME, et al. Somatic mutation profiles of clear cell endometrial tumors revealed by whole exome and targeted gene sequencing. *Cancer* 2017;123(17):3261-68. doi: 10.1002/cncr.30745 [published Online First: 2017/05/10]
50. DeLair DF, Burke KA, Selenica P, et al. The genetic landscape of endometrial clear cell carcinomas. *J Pathol* 2017;243(2):230-41. doi: 10.1002/path.4947 [published Online First: 2017/07/19]
51. An HJ, Logani S, Isacson C, et al. Molecular characterization of uterine clear cell carcinoma. *Mod Pathol* 2004;17(5):530-7. doi: 10.1038/modpathol.3800057 [published Online First: 2004/02/21]
52. Hoang LN, McConechy MK, Meng B, et al. Targeted mutation analysis of endometrial clear cell carcinoma. *Histopathology* 2015;66(5):664-74. doi: 10.1111/his.12581 [published Online First: 2014/10/14]

53. Cherniack AD, Shen H, Walter V, et al. Integrated Molecular Characterization of Uterine Carcinosarcoma. *Cancer Cell* 2017;31(3):411-23. doi: 10.1016/j.ccell.2017.02.010 [published Online First: 2017/03/16]
54. Le Gallo M, Rudd ML, Urick ME, et al. The FOXA2 transcription factor is frequently somatically mutated in uterine carcinosarcomas and carcinomas. *Cancer* 2018;124(1):65-73. doi: 10.1002/cncr.30971 [published Online First: 2017/09/25]
55. Jones S, Stransky N, McCord CL, et al. Genomic analyses of gynaecologic carcinosarcomas reveal frequent mutations in chromatin remodelling genes. *Nat Commun* 2014;5:5006. doi: 10.1038/ncomms6006 [published Online First: 2014/09/23]
56. Zhao S, Bellone S, Lopez S, et al. Mutational landscape of uterine and ovarian carcinosarcomas implicates histone genes in epithelial-mesenchymal transition. *Proc Natl Acad Sci U S A* 2016;113(43):12238-43. doi: 10.1073/pnas.1614120113 [published Online First: 2016/10/30]
57. McConechy MK, Hoang LN, Chui MH, et al. In-depth molecular profiling of the biphasic components of uterine carcinosarcomas. *J Pathol Clin Res* 2015;1(3):173-85. doi: 10.1002/cjp2.18 [published Online First: 2016/08/09]
58. Gotoh O, Sugiyama Y, Takazawa Y, et al. Clinically relevant molecular subtypes and genomic alteration-independent differentiation in gynecologic carcinosarcoma. *Nat Commun* 2019;10(1):4965. doi: 10.1038/s41467-019-12985-x [published Online First: 2019/11/02]
59. Matias-Guiu X, Catasus L, Bussaglia E, et al. Molecular pathology of endometrial hyperplasia and carcinoma. *Hum Pathol* 2001;32(6):569-77. doi: 10.1053/hupa.2001.25929 [published Online First: 2001/06/30]
60. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* 2006;7(8):606-19. doi: 10.1038/nrg1879 [published Online First: 2006/07/19]
61. Bader AG, Kang S, Zhao L, et al. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 2005;5(12):921-9. doi: 10.1038/nrc1753 [published Online First: 2005/12/13]
62. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2(7):489-501. doi: 10.1038/nrc839 [published Online First: 2002/07/03]
63. Liu P, Cheng H, Roberts TM, et al. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov* 2009;8(8):627-44. doi: 10.1038/nrd2926 [published Online First: 2009/08/01]
64. Whitman M, Downes CP, Keeler M, et al. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* 1988;332(6165):644-6. doi: 10.1038/332644a0 [published Online First: 1988/04/14]
65. Stephens L, Anderson K, Stokoe D, et al. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 1998;279(5351):710-4. doi: 10.1126/science.279.5351.710 [published Online First: 1998/02/21]

66. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* 2010;11(1):9-22. doi: 10.1038/nrm2822 [published Online First: 2009/12/23]
67. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 2009;10(5):307-18. doi: 10.1038/nrm2672 [published Online First: 2009/04/03]
68. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998;273(22):13375-8. doi: 10.1074/jbc.273.22.13375 [published Online First: 1998/06/05]
69. Stambolic V, Suzuki A, de la Pompa JL, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998;95(1):29-39. doi: 10.1016/s0092-8674(00)81780-8 [published Online First: 1998/10/20]
70. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci* 2011;36(6):320-8. doi: 10.1016/j.tibs.2011.03.006 [published Online First: 2011/05/03]
71. Alessi DR, Saito Y, Campbell DG, et al. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J* 1994;13(7):1610-9. [published Online First: 1994/04/01]
72. McCubrey JA, Steelman LS, Chappell WH, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* 2007;1773(8):1263-84. doi: 10.1016/j.bbamcr.2006.10.001 [published Online First: 2006/11/28]
73. Axelrad JE, Lichtiger S, Yajnik V. Inflammatory bowel disease and cancer: The role of inflammation, immunosuppression, and cancer treatment. *World J Gastroenterol* 2016;22(20):4794-801. doi: 10.3748/wjg.v22.i20.4794 [published Online First: 2016/05/31]
74. Herder C, Schneitler S, Rathmann W, et al. Low-grade inflammation, obesity, and insulin resistance in adolescents. *J Clin Endocrinol Metab* 2007;92(12):4569-74. doi: 10.1210/jc.2007-0955 [published Online First: 2007/10/04]
75. Dossus L, Becker S, Rinaldi S, et al. Tumor necrosis factor (TNF)-alpha, soluble TNF receptors and endometrial cancer risk: the EPIC study. *Int J Cancer* 2011;129(8):2032-7. doi: 10.1002/ijc.25840 [published Online First: 2010/12/15]
76. Delahanty RJ, Xiang YB, Spurdle A, et al. Polymorphisms in inflammation pathway genes and endometrial cancer risk. *Cancer Epidemiol Biomarkers Prev* 2013;22(2):216-23. doi: 10.1158/1055-9965.EPI-12-0903 [published Online First: 2012/12/12]
77. Modugno F, Ness RB, Chen C, et al. Inflammation and endometrial cancer: a hypothesis. *Cancer Epidemiol Biomarkers Prev* 2005;14(12):2840-7. doi: 10.1158/1055-9965.EPI-05-0493 [published Online First: 2005/12/21]
78. Vaskivuo TE, Stenback F, Tapanainen JS. Apoptosis and apoptosis-related factors Bcl-2, Bax, tumor necrosis factor-alpha, and NF-kappaB in human endometrial hyperplasia and carcinoma. *Cancer* 2002;95(7):1463-71. doi: 10.1002/cncr.10876 [published Online First: 2002/09/19]
79. Ferrandina G, Legge F, Ranelletti FO, et al. Cyclooxygenase-2 expression in endometrial carcinoma: correlation with clinicopathologic parameters and

clinical outcome. *Cancer* 2002;95(4):801-7. doi: 10.1002/cncr.10736 [published Online First: 2002/09/05]
80. Tamura M, Sebastian S, Yang S, et al. Up-regulation of cyclooxygenase-2 expression and prostaglandin synthesis in endometrial stromal cells by malignant endometrial epithelial cells. A paracrine effect mediated by prostaglandin E2 and nuclear factor-kappa B. *J Biol Chem* 2002;277(29):26208-16. doi: 10.1074/jbc.M201347200 [published Online First: 2002/05/15]
81. Arango HA, Icely S, Roberts WS, et al. Aspirin effects on endometrial cancer cell growth. *Obstet Gynecol* 2001;97(3):423-7. doi: 10.1016/s0029-7844(00)01161-3 [published Online First: 2001/03/10]
82. Gao J, Niwa K, Sun W, et al. Non-steroidal anti-inflammatory drugs inhibit cellular proliferation and upregulate cyclooxygenase-2 protein expression in endometrial cancer cells. *Cancer Sci* 2004;95(11):901-7. doi: 10.1111/j.1349-7006.2004.tb02200.x [published Online First: 2004/11/18]
83. Li HL, Zhang HW, Chen DD, et al. JTE-522, a selective COX-2 inhibitor, inhibits cell proliferation and induces apoptosis in RL95-2 cells. *Acta Pharmacol Sin* 2002;23(7):631-7. [published Online First: 2002/07/09]
84. Muzio M, Ni J, Feng P, et al. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* 1997;278(5343):1612-5. doi: 10.1126/science.278.5343.1612 [published Online First: 1997/12/31]
85. Janssens S, Beyaert R. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol Cell* 2003;11(2):293-302. doi: 10.1016/s1097-2765(03)00053-4 [published Online First: 2003/03/07]
86. Bradley JR, Pober JS. Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene* 2001;20(44):6482-91. doi: 10.1038/sj.onc.1204788 [published Online First: 2001/10/19]
87. Takaesu G, Surabhi RM, Park KJ, et al. TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. *J Mol Biol* 2003;326(1):105-15. doi: 10.1016/s0022-2836(02)01404-3 [published Online First: 2003/01/28]
88. Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Genet Dev* 2002;12(1):14-21. doi: 10.1016/s0959-437x(01)00258-1 [published Online First: 2002/01/16]
89. Hoesel B, Schmid JA. The complexity of NF-kappaB signaling in inflammation and cancer. *Mol Cancer* 2013;12:86. doi: 10.1186/1476-4598-12-86 [published Online First: 2013/08/07]
90. Gallagher EJ, LeRoith D. The proliferating role of insulin and insulin-like growth factors in cancer. *Trends Endocrinol Metab* 2010;21(10):610-8. doi: 10.1016/j.tem.2010.06.007 [published Online First: 2010/07/29]
91. Belfiore A, Frasca F, Pandini G, et al. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009;30(6):586-623. doi: 10.1210/er.2008-0047 [published Online First: 2009/09/16]
92. Lawlor MA, Alessi DR. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci* 2001;114(Pt 16):2903-10. [published Online First: 2001/11/01]

93. Prisco M, Romano G, Peruzzi F, et al. Insulin and IGF-I receptors signaling in protection from apoptosis. *Horm Metab Res* 1999;31(2-3):80-9. doi: 10.1055/s-2007-978703 [published Online First: 1999/05/05]
94. Ish-Shalom D, Christoffersen CT, Vorwerk P, et al. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. *Diabetologia* 1997;40 Suppl 2:S25-31. doi: 10.1007/s001250051393 [published Online First: 1997/07/01]
95. Le Roith D. Regulation of proliferation and apoptosis by the insulin-like growth factor I receptor. *Growth Horm IGF Res* 2000;10 Suppl A:S12-3. doi: 10.1016/s1096-6374(00)90005-4 [published Online First: 2000/09/13]
96. Khandwala HM, McCutcheon IE, Flyvbjerg A, et al. The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocr Rev* 2000;21(3):215-44. doi: 10.1210/edrv.21.3.0399 [published Online First: 2000/06/17]
97. Russell-Jones DL, Umpleby AM, Shojaee-Moradie F, et al. The effect of an intravenous infusion of IGF-I and insulin on IGFBP-1, IGFBP-3, acid labile subunit, free and bound IGF-I, catecholamines and potassium in normal volunteers during an amino acid and glucose clamp. *Clin Endocrinol (Oxf)* 1997;47(6):685-91. doi: 10.1046/j.1365-2265.1997.3161133.x [published Online First: 1998/03/14]
98. Owuor TO, Reid M, Reschke L, et al. Maternal obesogenic diet induces endometrial hyperplasia, an early hallmark of endometrial cancer, in a diethylstilbestrol mouse model. *PLoS One* 2018;13(5):e0186390. doi: 10.1371/journal.pone.0186390 [published Online First: 2018/05/19]
99. Shafie SM, Grantham FH. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. *J Natl Cancer Inst* 1981;67(1):51-6. [published Online First: 1981/07/01]
100. Shafie SM, Hilf R. Insulin receptor levels and magnitude of insulin-induced responses in 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats. *Cancer Res* 1981;41(3):826-9. [published Online First: 1981/03/01]
101. Heuson JC, Legros N. Effect of insulin and of alloxan diabetes on growth of the rat mammary carcinoma in vivo. *Eur J Cancer* 1970;6(4):349-51. doi: 10.1016/0014-2964(70)90100-3 [published Online First: 1970/08/01]
102. Heuson JC, Legros N. Influence of insulin deprivation on growth of the 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction. *Cancer Res* 1972;32(2):226-32. [published Online First: 1972/02/01]
103. Cocca C, Martin G, Rivera E, et al. An experimental model of diabetes and cancer in rats. *Eur J Cancer* 1998;34(6):889-94. doi: 10.1016/s0959-8049(97)10077-6 [published Online First: 1998/11/03]
104. Cocca C, Gutierrez A, Nunez M, et al. Suppression of mammary gland tumorigenesis in diabetic rats. *Cancer Detect Prev* 2003;27(1):37-46. doi: 10.1016/s0361-090x(02)00130-7 [published Online First: 2003/02/26]
105. LeRoith D, Roberts CT, Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 2003;195(2):127-37. doi: 10.1016/s0304-3835(03)00159-9 [published Online First: 2003/05/28]

106. Calle EE, Thun MJ. Obesity and cancer. *Oncogene* 2004;23(38):6365-78. doi: 10.1038/sj.onc.1207751 [published Online First: 2004/08/24]
107. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993;259(5091):87-91. doi: 10.1126/science.7678183 [published Online First: 1993/01/01]
108. Hotamisligil GS, Peraldi P, Budavari A, et al. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996;271(5249):665-8. doi: 10.1126/science.271.5249.665 [published Online First: 1996/02/02]
109. Aguirre V, Uchida T, Yenush L, et al. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 2000;275(12):9047-54. doi: 10.1074/jbc.275.12.9047 [published Online First: 2000/03/18]
110. Aguirre V, Werner ED, Giraud J, et al. Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J Biol Chem* 2002;277(2):1531-7. doi: 10.1074/jbc.M101521200 [published Online First: 2001/10/19]
111. Paz K, Hemi R, LeRoith D, et al. A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* 1997;272(47):29911-8. doi: 10.1074/jbc.272.47.29911 [published Online First: 1997/12/31]
112. Peruzzi F, Prisco M, Dews M, et al. Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Mol Cell Biol* 1999;19(10):7203-15. doi: 10.1128/MCB.19.10.7203 [published Online First: 1999/09/22]
113. Draznin B. Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85alpha: the two sides of a coin. *Diabetes* 2006;55(8):2392-7. doi: 10.2337/db06-0391 [published Online First: 2006/07/29]
114. Boura-Halfon S, Zick Y. Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab* 2009;296(4):E581-91. doi: 10.1152/ajpendo.90437.2008 [published Online First: 2008/08/30]
115. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;444(7121):860-7. doi: 10.1038/nature05485 [published Online First: 2006/12/15]
116. Lederberg J, AT M. 'Ome Sweet 'Omics—a genealogical treasury of words. *Scientist* 2001;15(8)
117. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol* 2016;14(8):e1002533. doi: 10.1371/journal.pbio.1002533 [published Online First: 2016/08/20]
118. Handelsman J. Metagenomics: application of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev* 2004;68(4):669-85. doi: 10.1128/MMBR.68.4.669-685.2004 [published Online First: 2004/12/14]

119. Tyson GW, Chapman J, Hugenholtz P, et al. Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 2004;428(6978):37-43. doi: 10.1038/nature02340 [published Online First: 2004/02/13]
120. Group NHW, Peterson J, Garges S, et al. The NIH Human Microbiome Project. *Genome Res* 2009;19(12):2317-23. doi: 10.1101/gr.096651.109 [published Online First: 2009/10/13]
121. Manor O, Dai CL, Kornilov SA, et al. Health and disease markers correlate with gut microbiome composition across thousands of people. *Nat Commun* 2020;11(1):5206. doi: 10.1038/s41467-020-18871-1 [published Online First: 2020/10/17]
122. Backhed F, Fraser CM, Ringel Y, et al. Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* 2012;12(5):611-22. doi: 10.1016/j.chom.2012.10.012 [published Online First: 2012/11/20]
123. Larsen B, Galask RP. Vaginal microbial flora: composition and influences of host physiology. *Ann Intern Med* 1982;96(6 Pt 2):926-30. doi: 10.7326/0003-4819-96-6-926 [published Online First: 1982/06/01]
124. Lepargneur JP, Rousseau V. [Protective role of the Doderlein flora]. *J Gynecol Obstet Biol Reprod (Paris)* 2002;31(5):485-94. [published Online First: 2002/10/16]
125. Huang B, Fettweis JM, Brooks JP, et al. The changing landscape of the vaginal microbiome. *Clin Lab Med* 2014;34(4):747-61. doi: 10.1016/j.cll.2014.08.006 [published Online First: 2014/12/03]
126. Ravel J, Gajer P, Abdo Z, et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 2011;108 Suppl 1:4680-7. doi: 10.1073/pnas.1002611107 [published Online First: 2010/06/11]
127. Ata B, Yildiz S, Turkgeldi E, et al. The Endobiota Study: Comparison of Vaginal, Cervical and Gut Microbiota Between Women with Stage 3/4 Endometriosis and Healthy Controls. *Sci Rep* 2019;9(1):2204. doi: 10.1038/s41598-019-39700-6 [published Online First: 2019/02/20]
128. Wang H, Ma Y, Li R, et al. Associations of Cervicovaginal Lactobacilli With High-Risk Human Papillomavirus Infection, Cervical Intraepithelial Neoplasia, and Cancer: A Systematic Review and Meta-Analysis. *J Infect Dis* 2019;220(8):1243-54. doi: 10.1093/infdis/jiz325 [published Online First: 2019/06/27]
129. Arokiyaraj S, Seo SS, Kwon M, et al. Association of cervical microbial community with persistence, clearance and negativity of Human Papillomavirus in Korean women: a longitudinal study. *Sci Rep* 2018;8(1):15479. doi: 10.1038/s41598-018-33750-y [published Online First: 2018/10/21]
130. Usyk M, Zolnik CP, Castle PE, et al. Cervicovaginal microbiome and natural history of HPV in a longitudinal study. *PLoS Pathog* 2020;16(3):e1008376. doi: 10.1371/journal.ppat.1008376 [published Online First: 2020/03/28]
131. Mitra A, MacIntyre DA, Ntritsos G, et al. The vaginal microbiota associates with the regression of untreated cervical intraepithelial neoplasia 2 lesions. *Nat Commun* 2020;11(1):1999. doi: 10.1038/s41467-020-15856-y [published Online First: 2020/04/26]

132. Caselli E, D'Accolti M, Santi E, et al. Vaginal Microbiota and Cytokine Microenvironment in HPV Clearance/Persistence in Women Surgically Treated for Cervical Intraepithelial Neoplasia: An Observational Prospective Study. *Front Cell Infect Microbiol* 2020;10:540900. doi: 10.3389/fcimb.2020.540900 [published Online First: 2020/12/01]
133. Kawahara R, Fujii T, Kukimoto I, et al. Changes to the cervicovaginal microbiota and cervical cytokine profile following surgery for cervical intraepithelial neoplasia. *Sci Rep* 2021;11(1):2156. doi: 10.1038/s41598-020-80176-6 [published Online First: 2021/01/27]
134. Zhang H, Lu J, Lu Y, et al. Cervical microbiome is altered in cervical intraepithelial neoplasia after loop electrosurgical excision procedure in china. *Sci Rep* 2018;8(1):4923. doi: 10.1038/s41598-018-23389-0 [published Online First: 2018/03/23]
135. Wiik J, Sengpiel V, Kyrgiou M, et al. Cervical microbiota in women with cervical intra-epithelial neoplasia, prior to and after local excisional treatment, a Norwegian cohort study. *BMC Womens Health* 2019;19(1):30. doi: 10.1186/s12905-019-0727-0 [published Online First: 2019/02/08]
136. Nene NR, Reisel D, Leimbach A, et al. Association between the cervicovaginal microbiome, BRCA1 mutation status, and risk of ovarian cancer: a case-control study. *Lancet Oncol* 2019;20(8):1171-82. doi: 10.1016/S1470-2045(19)30340-7 [published Online First: 2019/07/14]
137. Ness RB, Goodman MT, Shen C, et al. Serologic evidence of past infection with Chlamydia trachomatis, in relation to ovarian cancer. *J Infect Dis* 2003;187(7):1147-52. doi: 10.1086/368380 [published Online First: 2003/03/28]
138. Tsementzi D, Pena-Gonzalez A, Bai J, et al. Comparison of vaginal microbiota in gynecologic cancer patients pre- and post-radiation therapy and healthy women. *Cancer Med* 2020;9(11):3714-24. doi: 10.1002/cam4.3027 [published Online First: 2020/04/03]
139. Bai J, Jhaney I, Daniel G, et al. Pilot Study of Vaginal Microbiome Using QIIME 2 in Women With Gynecologic Cancer Before and After Radiation Therapy. *Oncol Nurs Forum* 2019;46(2):E48-E59. doi: 10.1188/19.ONF.E48-E59 [published Online First: 2019/02/16]
140. Jacobson D, Moore K, Gunderson C, et al. Shifts in gut and vaginal microbiomes are associated with cancer recurrence time in women with ovarian cancer. *PeerJ* 2021;9:e11574. doi: 10.7717/peerj.11574 [published Online First: 2021/06/29]
141. Chen C, Song X, Wei W, et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun* 2017;8(1):875. doi: 10.1038/s41467-017-00901-0 [published Online First: 2017/10/19]
142. Mitchell CM, Haick A, Nkwopara E, et al. Colonization of the upper genital tract by vaginal bacterial species in nonpregnant women. *Am J Obstet Gynecol* 2015;212(5):611 e1-9. doi: 10.1016/j.ajog.2014.11.043 [published Online First: 2014/12/20]
143. Verstraelen H, Vilchez-Vargas R, Desimpel F, et al. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing

of the V1-2 region of the 16S rRNA gene. *PeerJ* 2016;4:e1602. doi: 10.7717/peerj.1602 [published Online First: 2016/01/30]
144. Moreno I, Codoner FM, Vilella F, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol* 2016;215(6):684-703. doi: 10.1016/j.ajog.2016.09.075 [published Online First: 2016/10/21]
145. Hyman RW, Fukushima M, Jiang H, et al. Diversity of the vaginal microbiome correlates with preterm birth. *Reprod Sci* 2014;21(1):32-40. doi: 10.1177/1933719113488838 [published Online First: 2013/05/30]
146. Jefferson KK. The bacterial etiology of preterm birth. *Adv Appl Microbiol* 2012;80:1-22. doi: 10.1016/B978-0-12-394381-1.00001-5 [published Online First: 2012/07/17]
147. Hecht JL, Onderdonk A, Delaney M, et al. Characterization of chorioamnionitis in 2nd-trimester C-section placentas and correlation with microorganism recovery from subamniotic tissues. *Pediatr Dev Pathol* 2008;11(1):15-22. doi: 10.2350/07-06-0285.1 [published Online First: 2008/02/02]
148. Prince AL, Ma J, Kannan PS, et al. The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. *Am J Obstet Gynecol* 2016;214(5):627 e1-27 e16. doi: 10.1016/j.ajog.2016.01.193 [published Online First: 2016/03/12]
149. Barak S, Oettinger-Barak O, Machtei EE, et al. Evidence of periopathogenic microorganisms in placentas of women with preeclampsia. *J Periodontol* 2007;78(4):670-6. doi: 10.1902/jop.2007.060362 [published Online First: 2007/04/03]
150. DiGiulio DB, Gervasi M, Romero R, et al. Microbial invasion of the amniotic cavity in preeclampsia as assessed by cultivation and sequence-based methods. *J Perinat Med* 2010;38(5):503-13. doi: 10.1515/jpm.2010.078 [published Online First: 2010/05/21]
151. Amarasekara R, Jayasekara RW, Senanayake H, et al. Microbiome of the placenta in pre-eclampsia supports the role of bacteria in the multifactorial cause of pre-eclampsia. *J Obstet Gynaecol Res* 2015;41(5):662-9. doi: 10.1111/jog.12619 [published Online First: 2014/12/11]
152. Cicinelli E, De Ziegler D, Nicoletti R, et al. Poor reliability of vaginal and endocervical cultures for evaluating microbiology of endometrial cavity in women with chronic endometritis. *Gynecol Obstet Invest* 2009;68(2):108-15. doi: 10.1159/000223819 [published Online First: 2009/06/13]
153. Kushnir VA, Solouki S, Sarig-Meth T, et al. Systemic Inflammation and Autoimmunity in Women with Chronic Endometritis. *Am J Reprod Immunol* 2016;75(6):672-7. doi: 10.1111/aji.12508 [published Online First: 2016/03/10]
154. Johnston-MacAnanny EB, Hartnett J, Engmann LL, et al. Chronic endometritis is a frequent finding in women with recurrent implantation failure after in vitro fertilization. *Fertil Steril* 2010;93(2):437-41. doi: 10.1016/j.fertnstert.2008.12.131 [published Online First: 2009/02/17]
155. Khan KN, Kitajima M, Hiraki K, et al. Escherichia coli contamination of menstrual blood and effect of bacterial endotoxin on endometriosis. *Fertil Steril* 2010;94(7):2860-3 e1-3. doi: 10.1016/j.fertnstert.2010.04.053 [published Online First: 2010/07/16]

156. Khan KN, Fujishita A, Kitajima M, et al. Intra-uterine microbial colonization and occurrence of endometritis in women with endometriosisdagger. *Hum Reprod* 2014;29(11):2446-56. doi: 10.1093/humrep/deu222 [published Online First: 2014/09/11]
157. Khan KN, Fujishita A, Masumoto H, et al. Molecular detection of intrauterine microbial colonization in women with endometriosis. *Eur J Obstet Gynecol Reprod Biol* 2016;199:69-75. doi: 10.1016/j.ejogrb.2016.01.040 [published Online First: 2016/02/24]
158. Walther-Antonio MR, Chen J, Multinu F, et al. Potential contribution of the uterine microbiome in the development of endometrial cancer. *Genome Med* 2016;8(1):122. doi: 10.1186/s13073-016-0368-y [published Online First: 2016/11/26]
159. Zhou B, Sun C, Huang J, et al. The biodiversity Composition of Microbiome in Ovarian Carcinoma Patients. *Sci Rep* 2019;9(1):1691. doi: 10.1038/s41598-018-38031-2 [published Online First: 2019/02/10]
160. Brewster W KE, O Keku T. An evaluation of the microbiota of the upper genital tract of women with benign changes and epithelial ovarian cancer. *Journal of Clinical Oncology* 2016;34:5568-68.
161. Banerjee S, Tian T, Wei Z, et al. The ovarian cancer oncobiome. *Oncotarget* 2017;8(22):36225-45. doi: 10.18632/oncotarget.16717 [published Online First: 2017/04/15]
162. Shanmughapriya S, Senthilkumar G, Vinodhini K, et al. Viral and bacterial aetiologies of epithelial ovarian cancer. *Eur J Clin Microbiol Infect Dis* 2012;31(9):2311-7. doi: 10.1007/s10096-012-1570-5 [published Online First: 2012/03/10]
163. Idahl A, Lundin E, Jurstrand M, et al. Chlamydia trachomatis and Mycoplasma genitalium plasma antibodies in relation to epithelial ovarian tumors. *Infect Dis Obstet Gynecol* 2011;2011:824627. doi: 10.1155/2011/824627 [published Online First: 2011/08/04]
164. Idahl A, Lundin E, Elgh F, et al. Chlamydia trachomatis, Mycoplasma genitalium, Neisseria gonorrhoeae, human papillomavirus, and polyomavirus are not detectable in human tissue with epithelial ovarian cancer, borderline tumor, or benign conditions. *Am J Obstet Gynecol* 2010;202(1):71 e1-6. doi: 10.1016/j.ajog.2009.07.042 [published Online First: 2009/09/22]
165. Miao R, Badger TC, Groesch K, et al. Assessment of peritoneal microbial features and tumor marker levels as potential diagnostic tools for ovarian cancer. *PLoS One* 2020;15(1):e0227707. doi: 10.1371/journal.pone.0227707 [published Online First: 2020/01/10]
166. Kim SI, Kang N, Leem S, et al. Metagenomic Analysis of Serum Microbe-Derived Extracellular Vesicles and Diagnostic Models to Differentiate Ovarian Cancer and Benign Ovarian Tumor. *Cancers (Basel)* 2020;12(5) doi: 10.3390/cancers12051309 [published Online First: 2020/05/28]
167. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010;464(7285):59-65. doi: 10.1038/nature08821 [published Online First: 2010/03/06]
168. Quaranta G, Sanguinetti M, Masucci L. Fecal Microbiota Transplantation: A Potential Tool for Treatment of Human Female Reproductive Tract Diseases.

Front Immunol 2019;10:2653. doi: 10.3389/fimmu.2019.02653 [published Online First: 2019/12/13]
169. Scarpellini E, Ianiro G, Attili F, et al. The human gut microbiota and virome: Potential therapeutic implications. *Dig Liver Dis* 2015;47(12):1007-12. doi: 10.1016/j.dld.2015.07.008 [published Online First: 2015/08/11]
170. Rinninella E, Raoul P, Cintoni M, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* 2019;7(1) doi: 10.3390/microorganisms7010014 [published Online First: 2019/01/13]
171. Cammarota G, Ianiro G, Cianci R, et al. The involvement of gut microbiota in inflammatory bowel disease pathogenesis: potential for therapy. *Pharmacol Ther* 2015;149:191-212. doi: 10.1016/j.pharmthera.2014.12.006 [published Online First: 2015/01/07]
172. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the human intestinal microbial flora. *Science* 2005;308(5728):1635-8. doi: 10.1126/science.1110591 [published Online First: 2005/04/16]
173. Mueller NT, Bakacs E, Combellick J, et al. The infant microbiome development: mom matters. *Trends Mol Med* 2015;21(2):109-17. doi: 10.1016/j.molmed.2014.12.002 [published Online First: 2015/01/13]
174. Carding S, Verbeke K, Vipond DT, et al. Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis* 2015;26:26191. doi: 10.3402/mehd.v26.26191 [published Online First: 2015/02/06]
175. Ley RE, Turnbaugh PJ, Klein S, et al. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006;444(7122):1022-3. doi: 10.1038/4441022a [published Online First: 2006/12/22]
176. Turnbaugh PJ, Backhed F, Fulton L, et al. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* 2008;3(4):213-23. doi: 10.1016/j.chom.2008.02.015 [published Online First: 2008/04/15]
177. Schwiertz A, Taras D, Schafer K, et al. Microbiota and SCFA in lean and overweight healthy subjects. *Obesity (Silver Spring)* 2010;18(1):190-5. doi: 10.1038/oby.2009.167 [published Online First: 2009/06/06]
178. Tims S, Derom C, Jonkers DM, et al. Microbiota conservation and BMI signatures in adult monozygotic twins. *ISME J* 2013;7(4):707-17. doi: 10.1038/ismej.2012.146 [published Online First: 2012/11/30]
179. Allin KH, Tremaroli V, Caesar R, et al. Aberrant intestinal microbiota in individuals with prediabetes. *Diabetologia* 2018;61(4):810-20. doi: 10.1007/s00125-018-4550-1 [published Online First: 2018/01/31]
180. Zhong H, Ren H, Lu Y, et al. Distinct gut metagenomics and metaproteomics signatures in prediabetics and treatment-naive type 2 diabetics. *EBioMedicine* 2019;47:373-83. doi: 10.1016/j.ebiom.2019.08.048 [published Online First: 2019/09/08]
181. Backhed F, Manchester JK, Semenkovich CF, et al. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci U S A* 2007;104(3):979-84. doi: 10.1073/pnas.0605374104 [published Online First: 2007/01/11]

182. Turnbaugh PJ, Ley RE, Mahowald MA, et al. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006;444(7122):1027-31. doi: 10.1038/nature05414 [published Online First: 2006/12/22]
183. Cani PD, Bibiloni R, Knauf C, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008;57(6):1470-81. doi: 10.2337/db07-1403 [published Online First: 2008/02/29]
184. Creely SJ, McTernan PG, Kusminski CM, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab* 2007;292(3):E740-7. doi: 10.1152/ajpendo.00302.2006 [published Online First: 2006/11/09]
185. Brun P, Castagliuolo I, Di Leo V, et al. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *Am J Physiol Gastrointest Liver Physiol* 2007;292(2):G518-25. doi: 10.1152/ajpgi.00024.2006 [published Online First: 2006/10/07]
186. Wang Z, Wang Q, Zhao J, et al. Altered diversity and composition of the gut microbiome in patients with cervical cancer. *AMB Express* 2019;9(1):40. doi: 10.1186/s13568-019-0763-z [published Online First: 2019/03/25]
187. Mori G, Orena BS, Cultrera I, et al. Gut Microbiota Analysis in Postoperative Lynch Syndrome Patients. *Front Microbiol* 2019;10:1746. doi: 10.3389/fmicb.2019.01746 [published Online First: 2019/08/17]
188. El Alam MB, Sims TT, Kouzy R, et al. A prospective study of the adaptive changes in the gut microbiome during standard-of-care chemoradiotherapy for gynecologic cancers. *PLoS One* 2021;16(3):e0247905. doi: 10.1371/journal.pone.0247905 [published Online First: 2021/03/05]
189. Mitra A, Grossman Biegert GW, Delgado AY, et al. Microbial Diversity and Composition Is Associated with Patient-Reported Toxicity during Chemoradiation Therapy for Cervical Cancer. *Int J Radiat Oncol Biol Phys* 2020;107(1):163-71. doi: 10.1016/j.ijrobp.2019.12.040 [published Online First: 2020/01/29]
190. Nam YD, Kim HJ, Seo JG, et al. Impact of pelvic radiotherapy on gut microbiota of gynecological cancer patients revealed by massive pyrosequencing. *PLoS One* 2013;8(12):e82659. doi: 10.1371/journal.pone.0082659 [published Online First: 2013/12/25]
191. Sims TT, El Alam MB, Karpinets TV, et al. Gut microbiome diversity is an independent predictor of survival in cervical cancer patients receiving chemoradiation. *Commun Biol* 2021;4(1):237. doi: 10.1038/s42003-021-01741-x [published Online First: 2021/02/24]
192. Muls A, Andreyev J, Lalondrelle S, et al. Systematic Review: The Impact of Cancer Treatment on the Gut and Vaginal Microbiome in Women With a Gynecological Malignancy. *Int J Gynecol Cancer* 2017;27(7):1550-59. doi: 10.1097/IGC.0000000000000999 [published Online First: 2017/06/08]
193. El Aila NA, Tency I, Saerens B, et al. Strong correspondence in bacterial loads between the vagina and rectum of pregnant women. *Res Microbiol* 2011;162(5):506-13. doi: 10.1016/j.resmic.2011.04.004 [published Online First: 2011/04/26]

194. Antonio MA, Rabe LK, Hillier SL. Colonization of the rectum by Lactobacillus species and decreased risk of bacterial vaginosis. *J Infect Dis* 2005;192(3):394-8. doi: 10.1086/430926 [published Online First: 2005/07/05]
195. Marrazzo JM, Antonio M, Agnew K, et al. Distribution of genital Lactobacillus strains shared by female sex partners. *J Infect Dis* 2009;199(5):680-3. doi: 10.1086/596632 [published Online First: 2009/02/10]
196. Mirmonsef P, Hotton AL, Gilbert D, et al. Free glycogen in vaginal fluids is associated with Lactobacillus colonization and low vaginal pH. *PLoS One* 2014;9(7):e102467. doi: 10.1371/journal.pone.0102467 [published Online First: 2014/07/18]
197. Plottel CS, Blaser MJ. Microbiome and malignancy. *Cell Host Microbe* 2011;10(4):324-35. doi: 10.1016/j.chom.2011.10.003 [published Online First: 2011/10/25]
198. Baker JM, Al-Nakkash L, Herbst-Kralovetz MM. Estrogen-gut microbiome axis: Physiological and clinical implications. *Maturitas* 2017;103:45-53. doi: 10.1016/j.maturitas.2017.06.025 [published Online First: 2017/08/06]
199. Kwa M, Plottel CS, Blaser MJ, et al. The Intestinal Microbiome and Estrogen Receptor-Positive Female Breast Cancer. *J Natl Cancer Inst* 2016;108(8) doi: 10.1093/jnci/djw029 [published Online First: 2016/04/24]
200. Ervin SM, Li H, Lim L, et al. Gut microbial beta-glucuronidases reactivate estrogens as components of the estrobolome that reactivate estrogens. *J Biol Chem* 2019;294(49):18586-99. doi: 10.1074/jbc.RA119.010950 [published Online First: 2019/10/23]
201. Sher A, Rahman MA. Enterohepatic recycling of estrogen and its relevance with female fertility. *Arch Pharm Res* 2000;23(5):513-7. doi: 10.1007/BF02976582 [published Online First: 2000/11/04]
202. Vree TB, Timmer CJ. Enterohepatic cycling and pharmacokinetics of oestradiol in postmenopausal women. *J Pharm Pharmacol* 1998;50(8):857-64. doi: 10.1111/j.2042-7158.1998.tb04000.x [published Online First: 1998/09/29]
203. Stewart-Tull DE. Evidence That Vaginal Lactobacilli Do Not Ferment Glycogen. *Am J Obstet Gynecol* 1964;88:676-9. doi: 10.1016/0002-9378(64)90898-1 [published Online First: 1964/03/01]
204. Martin R, Soberon N, Vaneechoutte M, et al. Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *Int Microbiol* 2008;11(4):261-6. doi: 10.2436/20.1501.01.70 [published Online First: 2009/02/11]
205. Spear GT, French AL, Gilbert D, et al. Human alpha-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by Lactobacillus. *J Infect Dis* 2014;210(7):1019-28. doi: 10.1093/infdis/jiu231 [published Online First: 2014/04/17]
206. Nasioudis D, Beghini J, Bongiovanni AM, et al. alpha-Amylase in Vaginal Fluid: Association With Conditions Favorable to Dominance of Lactobacillus. *Reprod Sci* 2015;22(11):1393-8. doi: 10.1177/1933719115581000 [published Online First: 2015/04/17]
207. Blaser MJ. Understanding microbe-induced cancers. *Cancer Prev Res (Phila)* 2008;1(1):15-20. doi: 10.1158/1940-6207.CAPR-08-0024 [published Online First: 2009/01/14]

208. Sepich-Poore GD, Zitvogel L, Straussman R, et al. The microbiome and human cancer. *Science* 2021;371(6536) doi: 10.1126/science.abc4552 [published Online First: 2021/03/27]
209. Wroblewski LE, Peek RM, Jr. Helicobacter pylori in gastric carcinogenesis: mechanisms. *Gastroenterol Clin North Am* 2013;42(2):285-98. doi: 10.1016/j.gtc.2013.01.006 [published Online First: 2013/05/04]
210. Koeppel M, Garcia-Alcalde F, Glowinski F, et al. Helicobacter pylori Infection Causes Characteristic DNA Damage Patterns in Human Cells. *Cell Rep* 2015;11(11):1703-13. doi: 10.1016/j.celrep.2015.05.030 [published Online First: 2015/06/16]
211. Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. *Nat Rev Cancer* 2013;13(2):123-35. doi: 10.1038/nrc3449 [published Online First: 2013/01/25]
212. Schiffman M, Wentzensen N, Wacholder S, et al. Human papillomavirus testing in the prevention of cervical cancer. *J Natl Cancer Inst* 2011;103(5):368-83. doi: 10.1093/jnci/djq562 [published Online First: 2011/02/02]
213. Helmink BA, Khan MAW, Hermann A, et al. The microbiome, cancer, and cancer therapy. *Nat Med* 2019;25(3):377-88. doi: 10.1038/s41591-019-0377-7 [published Online First: 2019/03/08]
214. Faghfoori Z, Faghfoori MH, Saber A, et al. Anticancer effects of bifidobacteria on colon cancer cell lines. *Cancer Cell Int* 2021;21(1):258. doi: 10.1186/s12935-021-01971-3 [published Online First: 2021/05/14]
215. Sacksteder MR. Occurrence of spontaneous tumors in the germfree F344 rat. *J Natl Cancer Inst* 1976;57(6):1371-3. doi: 10.1093/jnci/57.6.1371 [published Online First: 1976/12/01]
216. Schreiber H, Nettesheim P, Lijinsky W, et al. Induction of lung cancer in germfree, specific-pathogen-free, and infected rats by N-nitrosoheptamethyleneimine: enhancement by respiratory infection. *J Natl Cancer Inst* 1972;49(4):1107-14. [published Online First: 1972/10/01]
217. Reddy BS, Narisawa T, Weisburger JH. Colon carcinogenesis in germ-free rats with intrarectal 1,2-dimethylhydrazine and subcutaneous azoxymethane. *Cancer Res* 1976;36(8):2874-6. [published Online First: 1976/08/01]
218. Reddy BS, Ohmori T. Effect of intestinal microflora and dietary fat on 3,2'-dimethyl-4-aminobiphenyl-induced colon carcinogenesis in F344 rats. *Cancer Res* 1981;41(4):1363-7. [published Online First: 1981/04/01]
219. Laqueur GL, Matsumoto H, Yamamoto RS. Comparison of the carcinogenicity of methylazoxymethanol-beta-D-glucosiduronic acid in conventional and germfree Sprague-Dawley rats. *J Natl Cancer Inst* 1981;67(5):1053-5. [published Online First: 1981/11/01]
220. Lofgren JL, Whary MT, Ge Z, et al. Lack of commensal flora in Helicobacter pylori-infected INS-GAS mice reduces gastritis and delays intraepithelial neoplasia. *Gastroenterology* 2011;140(1):210-20. doi: 10.1053/j.gastro.2010.09.048 [published Online First: 2010/10/19]
221. Li Y, Kundu P, Seow SW, et al. Gut microbiota accelerate tumor growth via c-jun and STAT3 phosphorylation in APCMin/+ mice. *Carcinogenesis* 2012;33(6):1231-8. doi: 10.1093/carcin/bgs137 [published Online First: 2012/03/31]

222. Vannucci L, Stepankova R, Kozakova H, et al. Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. *Int J Oncol* 2008;32(3):609-17. [published Online First: 2008/02/23]
223. Chen GY, Shaw MH, Redondo G, et al. The innate immune receptor Nod1 protects the intestine from inflammation-induced tumorigenesis. *Cancer Res* 2008;68(24):10060-7. doi: 10.1158/0008-5472.CAN-08-2061 [published Online First: 2008/12/17]
224. Yu LX, Yan HX, Liu Q, et al. Endotoxin accumulation prevents carcinogen-induced apoptosis and promotes liver tumorigenesis in rodents. *Hepatology* 2010;52(4):1322-33. doi: 10.1002/hep.23845 [published Online First: 2010/08/31]
225. Grivennikov SI, Wang K, Mucida D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* 2012;491(7423):254-8. doi: 10.1038/nature11465 [published Online First: 2012/10/05]
226. Klimesova K, Kverka M, Zakostelska Z, et al. Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M-deficient mice. *Inflamm Bowel Dis* 2013;19(6):1266-77. doi: 10.1097/MIB.0b013e318281330a [published Online First: 2013/04/10]
227. Lee CW, Rickman B, Rogers AB, et al. Helicobacter pylori eradication prevents progression of gastric cancer in hypergastrinemic INS-GAS mice. *Cancer Res* 2008;68(9):3540-8. doi: 10.1158/0008-5472.CAN-07-6786 [published Online First: 2008/04/29]
228. Dapito DH, Mencin A, Gwak GY, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell* 2012;21(4):504-16. doi: 10.1016/j.ccr.2012.02.007 [published Online First: 2012/04/21]
229. Ochi A, Nguyen AH, Bedrosian AS, et al. MyD88 inhibition amplifies dendritic cell capacity to promote pancreatic carcinogenesis via Th2 cells. *J Exp Med* 2012;209(9):1671-87. doi: 10.1084/jem.20111706 [published Online First: 2012/08/22]
230. Kadosh E, Snir-Alkalay I, Venkatachalam A, et al. The gut microbiome switches mutant p53 from tumour-suppressive to oncogenic. *Nature* 2020;586(7827):133-38. doi: 10.1038/s41586-020-2541-0 [published Online First: 2020/07/31]
231. Sobhani I, Bergsten E, Couffin S, et al. Colorectal cancer-associated microbiota contributes to oncogenic epigenetic signatures. *Proc Natl Acad Sci U S A* 2019;116(48):24285-95. doi: 10.1073/pnas.1912129116 [published Online First: 2019/11/13]
232. Schwabe RF, Jobin C. The microbiome and cancer. *Nat Rev Cancer* 2013;13(11):800-12. doi: 10.1038/nrc3610 [published Online First: 2013/10/18]
233. Mittal D, Saccheri F, Venereau E, et al. TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells. *EMBO J* 2010;29(13):2242-52. doi: 10.1038/emboj.2010.94 [published Online First: 2010/06/08]
234. Fukata M, Chen A, Vamadevan AS, et al. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. *Gastroenterology*

2007;133(6):1869-81. doi: 10.1053/j.gastro.2007.09.008 [published Online First: 2007/12/07]
235. Fukata M, Shang L, Santaolalla R, et al. Constitutive activation of epithelial TLR4 augments inflammatory responses to mucosal injury and drives colitis-associated tumorigenesis. *Inflamm Bowel Dis* 2011;17(7):1464-73. doi: 10.1002/ibd.21527 [published Online First: 2011/06/16]
236. Tye H, Kennedy CL, Najdovska M, et al. STAT3-driven upregulation of TLR2 promotes gastric tumorigenesis independent of tumor inflammation. *Cancer Cell* 2012;22(4):466-78. doi: 10.1016/j.ccr.2012.08.010 [published Online First: 2012/10/20]
237. Travaglione S, Fabbri A, Fiorentini C. The Rho-activating CNF1 toxin from pathogenic E. coli: a risk factor for human cancer development? *Infect Agent Cancer* 2008;3:4. doi: 10.1186/1750-9378-3-4 [published Online First: 2008/03/14]
238. Nesic D, Hsu Y, Stebbins CE. Assembly and function of a bacterial genotoxin. *Nature* 2004;429(6990):429-33. doi: 10.1038/nature02532 [published Online First: 2004/05/28]
239. Smith JL, Bayles DO. The contribution of cytolethal distending toxin to bacterial pathogenesis. *Crit Rev Microbiol* 2006;32(4):227-48. doi: 10.1080/10408410601023557 [published Online First: 2006/11/25]
240. Philipp B. Bacterial degradation of bile salts. *Appl Microbiol Biotechnol* 2011;89(4):903-15. doi: 10.1007/s00253-010-2998-0 [published Online First: 2010/11/23]
241. Gill SR, Pop M, Deboy RT, et al. Metagenomic analysis of the human distal gut microbiome. *Science* 2006;312(5778):1355-9. doi: 10.1126/science.1124234 [published Online First: 2006/06/03]
242. Nyangale EP, Mottram DS, Gibson GR. Gut microbial activity, implications for health and disease: the potential role of metabolite analysis. *J Proteome Res* 2012;11(12):5573-85. doi: 10.1021/pr300637d [published Online First: 2012/11/03]
243. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. *Cell Res* 2020;30(6):492-506. doi: 10.1038/s41422-020-0332-7 [published Online First: 2020/05/21]
244. Zhang Z, Tang H, Chen P, et al. Demystifying the manipulation of host immunity, metabolism, and extraintestinal tumors by the gut microbiome. *Signal Transduct Target Ther* 2019;4:41. doi: 10.1038/s41392-019-0074-5 [published Online First: 2019/10/23]
245. Seth P, Hsieh PN, Jamal S, et al. Regulation of MicroRNA Machinery and Development by Interspecies S-Nitrosylation. *Cell* 2019;176(5):1014-25 e12. doi: 10.1016/j.cell.2019.01.037 [published Online First: 2019/02/23]
246. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, et al. Mapping human microbiome drug metabolism by gut bacteria and their genes. *Nature* 2019;570(7762):462-67. doi: 10.1038/s41586-019-1291-3 [published Online First: 2019/06/04]
247. Rosshart SP, Vassallo BG, Angeletti D, et al. Wild Mouse Gut Microbiota Promotes Host Fitness and Improves Disease Resistance. *Cell*

2017;171(5):1015-28 e13. doi: 10.1016/j.cell.2017.09.016 [published Online First: 2017/10/24]
248. Hearps AC, Tyssen D, Srbinovski D, et al. Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. *Mucosal Immunol* 2017;10(6):1480-90. doi: 10.1038/mi.2017.27 [published Online First: 2017/04/13]
249. Wagner W, Ciszewski WM, Kania KD. L- and D-lactate enhance DNA repair and modulate the resistance of cervical carcinoma cells to anticancer drugs via histone deacetylase inhibition and hydroxycarboxylic acid receptor 1 activation. *Cell Commun Signal* 2015;13:36. doi: 10.1186/s12964-015-0114-x [published Online First: 2015/07/26]
250. Tukenmez U, Aktas B, Aslim B, et al. The relationship between the structural characteristics of lactobacilli-EPS and its ability to induce apoptosis in colon cancer cells in vitro. *Sci Rep* 2019;9(1):8268. doi: 10.1038/s41598-019-44753-8 [published Online First: 2019/06/06]
251. An J, Ha EM. Combination Therapy of Lactobacillus plantarum Supernatant and 5-Fluouracil Increases Chemosensitivity in Colorectal Cancer Cells. *J Microbiol Biotechnol* 2016;26(8):1490-503. doi: 10.4014/jmb.1605.05024 [published Online First: 2016/05/26]
252. Burrows MP, Volchkov P, Kobayashi KS, et al. Microbiota regulates type 1 diabetes through Toll-like receptors. *Proc Natl Acad Sci U S A* 2015;112(32):9973-7. doi: 10.1073/pnas.1508740112 [published Online First: 2015/07/29]
253. Caricilli AM, Saad MJ. The role of gut microbiota on insulin resistance. *Nutrients* 2013;5(3):829-51. doi: 10.3390/nu5030829 [published Online First: 2013/03/14]
254. Lee CJ, Sears CL, Maruthur N. Gut microbiome and its role in obesity and insulin resistance. *Ann N Y Acad Sci* 2020;1461(1):37-52. doi: 10.1111/nyas.14107 [published Online First: 2019/05/16]
255. Zhang Y, Guo X, Guo J, et al. Lactobacillus casei reduces susceptibility to type 2 diabetes via microbiota-mediated body chloride ion influx. *Sci Rep* 2014;4:5654. doi: 10.1038/srep05654 [published Online First: 2014/08/19]
256. Chen YT, Yang NS, Lin YC, et al. A combination of Lactobacillus mali APS1 and dieting improved the efficacy of obesity treatment via manipulating gut microbiome in mice. *Sci Rep* 2018;8(1):6153. doi: 10.1038/s41598-018-23844-y [published Online First: 2018/04/20]
257. Zitvogel L, Daillere R, Roberti MP, et al. Anticancer effects of the microbiome and its products. *Nat Rev Microbiol* 2017;15(8):465-78. doi: 10.1038/nrmicro.2017.44 [published Online First: 2017/05/23]
258. Sears CL, Pardoll DM. The intestinal microbiome influences checkpoint blockade. *Nat Med* 2018;24(3):254-55. doi: 10.1038/nm.4511 [published Online First: 2018/03/07]
259. Nussing S, Trapani JA, Parish IA. Revisiting T Cell Tolerance as a Checkpoint Target for Cancer Immunotherapy. *Front Immunol* 2020;11:589641. doi: 10.3389/fimmu.2020.589641 [published Online First: 2020/10/20]

260. Darvin P, Toor SM, Sasidharan Nair V, et al. Immune checkpoint inhibitors: recent progress and potential biomarkers. *Exp Mol Med* 2018;50(12):1-11. doi: 10.1038/s12276-018-0191-1 [published Online First: 2018/12/14]
261. Sivan A, Corrales L, Hubert N, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* 2015;350(6264):1084-9. doi: 10.1126/science.aac4255 [published Online First: 2015/11/07]
262. Vetizou M, Pitt JM, Daillere R, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* 2015;350(6264):1079-84. doi: 10.1126/science.aad1329 [published Online First: 2015/11/07]
263. Routy B, Le Chatelier E, Derosa L, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* 2018;359(6371):91-97. doi: 10.1126/science.aan3706 [published Online First: 2017/11/04]
264. Gopalakrishnan V, Spencer CN, Nezi L, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* 2018;359(6371):97-103. doi: 10.1126/science.aan4236 [published Online First: 2017/11/04]
265. Matson V, Fessler J, Bao R, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* 2018;359(6371):104-08. doi: 10.1126/science.aao3290 [published Online First: 2018/01/06]
266. Boursi B, Mamtani R, Haynes K, et al. Recurrent antibiotic exposure may promote cancer formation--Another step in understanding the role of the human microbiota? *Eur J Cancer* 2015;51(17):2655-64. doi: 10.1016/j.ejca.2015.08.015 [published Online First: 2015/09/05]
267. Pinato DJ, Howlett S, Ottaviani D, et al. Association of Prior Antibiotic Treatment With Survival and Response to Immune Checkpoint Inhibitor Therapy in Patients With Cancer. *JAMA Oncol* 2019;5(12):1774-78. doi: 10.1001/jamaoncol.2019.2785 [published Online First: 2019/09/13]
268. Di Tucci C, Capone C, Galati G, et al. Immunotherapy in endometrial cancer: new scenarios on the horizon. *J Gynecol Oncol* 2019;30(3):e46. doi: 10.3802/jgo.2019.30.e46 [published Online First: 2019/03/20]
269. Doroshow DB, Bhalla S, Beasley MB, et al. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. *Nat Rev Clin Oncol* 2021;18(6):345-62. doi: 10.1038/s41571-021-00473-5 [published Online First: 2021/02/14]
270. Zhao P, Li L, Jiang X, et al. Mismatch repair deficiency/microsatellite instability-high as a predictor for anti-PD-1/PD-L1 immunotherapy efficacy. *J Hematol Oncol* 2019;12(1):54. doi: 10.1186/s13045-019-0738-1 [published Online First: 2019/06/04]
271. Marabelle A, Fakih M, Lopez J, et al. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. *Lancet Oncol* 2020;21(10):1353-65. doi: 10.1016/S1470-2045(20)30445-9 [published Online First: 2020/09/14]

272. Maqsood MI, Matin MM, Bahrami AR, et al. Immortality of cell lines: challenges and advantages of establishment. *Cell Biol Int* 2013;37(10):1038-45. doi: 10.1002/cbin.10137 [published Online First: 2013/06/01]
273. Huang Y, Liu Y, Zheng C, et al. Investigation of Cross-Contamination and Misidentification of 278 Widely Used Tumor Cell Lines. *PLoS One* 2017;12(1):e0170384. doi: 10.1371/journal.pone.0170384 [published Online First: 2017/01/21]
274. Ben-David U, Siranosian B, Ha G, et al. Genetic and transcriptional evolution alters cancer cell line drug response. *Nature* 2018;560(7718):325-30. doi: 10.1038/s41586-018-0409-3 [published Online First: 2018/08/10]
275. Geraghty RJ, Capes-Davis A, Davis JM, et al. Guidelines for the use of cell lines in biomedical research. *Br J Cancer* 2014;111(6):1021-46. doi: 10.1038/bjc.2014.166 [published Online First: 2014/08/15]
276. Duval K, Grover H, Han LH, et al. Modeling Physiological Events in 2D vs. 3D Cell Culture. *Physiology (Bethesda)* 2017;32(4):266-77. doi: 10.1152/physiol.00036.2016 [published Online First: 2017/06/16]
277. Walker C, Mojares E, Del Rio Hernandez A. Role of Extracellular Matrix in Development and Cancer Progression. *Int J Mol Sci* 2018;19(10) doi: 10.3390/ijms19103028 [published Online First: 2018/10/06]
278. Fung C, Lock R, Gao S, et al. Induction of autophagy during extracellular matrix detachment promotes cell survival. *Mol Biol Cell* 2008;19(3):797-806. doi: 10.1091/mbc.e07-10-1092 [published Online First: 2007/12/21]
279. Boretto M, Cox B, Noben M, et al. Development of organoids from mouse and human endometrium showing endometrial epithelium physiology and long-term expandability. *Development* 2017;144(10):1775-86. doi: 10.1242/dev.148478 [published Online First: 2017/04/27]
280. Turco MY, Gardner L, Hughes J, et al. Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium. *Nat Cell Biol* 2017;19(5):568-77. doi: 10.1038/ncb3516 [published Online First: 2017/04/11]
281. Haider S, Gamperl M, Burkard TR, et al. Estrogen Signaling Drives Ciliogenesis in Human Endometrial Organoids. *Endocrinology* 2019;160(10):2282-97. doi: 10.1210/en.2019-00314 [published Online First: 2019/07/11]
282. Boretto M, Maenhoudt N, Luo X, et al. Patient-derived organoids from endometrial disease capture clinical heterogeneity and are amenable to drug screening. *Nat Cell Biol* 2019;21(8):1041-51. doi: 10.1038/s41556-019-0360-z [published Online First: 2019/08/03]
283. Kopper O, de Witte CJ, Lohmussaar K, et al. An organoid platform for ovarian cancer captures intra- and interpatient heterogeneity. *Nat Med* 2019;25(5):838-49. doi: 10.1038/s41591-019-0422-6 [published Online First: 2019/04/24]
284. Hill SJ, Decker B, Roberts EA, et al. Prediction of DNA Repair Inhibitor Response in Short-Term Patient-Derived Ovarian Cancer Organoids. *Cancer Discov* 2018;8(11):1404-21. doi: 10.1158/2159-8290.CD-18-0474 [published Online First: 2018/09/15]
285. Kessler M, Hoffmann K, Brinkmann V, et al. The Notch and Wnt pathways regulate stemness and differentiation in human fallopian tube organoids. *Nat Commun* 2015;6:8989. doi: 10.1038/ncomms9989 [published Online First: 2015/12/09]

286. Kessler M, Hoffmann K, Fritsche K, et al. Chronic Chlamydia infection in human organoids increases stemness and promotes age-dependent CpG methylation. *Nat Commun* 2019;10(1):1194. doi: 10.1038/s41467-019-09144-7 [published Online First: 2019/03/20]
287. Ogawa-Tominaga M, Umezu T, Nakajima T, et al. Stratification of mouse vaginal epithelium. 1. Development of three-dimensional models in vitro with clonal cell lines. *Biol Reprod* 2018;99(4):718-26. doi: 10.1093/biolre/ioy114 [published Online First: 2018/05/17]
288. Zhao Y, Yao R, Ouyang L, et al. Three-dimensional printing of Hela cells for cervical tumor model in vitro. *Biofabrication* 2014;6(3):035001. doi: 10.1088/1758-5082/6/3/035001 [published Online First: 2014/04/12]
289. Lee JM, Park DY, Yang L, et al. Generation of uniform-sized multicellular tumor spheroids using hydrogel microwells for advanced drug screening. *Sci Rep* 2018;8(1):17145. doi: 10.1038/s41598-018-35216-7 [published Online First: 2018/11/23]
290. Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* 2008;8(5):387-98. doi: 10.1038/nrc2389 [published Online First: 2008/04/25]
291. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009;459(7244):262-5. doi: 10.1038/nature07935 [published Online First: 2009/03/31]
292. Eiraku M, Takata N, Ishibashi H, et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* 2011;472(7341):51-6. doi: 10.1038/nature09941 [published Online First: 2011/04/09]
293. Vidimar V, Chakravarti D, Bulun SE, et al. The AKT/BCL-2 Axis Mediates Survival of Uterine Leiomyoma in a Novel 3D Spheroid Model. *Endocrinology* 2018;159(3):1453-62. doi: 10.1210/en.2017-03191 [published Online First: 2018/01/31]
294. Xie J, Xu X, Yin P, et al. Application of ex-vivo spheroid model system for the analysis of senescence and senolytic phenotypes in uterine leiomyoma. *Lab Invest* 2018;98(12):1575-87. doi: 10.1038/s41374-018-0117-5 [published Online First: 2018/09/13]
295. Domnina A, Novikova P, Obidina J, et al. Human mesenchymal stem cells in spheroids improve fertility in model animals with damaged endometrium. *Stem Cell Res Ther* 2018;9(1):50. doi: 10.1186/s13287-018-0801-9 [published Online First: 2018/02/28]
296. Haeger JD, Hambruch N, Dantzer V, et al. Changes in endometrial ezrin and cytokeratin 18 expression during bovine implantation and in caruncular endometrial spheroids in vitro. *Placenta* 2015;36(8):821-31. doi: 10.1016/j.placenta.2015.06.001 [published Online First: 2015/06/29]
297. Seishima R, Leung C, Yada S, et al. Neonatal Wnt-dependent Lgr5 positive stem cells are essential for uterine gland development. *Nat Commun* 2019;10(1):5378. doi: 10.1038/s41467-019-13363-3 [published Online First: 2019/11/28]
298. Girda E, Huang EC, Leiserowitz GS, et al. The Use of Endometrial Cancer Patient-Derived Organoid Culture for Drug Sensitivity Testing Is Feasible. *Int J*

Gynecol Cancer 2017;27(8):1701-07. doi: 10.1097/IGC.0000000000001061 [published Online First: 2017/07/07]
299. Kiyohara Y, Yoshino K, Kubota S, et al. Drug screening and grouping by sensitivity with a panel of primary cultured cancer spheroids derived from endometrial cancer. *Cancer Sci* 2016;107(4):452-60. doi: 10.1111/cas.12898 [published Online First: 2016/01/31]
300. Maru Y, Tanaka N, Itami M, et al. Efficient use of patient-derived organoids as a preclinical model for gynecologic tumors. *Gynecol Oncol* 2019;154(1):189-98. doi: 10.1016/j.ygyno.2019.05.005 [published Online First: 2019/05/19]
301. Li X, Francies HE, Secrier M, et al. Organoid cultures recapitulate esophageal adenocarcinoma heterogeneity providing a model for clonality studies and precision therapeutics. *Nat Commun* 2018;9(1):2983. doi: 10.1038/s41467-018-05190-9 [published Online First: 2018/08/01]
302. Gao D, Vela I, Sboner A, et al. Organoid cultures derived from patients with advanced prostate cancer. *Cell* 2014;159(1):176-87. doi: 10.1016/j.cell.2014.08.016 [published Online First: 2014/09/10]
303. Lawrenson K, Notaridou M, Lee N, et al. In vitro three-dimensional modeling of fallopian tube secretory epithelial cells. *BMC Cell Biol* 2013;14:43. doi: 10.1186/1471-2121-14-43 [published Online First: 2013/09/28]
304. Semertzidou A, Brosens JJ, McNeish I, et al. Organoid models in gynaecological oncology research. *Cancer Treat Rev* 2020;90:102103. doi: 10.1016/j.ctrv.2020.102103 [published Online First: 2020/09/16]
305. O'Hara AJ, Bell DW. The genomics and genetics of endometrial cancer. *Adv Genomics Genet* 2012;2012(2):33-47. doi: 10.2147/AGG.S28953 [published Online First: 2012/08/14]
306. Eritja N, Llobet D, Domingo M, et al. A novel three-dimensional culture system of polarized epithelial cells to study endometrial carcinogenesis. *Am J Pathol* 2010;176(6):2722-31. doi: 10.2353/ajpath.2010.090974 [published Online First: 2010/04/17]
307. Hennes A, Held K, Boretto M, et al. Functional expression of the mechanosensitive PIEZO1 channel in primary endometrial epithelial cells and endometrial organoids. *Sci Rep* 2019;9(1):1779. doi: 10.1038/s41598-018-38376-8 [published Online First: 2019/02/12]
308. Walsh DM, Hokenstad AN, Chen J, et al. Postmenopause as a key factor in the composition of the Endometrial Cancer Microbiome (ECbiome). *Sci Rep* 2019;9(1):19213. doi: 10.1038/s41598-019-55720-8 [published Online First: 2019/12/18]
309. Raglan O, MacIntyre DA, Mitra A, et al. The association between obesity and weight loss after bariatric surgery on the vaginal microbiota. *Microbiome* 2021;9(1):124. doi: 10.1186/s40168-021-01011-2 [published Online First: 2021/05/30]
310. Norenhag J, Du J, Olovsson M, et al. The vaginal microbiota, human papillomavirus and cervical dysplasia: a systematic review and network meta-analysis. *BJOG* 2020;127(2):171-80. doi: 10.1111/1471-0528.15854 [published Online First: 2019/06/27]

311. Reilly SM, Saltiel AR. Adapting to obesity with adipose tissue inflammation. *Nat Rev Endocrinol* 2017;13(11):633-43. doi: 10.1038/nrendo.2017.90 [published Online First: 2017/08/12]
312. Chelakkot C, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. *Exp Mol Med* 2018;50(8):1-9. doi: 10.1038/s12276-018-0126-x [published Online First: 2018/08/18]
313. Cristofori F, Dargenio VN, Dargenio C, et al. Anti-Inflammatory and Immunomodulatory Effects of Probiotics in Gut Inflammation: A Door to the Body. *Front Immunol* 2021;12:578386. doi: 10.3389/fimmu.2021.578386 [published Online First: 2021/03/16]
314. Orlando A, Refolo MG, Messa C, et al. Antiproliferative and proapoptotic effects of viable or heat-killed Lactobacillus paracasei IMPC2.1 and Lactobacillus rhamnosus GG in HGC-27 gastric and DLD-1 colon cell lines. *Nutr Cancer* 2012;64(7):1103-11. doi: 10.1080/01635581.2012.717676 [published Online First: 2012/10/16]
315. Nowak A, Paliwoda A, Blasiak J. Anti-proliferative, pro-apoptotic and anti-oxidative activity of Lactobacillus and Bifidobacterium strains: A review of mechanisms and therapeutic perspectives. *Crit Rev Food Sci Nutr* 2019;59(21):3456-67. doi: 10.1080/10408398.2018.1494539 [published Online First: 2018/07/17]
316. Sterne JA, Davey Smith G. Sifting the evidence-what's wrong with significance tests? *BMJ* 2001;322(7280):226-31. doi: 10.1136/bmj.322.7280.226 [published Online First: 2001/02/13]
317. Johnson VE. Revised standards for statistical evidence. *Proc Natl Acad Sci U S A* 2013;110(48):19313-7. doi: 10.1073/pnas.1313476110 [published Online First: 2013/11/13]
318. Ioannidis JP, Tarone R, McLaughlin JK. The false-positive to false-negative ratio in epidemiologic studies. *Epidemiology* 2011;22(4):450-6. doi: 10.1097/EDE.0b013e31821b506e [published Online First: 2011/04/15]
319. Kalliala I, Markozannes G, Gunter MJ, et al. Obesity and gynaecological and obstetric conditions: umbrella review of the literature. *BMJ* 2017;359:j4511. doi: 10.1136/bmj.j4511 [published Online First: 2017/10/28]
320. Kyrgiou M, Kalliala I, Markozannes G, et al. Adiposity and cancer at major anatomical sites: umbrella review of the literature. *BMJ* 2017;356:j477. doi: 10.1136/bmj.j477 [published Online First: 2017/03/02]
321. Shea BJ, Reeves BC, Wells G, et al. AMSTAR 2: a critical appraisal tool for systematic reviews that include randomised or non-randomised studies of healthcare interventions, or both. *BMJ* 2017;358:j4008. doi: 10.1136/bmj.j4008 [published Online First: 2017/09/25]
322. Shea BJ, Grimshaw JM, Wells GA, et al. Development of AMSTAR: a measurement tool to assess the methodological quality of systematic reviews. *BMC Med Res Methodol* 2007;7:10. doi: 10.1186/1471-2288-7-10 [published Online First: 2007/02/17]
323. Shea BJ, Hamel C, Wells GA, et al. AMSTAR is a reliable and valid measurement tool to assess the methodological quality of systematic reviews. *J Clin Epidemiol* 2009;62(10):1013-20. doi: 10.1016/j.jclinepi.2008.10.009 [published Online First: 2009/02/24]

324. Savovic J, Weeks L, Sterne JA, et al. Evaluation of the Cochrane Collaboration's tool for assessing the risk of bias in randomized trials: focus groups, online survey, proposed recommendations and their implementation. *Syst Rev* 2014;3:37. doi: 10.1186/2046-4053-3-37 [published Online First: 2014/04/16]
325. Sterne JA, Hernan MA, Reeves BC, et al. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. *BMJ* 2016;355:i4919. doi: 10.1136/bmj.i4919 [published Online First: 2016/10/14]
326. DerSimonian R, Laird N. Meta-analysis in clinical trials revisited. *Contemp Clin Trials* 2015;45(Pt A):139-45. doi: 10.1016/j.cct.2015.09.002 [published Online First: 2015/09/08]
327. Higgins JP, Thompson SG, Deeks JJ, et al. Measuring inconsistency in meta-analyses. *BMJ* 2003;327(7414):557-60. doi: 10.1136/bmj.327.7414.557 [published Online First: 2003/09/06]
328. Ioannidis JP, Trikalinos TA. An exploratory test for an excess of significant findings. *Clin Trials* 2007;4(3):245-53. doi: 10.1177/1740774507079441 [published Online First: 2007/08/24]
329. IntHout J, Ioannidis JP, Rovers MM, et al. Plea for routinely presenting prediction intervals in meta-analysis. *BMJ Open* 2016;6(7):e010247. doi: 10.1136/bmjopen-2015-010247 [published Online First: 2016/07/14]
330. Riley RD, Higgins JP, Deeks JJ. Interpretation of random effects meta-analyses. *BMJ* 2011;342:d549. doi: 10.1136/bmj.d549 [published Online First: 2011/02/12]
331. Nuesch E, Trelle S, Reichenbach S, et al. Small study effects in meta-analyses of osteoarthritis trials: meta-epidemiological study. *BMJ* 2010;341:c3515. doi: 10.1136/bmj.c3515 [published Online First: 2010/07/20]
332. Tsilidis KK, Kasimis JC, Lopez DS, et al. Type 2 diabetes and cancer: umbrella review of meta-analyses of observational studies. *BMJ* 2015;350:g7607. doi: 10.1136/bmj.g7607 [published Online First: 2015/01/06]
333. Belbasis L, Bellou V, Evangelou E, et al. Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. *Lancet Neurol* 2015;14(3):263-73. doi: 10.1016/S1474-4422(14)70267-4 [published Online First: 2015/02/11]
334. Markozannes G, Tzoulaki I, Karli D, et al. Diet, body size, physical activity and risk of prostate cancer: An umbrella review of the evidence. *Eur J Cancer* 2016;69:61-69. doi: 10.1016/j.ejca.2016.09.026 [published Online First: 2016/11/07]
335. Bellou V, Belbasis L, Tzoulaki I, et al. Environmental risk factors and Parkinson's disease: An umbrella review of meta-analyses. *Parkinsonism Relat Disord* 2016;23:1-9. doi: 10.1016/j.parkreldis.2015.12.008 [published Online First: 2016/01/08]
336. Salanti G, Ioannidis JP. Synthesis of observational studies should consider credibility ceilings. *J Clin Epidemiol* 2009;62(2):115-22. doi: 10.1016/j.jclinepi.2008.05.014 [published Online First: 2009/01/10]
337. Papatheodorou SI, Tsilidis KK, Evangelou E, et al. Application of credibility ceilings probes the robustness of meta-analyses of biomarkers and cancer risk. *J Clin Epidemiol* 2015;68(2):163-74. doi: 10.1016/j.jclinepi.2014.09.004 [published Online First: 2014/12/01]

338. Liu CM, Aziz M, Kachur S, et al. BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. *BMC Microbiol* 2012;12:56. doi: 10.1186/1471-2180-12-56 [published Online First: 2012/04/19]
339. Kozich JJ, Westcott SL, Baxter NT, et al. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013;79(17):5112-20. doi: 10.1128/AEM.01043-13 [published Online First: 2013/06/25]
340. Wang Q, Garrity GM, Tiedje JM, et al. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;73(16):5261-7. doi: 10.1128/AEM.00062-07 [published Online First: 2007/06/26]
341. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26(19):2460-1. doi: 10.1093/bioinformatics/btq461 [published Online First: 2010/08/17]
342. Fettweis JM, Serrano MG, Sheth NU, et al. Species-level classification of the vaginal microbiome. *BMC Genomics* 2012;13 Suppl 8:S17. doi: 10.1186/1471-2164-13-S8-S17 [published Online First: 2013/01/11]
343. Davis NM, Proctor DM, Holmes SP, et al. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 2018;6(1):226. doi: 10.1186/s40168-018-0605-2 [published Online First: 2018/12/19]
344. Parks DH, Tyson GW, Hugenholtz P, et al. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 2014;30(21):3123-4. doi: 10.1093/bioinformatics/btu494 [published Online First: 2014/07/26]
345. Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res* 2015;43(W1):W566-70. doi: 10.1093/nar/gkv468 [published Online First: 2015/05/15]
346. Chong J, Liu P, Zhou G, et al. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Protoc* 2020;15(3):799-821. doi: 10.1038/s41596-019-0264-1 [published Online First: 2020/01/17]
347. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12(6):R60. doi: 10.1186/gb-2011-12-6-r60 [published Online First: 2011/06/28]
348. Federation ID. [Available from: https://www.idf.org/our-activities/care-prevention/gdm accessed 22 July 2021]
349. Diabetes UK [Available from: https://www.diabetes.org.uk/ accessed 22 July 2021]
350. Saed L, Varse F, Baradaran HR, et al. The effect of diabetes on the risk of endometrial Cancer: an updated a systematic review and meta-analysis. *BMC Cancer* 2019;19(1):527. doi: 10.1186/s12885-019-5748-4 [published Online First: 2019/06/04]
351. Wang L, Wang L, Zhang J, et al. Association between diabetes mellitus and subsequent ovarian cancer in women: A systematic review and meta-analysis

of cohort studies. *Medicine (Baltimore)* 2017;96(16):e6396. doi: 10.1097/MD.0000000000006396 [published Online First: 2017/04/20]
352. Zhang D, Li N, Xi Y, et al. Diabetes mellitus and risk of ovarian cancer. A systematic review and meta-analysis of 15 cohort studies. *Diabetes Res Clin Pract* 2017;130:43-52. doi: 10.1016/j.diabres.2017.04.005 [published Online First: 2017/05/30]
353. Raffone A, Travaglino A, Saccone G, et al. Diabetes mellitus and responsiveness of endometrial hyperplasia and early endometrial cancer to conservative treatment. *Gynecol Endocrinol* 2019;35(11):932-37. doi: 10.1080/09513590.2019.1624716 [published Online First: 20190605]
354. Tian J, Liang Y, Qu P. Antidiabetic Medications and the Risk of Endometrial Cancer in Patients. *Gynecol Obstet Invest* 2019;84(5):455-62. doi: 10.1159/000497202 [published Online First: 20190226]
355. Chen L, Yang T, Chen L, et al. Risk of congenital heart defects in offspring exposed to maternal diabetes mellitus: an updated systematic review and meta-analysis. *Arch Gynecol Obstet* 2019;300(6):1491-506. doi: 10.1007/s00404-019-05376-6 [published Online First: 2019/11/13]
356. Flenady V, Koopmans L, Middleton P, et al. Major risk factors for stillbirth in high-income countries: a systematic review and meta-analysis. *Lancet* 2011;377(9774):1331-40. doi: 10.1016/S0140-6736(10)62233-7 [published Online First: 2011/04/19]
357. Inkster ME, Fahey TP, Donnan PT, et al. Poor glycated haemoglobin control and adverse pregnancy outcomes in type 1 and type 2 diabetes mellitus: systematic review of observational studies. *BMC Pregnancy Childbirth* 2006;6:30. doi: 10.1186/1471-2393-6-30 [published Online First: 2006/11/01]
358. Li Y, Wang W, Zhang D. Maternal diabetes mellitus and risk of neonatal respiratory distress syndrome: a meta-analysis. *Acta Diabetol* 2019;56(7):729-40. doi: 10.1007/s00592-019-01327-4 [published Online First: 20190406]
359. Manerkar K, Harding J, Conlon C, et al. Maternal gestational diabetes and infant feeding, nutrition and growth: a systematic review and meta-analysis. *Br J Nutr* 2020;123(11):1201-15. doi: 10.1017/S0007114520000264 [published Online First: 2020/01/23]
360. Shu LP, Zhang RH, Cai YH, et al. Maternal Diabetes Mellitus and Persistent Pulmonary Hypertension of the Newborn: Accumulated Evidence From Observational Studies. *Can J Diabetes* 2020;44(4):327-34 e3. doi: 10.1016/j.jcjd.2019.10.002 [published Online First: 2020/01/07]
361. Simeone RM, Devine OJ, Marcinkevage JA, et al. Diabetes and congenital heart defects: a systematic review, meta-analysis, and modeling project. *Am J Prev Med* 2015;48(2):195-204. doi: 10.1016/j.amepre.2014.09.002 [published Online First: 2014/10/19]
362. Van der Looven R, Le Roy L, Tanghe E, et al. Risk factors for neonatal brachial plexus palsy: a systematic review and meta-analysis. *Dev Med Child Neurol* 2020;62(6):673-83. doi: 10.1111/dmcn.14381 [published Online First: 2019/11/02]
363. Wendland EM, Torloni MR, Falavigna M, et al. Gestational diabetes and pregnancy outcomes--a systematic review of the World Health Organization (WHO) and the International Association of Diabetes in Pregnancy Study

Groups (IADPSG) diagnostic criteria. *BMC Pregnancy Childbirth* 2012;12:23. doi: 10.1186/1471-2393-12-23 [published Online First: 2012/04/03]
364. Wilson CA, Newham J, Rankin J, et al. Is there an increased risk of perinatal mental disorder in women with gestational diabetes? A systematic review and meta-analysis. *Diabet Med* 2020;37(4):602-22. doi: 10.1111/dme.14170 [published Online First: 2019/11/07]
365. Zhang L, Wang XH, Zheng XM, et al. Maternal gestational smoking, diabetes, alcohol drinking, pre-pregnancy obesity and the risk of cryptorchidism: a systematic review and meta-analysis of observational studies. *PLoS One* 2015;10(3):e0119006. doi: 10.1371/journal.pone.0119006 [published Online First: 20150323]
366. Ioannidis JP. Why most discovered true associations are inflated. *Epidemiology* 2008;19(5):640-8. doi: 10.1097/EDE.0b013e31818131e7 [published Online First: 2008/07/18]
367. Ioannidis JP. Why most published research findings are false. *PLoS Med* 2005;2(8):e124. doi: 10.1371/journal.pmed.0020124 [published Online First: 2005/08/03]
368. Dwan K, Gamble C, Williamson PR, et al. Systematic review of the empirical evidence of study publication bias and outcome reporting bias - an updated review. *PLoS One* 2013;8(7):e66844. doi: 10.1371/journal.pone.0066844 [published Online First: 2013/07/19]
369. Balsells M, Garcia-Patterson A, Gich I, et al. Maternal and fetal outcome in women with type 2 versus type 1 diabetes mellitus: a systematic review and metaanalysis. *J Clin Endocrinol Metab* 2009;94(11):4284-91. doi: 10.1210/jc.2009-1231 [published Online First: 2009/10/08]
370. Bhatia D, Lega IC, Wu W, et al. Breast, cervical and colorectal cancer screening in adults with diabetes: a systematic review and meta-analysis. *Diabetologia* 2020;63(1):34-48. doi: 10.1007/s00125-019-04995-7 [published Online First: 2019/10/28]
371. Pergialiotis V, Prodromidou A, Siotos C, et al. Systemic hypertension and diabetes mellitus as predictors of malignancy among women with endometrial polyps: a meta-analysis of observational studies. *Menopause* 2016;23(6):691-7. doi: 10.1097/GME.0000000000000602 [published Online First: 2016/03/10]
372. Tabrizi R, Asemi Z, Lankarani KB, et al. Gestational diabetes mellitus in association with macrosomia in Iran: a meta-analysis. *J Diabetes Metab Disord* 2019;18(1):41-50. doi: 10.1007/s40200-019-00388-0 [published Online First: 2019/07/06]
373. Wang L, Zhong L, Xu B, et al. Diabetes mellitus and the risk of ovarian cancer: a systematic review and meta-analysis of cohort and case-control studies. *BMJ Open* 2020;10(12):e040137. doi: 10.1136/bmjopen-2020-040137 [published Online First: 2020/12/31]
374. Wang Y, Yan P, Fu T, et al. The association between gestational diabetes mellitus and cancer in women: A systematic review and meta-analysis of observational studies. *Diabetes Metab* 2020;46(6):461-71. doi: 10.1016/j.diabet.2020.02.003 [published Online First: 2020/02/26]
375. Zhao E, Zhang Y, Zeng X, et al. Association between maternal diabetes mellitus and the risk of congenital malformations: A meta-analysis of cohort studies.

Drug Discov Ther 2015;9(4):274-81. doi: 10.5582/ddt.2015.01044 [published Online First: 2015/09/16]

376. Alqudah A, McKinley MC, McNally R, et al. Risk of pre-eclampsia in women taking metformin: a systematic review and meta-analysis. *Diabet Med* 2018;35(2):160-72. doi: 10.1111/dme.13523 [published Online First: 2017/10/19]
377. Gonzalez Blanco C, Chico Ballesteros A, Gich Saladich I, et al. Glycemic control and pregnancy outcomes in women with type 1 diabetes mellitus using lispro versus regular insulin: a systematic review and meta-analysis. *Diabetes Technol Ther* 2011;13(9):907-11. doi: 10.1089/dia.2011.0032 [published Online First: 2011/07/01]
378. Chu D, Wu J, Wang K, et al. Effect of metformin use on the risk and prognosis of endometrial cancer: a systematic review and meta-analysis. *BMC Cancer* 2018;18(1):438. doi: 10.1186/s12885-018-4334-5 [published Online First: 2018/04/20]
379. Gilbert C, Valois M, Koren G. Pregnancy outcome after first-trimester exposure to metformin: a meta-analysis. *Fertil Steril* 2006;86(3):658-63. doi: 10.1016/j.fertnstert.2006.02.098 [published Online First: 2006/08/02]
380. Lepercq J, Lin J, Hall GC, et al. Meta-Analysis of Maternal and Neonatal Outcomes Associated with the Use of Insulin Glargine versus NPH Insulin during Pregnancy. *Obstet Gynecol Int* 2012;2012:649070. doi: 10.1155/2012/649070 [published Online First: 2012/06/12]
381. Lv S, Wang J, Xu Y. Safety of insulin analogs during pregnancy: a meta-analysis. *Arch Gynecol Obstet* 2015;292(4):749-56. doi: 10.1007/s00404-015-3692-3 [published Online First: 2015/04/10]
382. Pollex E, Moretti ME, Koren G, et al. Safety of insulin glargine use in pregnancy: a systematic review and meta-analysis. *Ann Pharmacother* 2011;45(1):9-16. doi: 10.1345/aph.1P327 [published Online First: 2011/01/06]
383. Ranasinghe PD, Maruthur NM, Nicholson WK, et al. Comparative effectiveness of continuous subcutaneous insulin infusion using insulin analogs and multiple daily injections in pregnant women with diabetes mellitus: a systematic review and meta-analysis. *J Womens Health (Larchmt)* 2015;24(3):237-49. doi: 10.1089/jwh.2014.4939 [published Online First: 2015/02/26]
384. Rys PM, Ludwig-Slomczynska AH, Cyganek K, et al. Continuous subcutaneous insulin infusion vs multiple daily injections in pregnant women with type 1 diabetes mellitus: a systematic review and meta-analysis of randomised controlled trials and observational studies. *Eur J Endocrinol* 2018;178(5):545-63. doi: 10.1530/EJE-17-0804 [published Online First: 2018/03/17]
385. Syed M, Javed H, Yakoob MY, et al. Effect of screening and management of diabetes during pregnancy on stillbirths. *BMC Public Health* 2011;11 Suppl 3:S2. doi: 10.1186/1471-2458-11-S3-S2 [published Online First: 2011/04/29]
386. Wahabi HA, Alzeidan RA, Bawazeer GA, et al. Preconception care for diabetic women for improving maternal and fetal outcomes: a systematic review and meta-analysis. *BMC Pregnancy Childbirth* 2010;10:63. doi: 10.1186/1471-2393-10-63 [published Online First: 2010/10/16]

387. Waugh N, Royle P, Clar C, et al. Screening for hyperglycaemia in pregnancy: a rapid update for the National Screening Committee. *Health Technol Assess* 2010;14(45):1-183. doi: 10.3310/hta14450 [published Online First: 2010/09/28]
388. Wen Q, Zhao Z, Wen J, et al. The association between metformin therapy and risk of gynecological cancer in patients: Two meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2019;237:33-41. doi: 10.1016/j.ejogrb.2019.03.029 [published Online First: 2019/04/23]
389. Zheng X, Liu Z, Zhang Y, et al. Relationship Between Myo-Inositol Supplementary and Gestational Diabetes Mellitus: A Meta-Analysis. *Medicine (Baltimore)* 2015;94(42):e1604. doi: 10.1097/MD.0000000000001604 [published Online First: 2015/10/27]
390. Alwan N, Tuffnell DJ, West J. Treatments for gestational diabetes. *Cochrane Database Syst Rev* 2009(3):CD003395. doi: 10.1002/14651858.CD003395.pub2 [published Online First: 2009/07/10]
391. Balsells M, Garcia-Patterson A, Gich I, et al. Ultrasound-guided compared to conventional treatment in gestational diabetes leads to improved birthweight but more insulin treatment: systematic review and meta-analysis. *Acta Obstet Gynecol Scand* 2014;93(2):144-51. doi: 10.1111/aogs.12291 [published Online First: 2014/01/01]
392. Bao LX, Shi WT, Han YX. Metformin versus insulin for gestational diabetes: a systematic review and meta-analysis. *J Matern Fetal Neonatal Med* 2021;34(16):2741-53. doi: 10.1080/14767058.2019.1670804 [published Online First: 2019/09/29]
393. Brown J, Grzeskowiak L, Williamson K, et al. Insulin for the treatment of women with gestational diabetes. *Cochrane Database Syst Rev* 2017;11:CD012037. doi: 10.1002/14651858.CD012037.pub2 [published Online First: 2017/11/06]
394. Brown J, Alwan NA, West J, et al. Lifestyle interventions for the treatment of women with gestational diabetes. *Cochrane Database Syst Rev* 2017;5:CD011970. doi: 10.1002/14651858.CD011970.pub2 [published Online First: 2017/05/05]
395. Brown J, Martis R, Hughes B, et al. Oral anti-diabetic pharmacological therapies for the treatment of women with gestational diabetes. *Cochrane Database Syst Rev* 2017;1:CD011967. doi: 10.1002/14651858.CD011967.pub2 [published Online First: 2017/01/26]
396. Butalia S, Gutierrez L, Lodha A, et al. Short- and long-term outcomes of metformin compared with insulin alone in pregnancy: a systematic review and meta-analysis. *Diabet Med* 2017;34(1):27-36. doi: 10.1111/dme.13150 [published Online First: 2016/05/07]
397. Ceysens G, Rouiller D, Boulvain M. Exercise for diabetic pregnant women. *Cochrane Database Syst Rev* 2006(3):CD004225. doi: 10.1002/14651858.CD004225.pub2 [published Online First: 2006/07/21]
398. Dhulkotia JS, Ola B, Fraser R, et al. Oral hypoglycemic agents vs insulin in management of gestational diabetes: a systematic review and metaanalysis. *Am J Obstet Gynecol* 2010;203(5):457 e1-9. doi: 10.1016/j.ajog.2010.06.044 [published Online First: 2010/08/27]
399. Falavigna M, Schmidt MI, Trujillo J, et al. Effectiveness of gestational diabetes treatment: a systematic review with quality of evidence assessment. *Diabetes*

Res Clin Pract 2012;98(3):396-405. doi: 10.1016/j.diabres.2012.09.002 [published Online First: 2012/10/04]
400. Farrar D, Tuffnell DJ, West J, et al. Continuous subcutaneous insulin infusion versus multiple daily injections of insulin for pregnant women with diabetes. *Cochrane Database Syst Rev* 2016(6):CD005542. doi: 10.1002/14651858.CD005542.pub3 [published Online First: 2016/06/09]
401. Gao L, Lin L, Shan N, et al. The impact of omega-3 fatty acid supplementation on glycemic control in patients with gestational diabetes: a systematic review and meta-analysis of randomized controlled studies. *J Matern Fetal Neonatal Med* 2020;33(10):1767-73. doi: 10.1080/14767058.2018.1526916 [published Online First: 2018/09/21]
402. Guo L, Ma J, Tang J, et al. Comparative Efficacy and Safety of Metformin, Glyburide, and Insulin in Treating Gestational Diabetes Mellitus: A Meta-Analysis. *J Diabetes Res* 2019;2019:9804708. doi: 10.1155/2019/9804708 [published Online First: 2019/11/30]
403. Han S, Crowther CA, Middleton P, et al. Different types of dietary advice for women with gestational diabetes mellitus. *Cochrane Database Syst Rev* 2013(3):CD009275. doi: 10.1002/14651858.CD009275.pub2 [published Online First: 2013/04/02]
404. Hartling L, Dryden DM, Guthrie A, et al. Benefits and harms of treating gestational diabetes mellitus: a systematic review and meta-analysis for the U.S. Preventive Services Task Force and the National Institutes of Health Office of Medical Applications of Research. *Ann Intern Med* 2013;159(2):123-9. doi: 10.7326/0003-4819-159-2-201307160-00661 [published Online First: 2013/05/29]
405. Horvath K, Koch K, Jeitler K, et al. Effects of treatment in women with gestational diabetes mellitus: systematic review and meta-analysis. *BMJ* 2010;340:c1395. doi: 10.1136/bmj.c1395 [published Online First: 2010/04/03]
406. Immanuel J, Simmons D. Screening and Treatment for Early-Onset Gestational Diabetes Mellitus: a Systematic Review and Meta-analysis. *Curr Diab Rep* 2017;17(11):115. doi: 10.1007/s11892-017-0943-7 [published Online First: 2017/10/04]
407. Jiang YF, Chen XY, Ding T, et al. Comparative efficacy and safety of OADs in management of GDM: network meta-analysis of randomized controlled trials. *J Clin Endocrinol Metab* 2015;100(5):2071-80. doi: 10.1210/jc.2014-4403 [published Online First: 2015/03/25]
408. Kalafat E, Sukur YE, Abdi A, et al. Metformin for prevention of hypertensive disorders of pregnancy in women with gestational diabetes or obesity: systematic review and meta-analysis of randomized trials. *Ultrasound Obstet Gynecol* 2018;52(6):706-14. doi: 10.1002/uog.19084 [published Online First: 2018/05/12]
409. Lau Y, Htun TP, Wong SN, et al. Efficacy of Internet-Based Self-Monitoring Interventions on Maternal and Neonatal Outcomes in Perinatal Diabetic Women: A Systematic Review and Meta-Analysis. *J Med Internet Res* 2016;18(8):e220. doi: 10.2196/jmir.6153 [published Online First: 2016/08/17]
410. Ming WK, Ding W, Zhang CJP, et al. The effect of exercise during pregnancy on gestational diabetes mellitus in normal-weight women: a systematic review and

meta-analysis. *BMC Pregnancy Childbirth* 2018;18(1):440. doi: 10.1186/s12884-018-2068-7 [published Online First: 2018/11/14]
411. Poolsup N, Suksomboon N, Amin M. Efficacy and safety of oral antidiabetic drugs in comparison to insulin in treating gestational diabetes mellitus: a meta-analysis. *PLoS One* 2014;9(10):e109985. doi: 10.1371/journal.pone.0109985 [published Online First: 2014/10/11]
412. Shi J, Liu B, Wang H, et al. Association of metformin use with ovarian cancer incidence and prognosis: a systematic review and meta-analysis. *Int J Gynecol Cancer* 2019;29(1):140-46. doi: 10.1136/ijgc-2018-000060 [published Online First: 2019/01/15]
413. Song R, Chen L, Chen Y, et al. Comparison of glyburide and insulin in the management of gestational diabetes: A meta-analysis. *PLoS One* 2017;12(8):e0182488. doi: 10.1371/journal.pone.0182488 [published Online First: 2017/08/05]
414. Su DF, Wang XY. Metformin vs insulin in the management of gestational diabetes: a systematic review and meta-analysis. *Diabetes Res Clin Pract* 2014;104(3):353-7. doi: 10.1016/j.diabres.2013.12.056 [published Online First: 2014/04/29]
415. Tarry-Adkins JL, Aiken CE, Ozanne SE. Neonatal, infant, and childhood growth following metformin versus insulin treatment for gestational diabetes: A systematic review and meta-analysis. *PLoS Med* 2019;16(8):e1002848. doi: 10.1371/journal.pmed.1002848 [published Online First: 20190806]
416. Tieu J, Coat S, Hague W, et al. Oral anti-diabetic agents for women with established diabetes/impaired glucose tolerance or previous gestational diabetes planning pregnancy, or pregnant women with pre-existing diabetes. *Cochrane Database Syst Rev* 2017;10:CD007724. doi: 10.1002/14651858.CD007724.pub3 [published Online First: 2017/10/19]
417. Tuffnell DJ, West J, Walkinshaw SA. Treatments for gestational diabetes and impaired glucose tolerance in pregnancy. *Cochrane Database Syst Rev* 2003(3):CD003395. doi: 10.1002/14651858.CD003395 [published Online First: 2003/08/15]
418. Viana LV, Gross JL, Azevedo MJ. Dietary intervention in patients with gestational diabetes mellitus: a systematic review and meta-analysis of randomized clinical trials on maternal and newborn outcomes. *Diabetes Care* 2014;37(12):3345-55. doi: 10.2337/dc14-1530 [published Online First: 2014/11/22]
419. Wei J, Heng W, Gao J. Effects of Low Glycemic Index Diets on Gestational Diabetes Mellitus: A Meta-Analysis of Randomized Controlled Clinical Trials. *Medicine (Baltimore)* 2016;95(22):e3792. doi: 10.1097/MD.0000000000003792 [published Online First: 2016/06/04]
420. Xu J, Ye S. Influence of low-glycemic index diet for gestational diabetes: a meta-analysis of randomized controlled trials. *J Matern Fetal Neonatal Med* 2020;33(4):687-92. doi: 10.1080/14767058.2018.1497595 [published Online First: 2018/09/21]
421. Yamamoto JM, Kellett JE, Balsells M, et al. Gestational Diabetes Mellitus and Diet: A Systematic Review and Meta-analysis of Randomized Controlled Trials Examining the Impact of Modified Dietary Interventions on Maternal Glucose

Control and Neonatal Birth Weight. *Diabetes Care* 2018;41(7):1346-61. doi: 10.2337/dc18-0102 [published Online First: 2018/06/24]
422. Zhu B, Zhang L, Fan YY, et al. Metformin versus insulin in gestational diabetes mellitus: a meta-analysis of randomized clinical trials. *Ir J Med Sci* 2016;185(2):371-81. doi: 10.1007/s11845-016-1414-x [published Online First: 2016/02/11]
423. Furlan AD, Pennick V, Bombardier C, et al. 2009 updated method guidelines for systematic reviews in the Cochrane Back Review Group. *Spine (Phila Pa 1976)* 2009;34(18):1929-41. doi: 10.1097/BRS.0b013e3181b1c99f [published Online First: 2009/08/15]
424. Green. Cochrane handbook for systematic reviews of interventions version 5.1. *Cochrane Collaboration* 2011
425. Al Faleh K, Al-Omran M. Reporting and methodologic quality of Cochrane Neonatal review group systematic reviews. *BMC Pediatr* 2009;9:38. doi: 10.1186/1471-2431-9-38 [published Online First: 2009/06/19]
426. Langendam MW, Akl EA, Dahm P, et al. Assessing and presenting summaries of evidence in Cochrane Reviews. *Syst Rev* 2013;2:81. doi: 10.1186/2046-4053-2-81 [published Online First: 2013/09/26]
427. 94. World Cancer Research Fund Continuous Update Project [Available from: https://www.wcrf.org/dietandcancer/endometrial-cancer/ accessed 22 July 2021]
428. Luo J, Beresford S, Chen C, et al. Association between diabetes, diabetes treatment and risk of developing endometrial cancer. *Br J Cancer* 2014;111(7):1432-9. doi: 10.1038/bjc.2014.407 [published Online First: 2014/07/23]
429. Nead KT, Sharp SJ, Thompson DJ, et al. Evidence of a Causal Association Between Insulinemia and Endometrial Cancer: A Mendelian Randomization Analysis. *J Natl Cancer Inst* 2015;107(9) doi: 10.1093/jnci/djv178 [published Online First: 2015/07/03]
430. Krzeslak A, Wojcik-Krowiranda K, Forma E, et al. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. *Pathol Oncol Res* 2012;18(3):721-8. doi: 10.1007/s12253-012-9500-5 [published Online First: 2012/01/25]
431. Byrne FL, Martin AR, Kosasih M, et al. The Role of Hyperglycemia in Endometrial Cancer Pathogenesis. *Cancers (Basel)* 2020;12(5) doi: 10.3390/cancers12051191 [published Online First: 2020/05/14]
432. Pierotti MA, Berrino F, Gariboldi M, et al. Targeting metabolism for cancer treatment and prevention: metformin, an old drug with multi-faceted effects. *Oncogene* 2013;32(12):1475-87. doi: 10.1038/onc.2012.181 [published Online First: 2012/06/06]
433. Pedersen J. Diabetes and pregnancy; blood sugar of newborn infants during fasting and glucose administration. *Nord Med* 1952;47(30):1049. [published Online First: 1952/07/25]
434. Gabbay-Benziv R, Reece EA, Wang F, et al. Birth defects in pregestational diabetes: Defect range, glycemic threshold and pathogenesis. *World J Diabetes* 2015;6(3):481-8. doi: 10.4239/wjd.v6.i3.481 [published Online First: 2015/04/22]

435. Nuzzo AM, Giuffrida D, Moretti L, et al. Placental and maternal sFlt1/PlGF expression in gestational diabetes mellitus. *Sci Rep* 2021;11(1):2312. doi: 10.1038/s41598-021-81785-5 [published Online First: 2021/01/29]
436. Tyrrell J, Richmond RC, Palmer TM, et al. Genetic Evidence for Causal Relationships Between Maternal Obesity-Related Traits and Birth Weight. *JAMA* 2016;315(11):1129-40. doi: 10.1001/jama.2016.1975 [published Online First: 2016/03/16]
437. Group HSCR, Metzger BE, Lowe LP, et al. Hyperglycemia and adverse pregnancy outcomes. *N Engl J Med* 2008;358(19):1991-2002. doi: 10.1056/NEJMoa0707943 [published Online First: 2008/05/09]
438. Wang F, Cao G, Yi W, et al. Effect of Metformin on a Preeclampsia-Like Mouse Model Induced by High-Fat Diet. *Biomed Res Int* 2019;2019:6547019. doi: 10.1155/2019/6547019 [published Online First: 2019/12/31]
439. Salomaki H, Heinaniemi M, Vahatalo LH, et al. Prenatal metformin exposure in a maternal high fat diet mouse model alters the transcriptome and modifies the metabolic responses of the offspring. *PLoS One* 2014;9(12):e115778. doi: 10.1371/journal.pone.0115778 [published Online First: 2014/12/30]
440. Kavvoura FK, Liberopoulos G, Ioannidis JP. Selection in reported epidemiological risks: an empirical assessment. *PLoS Med* 2007;4(3):e79. doi: 10.1371/journal.pmed.0040079 [published Online First: 2007/03/08]
441. Ioannidis JP. The importance of potential studies that have not existed and registration of observational data sets. *JAMA* 2012;308(6):575-6. doi: 10.1001/jama.2012.8144 [published Online First: 2012/08/09]
442. Norgaard M, Ehrenstein V, Vandenbroucke JP. Confounding in observational studies based on large health care databases: problems and potential solutions - a primer for the clinician. *Clin Epidemiol* 2017;9:185-93. doi: 10.2147/CLEP.S129879 [published Online First: 2017/04/14]
443. Lu CY. Observational studies: a review of study designs, challenges and strategies to reduce confounding. *Int J Clin Pract* 2009;63(5):691-7. doi: 10.1111/j.1742-1241.2009.02056.x [published Online First: 2009/04/28]
444. Xing Y, Tan KC. Aspirin for primary prevention of cardiovascular disease in diabetes. *J Diabetes Investig* 2019;10(4):899-901. doi: 10.1111/jdi.13006 [published Online First: 2019/01/15]
445. Okura Y, Urban LH, Mahoney DW, et al. Agreement between self-report questionnaires and medical record data was substantial for diabetes, hypertension, myocardial infarction and stroke but not for heart failure. *J Clin Epidemiol* 2004;57(10):1096-103. doi: 10.1016/j.jclinepi.2004.04.005 [published Online First: 2004/11/06]
446. Bakken LR. Separation and purification of bacteria from soil. *Appl Environ Microbiol* 1985;49(6):1482-7. doi: 10.1128/aem.49.6.1482-1487.1985 [published Online First: 1985/06/01]
447. Perez-Munoz ME, Arrieta MC, Ramer-Tait AE, et al. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome* 2017;5(1):48. doi: 10.1186/s40168-017-0268-4 [published Online First: 20170428]

448. Winters AD, Romero R, Gervasi MT, et al. Does the endometrial cavity have a molecular microbial signature? *Sci Rep* 2019;9(1):9905. doi: 10.1038/s41598-019-46173-0 [published Online First: 2019/07/11]
449. Miles SM, Hardy BL, Merrell DS. Investigation of the microbiota of the reproductive tract in women undergoing a total hysterectomy and bilateral salpingo-oopherectomy. *Fertil Steril* 2017;107(3):813-20 e1. doi: 10.1016/j.fertnstert.2016.11.028 [published Online First: 2017/01/11]
450. Pelzer ES, Willner D, Buttini M, et al. A role for the endometrial microbiome in dysfunctional menstrual bleeding. *Antonie Van Leeuwenhoek* 2018;111(6):933-43. doi: 10.1007/s10482-017-0992-6 [published Online First: 2018/01/05]
451. Kyono K, Hashimoto T, Nagai Y, et al. Analysis of endometrial microbiota by 16S ribosomal RNA gene sequencing among infertile patients: a single-center pilot study. *Reprod Med Biol* 2018;17(3):297-306. doi: 10.1002/rmb2.12105 [published Online First: 2018/07/18]
452. Moreno I, Cicinelli E, Garcia-Grau I, et al. The diagnosis of chronic endometritis in infertile asymptomatic women: a comparative study of histology, microbial cultures, hysteroscopy, and molecular microbiology. *Am J Obstet Gynecol* 2018;218(6):602 e1-02 e16. doi: 10.1016/j.ajog.2018.02.012 [published Online First: 2018/02/27]
453. Lauder AP, Roche AM, Sherrill-Mix S, et al. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. *Microbiome* 2016;4(1):29. doi: 10.1186/s40168-016-0172-3 [published Online First: 2016/06/25]
454. Eisenhofer R, Minich JJ, Marotz C, et al. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. *Trends Microbiol* 2019;27(2):105-17. doi: 10.1016/j.tim.2018.11.003 [published Online First: 2018/12/01]
455. Salter SJ, Cox MJ, Turek EM, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol* 2014;12:87. doi: 10.1186/s12915-014-0087-z [published Online First: 2014/11/13]
456. Fang RL, Chen LX, Shu WS, et al. Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am J Transl Res* 2016;8(3):1581-92. [published Online First: 2016/05/18]
457. Franasiak JM, Werner MD, Juneau CR, et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *J Assist Reprod Genet* 2016;33(1):129-36. doi: 10.1007/s10815-015-0614-z [published Online First: 2015/11/09]
458. Wee BA, Thomas M, Sweeney EL, et al. A retrospective pilot study to determine whether the reproductive tract microbiota differs between women with a history of infertility and fertile women. *Aust N Z J Obstet Gynaecol* 2018;58(3):341-48. doi: 10.1111/ajo.12754 [published Online First: 2017/12/28]
459. Liu Y, Wong KK, Ko EY, et al. Systematic Comparison of Bacterial Colonization of Endometrial Tissue and Fluid Samples in Recurrent Miscarriage Patients: Implications for Future Endometrial Microbiome Studies. *Clin Chem* 2018;64(12):1743-52. doi: 10.1373/clinchem.2018.289306 [published Online First: 2018/09/22]

460. Amabebe E, Anumba DOC. Female Gut and Genital Tract Microbiota-Induced Crosstalk and Differential Effects of Short-Chain Fatty Acids on Immune Sequelae. *Front Immunol* 2020;11:2184. doi: 10.3389/fimmu.2020.02184 [published Online First: 2020/10/06]
461. El Aila NA, Tency I, Claeys G, et al. Genotyping of Streptococcus agalactiae (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy. *BMC Infect Dis* 2009;9:153. doi: 10.1186/1471-2334-9-153 [published Online First: 2009/09/15]
462. El Aila NA, Tency I, Claeys G, et al. Identification and genotyping of bacteria from paired vaginal and rectal samples from pregnant women indicates similarity between vaginal and rectal microflora. *BMC Infect Dis* 2009;9:167. doi: 10.1186/1471-2334-9-167 [published Online First: 2009/10/16]
463. Prast-Nielsen S, Tobin AM, Adamzik K, et al. Investigation of the skin microbiome: swabs vs. biopsies. *Br J Dermatol* 2019;181(3):572-79. doi: 10.1111/bjd.17691 [published Online First: 2019/01/30]
464. Araujo-Perez F, McCoy AN, Okechukwu C, et al. Differences in microbial signatures between rectal mucosal biopsies and rectal swabs. *Gut Microbes* 2012;3(6):530-5. doi: 10.4161/gmic.22157 [published Online First: 2012/10/13]
465. Liu AQ, Vogtmann E, Shao DT, et al. A Comparison of Biopsy and Mucosal Swab Specimens for Examining the Microbiota of Upper Gastrointestinal Carcinoma. *Cancer Epidemiol Biomarkers Prev* 2019;28(12):2030-37. doi: 10.1158/1055-9965.EPI-18-1210 [published Online First: 2019/09/15]
466. Dai W, Du H, Li S, et al. Cervicovaginal Microbiome Factors in Clearance of Human Papillomavirus Infection. *Front Oncol* 2021;11:722639. doi: 10.3389/fonc.2021.722639 [published Online First: 2021/08/17]
467. Zhou Z, Zeng F, Yuan J, et al. Pelvic inflammatory disease and the risk of ovarian cancer: a meta-analysis. *Cancer Causes Control* 2017;28(5):415-28. doi: 10.1007/s10552-017-0873-3 [published Online First: 2017/03/28]
468. Piao J, Lee EJ, Lee M. Association between pelvic inflammatory disease and risk of ovarian cancer: An updated meta-analysis. *Gynecol Oncol* 2020;157(2):542-48. doi: 10.1016/j.ygyno.2020.02.002 [published Online First: 2020/02/11]
469. Chang CY, Lin KY, Huang CC, et al. Association of pelvic inflammatory disease (PID) with ovarian cancer: a nationwide population-based retrospective cohort study from Taiwan. *BMC Womens Health* 2021;21(1):274. doi: 10.1186/s12905-021-01413-2 [published Online First: 2021/07/30]
470. Rasmussen CB, Kjaer SK, Albieri V, et al. Pelvic Inflammatory Disease and the Risk of Ovarian Cancer and Borderline Ovarian Tumors: A Pooled Analysis of 13 Case-Control Studies. *Am J Epidemiol* 2017;185(1):8-20. doi: 10.1093/aje/kww161 [published Online First: 2016/12/13]
471. Rasmussen CB, Faber MT, Jensen A, et al. Pelvic inflammatory disease and risk of invasive ovarian cancer and ovarian borderline tumors. *Cancer Causes Control* 2013;24(7):1459-64. doi: 10.1007/s10552-013-0216-y [published Online First: 2013/04/26]
472. Wang Q, Zhao L, Han L, et al. The differential distribution of bacteria between cancerous and noncancerous ovarian tissues in situ. *J Ovarian Res* 2020;13(1):8. doi: 10.1186/s13048-019-0603-4 [published Online First: 2020/01/20]

473. Lu W, He F, Lin Z, et al. Dysbiosis of the endometrial microbiota and its association with inflammatory cytokines in endometrial cancer. *Int J Cancer* 2021;148(7):1708-16. doi: 10.1002/ijc.33428 [published Online First: 2020/12/08]
474. Gonzalez-Bosquet J, Pedra-Nobre S, Devor EJ, et al. Bacterial, Archaea, and Viral Transcripts (BAVT) Expression in Gynecological Cancers and Correlation with Regulatory Regions of the Genome. *Cancers (Basel)* 2021;13(5) doi: 10.3390/cancers13051109 [published Online First: 2021/04/04]
475. Al-Memar M, Bobdiwala S, Fourie H, et al. The association between vaginal bacterial composition and miscarriage: a nested case-control study. *BJOG* 2020;127(2):264-74. doi: 10.1111/1471-0528.15972 [published Online First: 2019/10/02]
476. Kindinger LM, Bennett PR, Lee YS, et al. The interaction between vaginal microbiota, cervical length, and vaginal progesterone treatment for preterm birth risk. *Microbiome* 2017;5(1):6. doi: 10.1186/s40168-016-0223-9 [published Online First: 2017/01/21]
477. Brusselaers N, Shrestha S, van de Wijgert J, et al. Vaginal dysbiosis and the risk of human papillomavirus and cervical cancer: systematic review and meta-analysis. *Am J Obstet Gynecol* 2019;221(1):9-18 e8. doi: 10.1016/j.ajog.2018.12.011 [published Online First: 2018/12/15]
478. Muhleisen AL, Herbst-Kralovetz MM. Menopause and the vaginal microbiome. *Maturitas* 2016;91:42-50. doi: 10.1016/j.maturitas.2016.05.015 [published Online First: 2016/07/28]
479. Brotman RM, He X, Gajer P, et al. Association between cigarette smoking and the vaginal microbiota: a pilot study. *BMC Infect Dis* 2014;14:471. doi: 10.1186/1471-2334-14-471 [published Online First: 2014/08/30]
480. Jespers V, van de Wijgert J, Cools P, et al. The significance of Lactobacillus crispatus and L. vaginalis for vaginal health and the negative effect of recent sex: a cross-sectional descriptive study across groups of African women. *BMC Infect Dis* 2015;15:115. doi: 10.1186/s12879-015-0825-z [published Online First: 2015/04/17]
481. Linhares IM, Summers PR, Larsen B, et al. Contemporary perspectives on vaginal pH and lactobacilli. *Am J Obstet Gynecol* 2011;204(2):120 e1-5. doi: 10.1016/j.ajog.2010.07.010 [published Online First: 2010/09/14]
482. Gliniewicz K, Schneider GM, Ridenhour BJ, et al. Comparison of the Vaginal Microbiomes of Premenopausal and Postmenopausal Women. *Front Microbiol* 2019;10:193. doi: 10.3389/fmicb.2019.00193 [published Online First: 20190214]
483. Srinivasan S, Hoffman NG, Morgan MT, et al. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* 2012;7(6):e37818. doi: 10.1371/journal.pone.0037818 [published Online First: 2012/06/22]
484. Jespers V, Menten J, Smet H, et al. Quantification of bacterial species of the vaginal microbiome in different groups of women, using nucleic acid amplification tests. *BMC Microbiol* 2012;12:83. doi: 10.1186/1471-2180-12-83 [published Online First: 2012/06/01]

485. Atashili J, Poole C, Ndumbe PM, et al. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *AIDS* 2008;22(12):1493-501. doi: 10.1097/QAD.0b013e3283021a37 [published Online First: 2008/07/11]
486. Ness RB, Kip KE, Hillier SL, et al. A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. *Am J Epidemiol* 2005;162(6):585-90. doi: 10.1093/aje/kwi243 [published Online First: 2005/08/12]
487. St Amant DC, Valentin-Bon IE, Jerse AE. Inhibition of Neisseria gonorrhoeae by Lactobacillus species that are commonly isolated from the female genital tract. *Infect Immun* 2002;70(12):7169-71. doi: 10.1128/IAI.70.12.7169-7171.2002 [published Online First: 2002/11/20]
488. Kalyoussef S, Nieves E, Dinerman E, et al. Lactobacillus proteins are associated with the bactericidal activity against E. coli of female genital tract secretions. *PLoS One* 2012;7(11):e49506. doi: 10.1371/journal.pone.0049506 [published Online First: 2012/11/28]
489. Santos CMA, Pires MCV, Leao TL, et al. Anti-inflammatory effect of two Lactobacillus strains during infection with Gardnerella vaginalis and Candida albicans in a HeLa cell culture model. *Microbiology (Reading)* 2018;164(3):349-58. doi: 10.1099/mic.0.000608 [published Online First: 2018/02/21]
490. Uchinaka A, Azuma N, Mizumoto H, et al. Anti-inflammatory effects of heat-killed Lactobacillus plantarum L-137 on cardiac and adipose tissue in rats with metabolic syndrome. *Sci Rep* 2018;8(1):8156. doi: 10.1038/s41598-018-26588-x [published Online First: 2018/05/29]
491. Brandi J, Cheri S, Manfredi M, et al. Exploring the wound healing, anti-inflammatory, anti-pathogenic and proteomic effects of lactic acid bacteria on keratinocytes. *Sci Rep* 2020;10(1):11572. doi: 10.1038/s41598-020-68483-4 [published Online First: 2020/07/16]
492. Menard S, Candalh C, Bambou JC, et al. Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. *Gut* 2004;53(6):821-8. doi: 10.1136/gut.2003.026252 [published Online First: 2004/05/13]
493. Takagi A, Ikemura H, Matsuzaki T, et al. Relationship between the in vitro response of dendritic cells to Lactobacillus and prevention of tumorigenesis in the mouse. *J Gastroenterol* 2008;43(9):661-9. doi: 10.1007/s00535-008-2212-7 [published Online First: 2008/09/23]
494. Aragon F, Carino S, Perdigon G, et al. Inhibition of Growth and Metastasis of Breast Cancer in Mice by Milk Fermented With Lactobacillus casei CRL 431. *J Immunother* 2015;38(5):185-96. doi: 10.1097/CJI.0000000000000079 [published Online First: 2015/05/12]
495. Baldwin C, Millette M, Oth D, et al. Probiotic Lactobacillus acidophilus and L. casei mix sensitize colorectal tumoral cells to 5-fluorouracil-induced apoptosis. *Nutr Cancer* 2010;62(3):371-8. doi: 10.1080/01635580903407197 [published Online First: 2010/04/02]
496. Li C, Gu Y, He Q, et al. Integrated Analysis of Microbiome and Transcriptome Data Reveals the Interplay Between Commensal Bacteria and Fibrin Degradation in Endometrial Cancer. *Front Cell Infect Microbiol*

2021;11:748558. doi: 10.3389/fcimb.2021.748558 [published Online First: 2021/10/09]
497. Cuzzolin L, Zambreri D, Donini M, et al. Influence of radiotherapy on intestinal microflora in cancer patients. *J Chemother* 1992;4(3):176-9. doi: 10.1080/1120009x.1992.11739160 [published Online First: 1992/06/01]
498. Bongso A, Gajra B, Lian NP, et al. Establishment of human endometrial cell cultures. *Hum Reprod* 1988;3(6):705-13. doi: 10.1093/oxfordjournals.humrep.a136770 [published Online First: 1988/08/01]
499. Fernandez-Shaw S, Shorter SC, Naish CE, et al. Isolation and purification of human endometrial stromal and glandular cells using immunomagnetic microspheres. *Hum Reprod* 1992;7(2):156-61. doi: 10.1093/oxfordjournals.humrep.a137609 [published Online First: 1992/02/01]
500. Qu W, Zhao Y, Wang X, et al. Culture characters, genetic background, estrogen/progesterone receptor expression, and tumorigenic activities of frequently used sixteen endometrial cancer cell lines. *Clin Chim Acta* 2019;489:225-32. doi: 10.1016/j.cca.2018.08.013 [published Online First: 2018/08/15]
501. Korch C, Spillman MA, Jackson TA, et al. DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. *Gynecol Oncol* 2012;127(1):241-8. doi: 10.1016/j.ygyno.2012.06.017 [published Online First: 2012/06/20]
502. Zhou X WZ, Zhao Y, Podratz K, Jiang S. Characterization of sixteen endometrial cancer cell lines. *AACR Annual Meeting-- Apr 14-18, 2007; Los Angeles, CA*

503. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol* 2014;15(12):786-801. doi: 10.1038/nrm3904 [published Online First: 2014/11/22]
504. Kratochvil MJ, Seymour AJ, Li TL, et al. Engineered materials for organoid systems. *Nat Rev Mater* 2019;4(9):606-22. doi: 10.1038/s41578-019-0129-9 [published Online First: 2019/09/01]
505. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 2011;141(5):1762-72. doi: 10.1053/j.gastro.2011.07.050 [published Online First: 2011/09/06]
506. Bui BN, Boretto M, Kobayashi H, et al. Organoids can be established reliably from cryopreserved biopsy catheter-derived endometrial tissue of infertile women. *Reprod Biomed Online* 2020;41(3):465-73. doi: 10.1016/j.rbmo.2020.03.019 [published Online First: 2020/07/06]
507. Dalseg AM, Gaardsvoll H, Bock E. Molecular biology of cadherins in the nervous system. *Mol Neurobiol* 1993;7(3-4):207-28. doi: 10.1007/BF02769176 [published Online First: 1993/01/01]
508. Behrens J. Cadherins as determinants of tissue morphology and suppressors of invasion. *Acta Anat (Basel)* 1994;149(3):165-9. doi: 10.1159/000147572 [published Online First: 1994/01/01]
509. MacCalman CD, Getsios S, Chen GT. Type 2 cadherins in the human endometrium and placenta: their putative roles in human implantation and

placentation. *Am J Reprod Immunol* 1998;39(2):96-107. doi: 10.1111/j.1600-0897.1998.tb00341.x [published Online First: 1998/03/20]
510. Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod Pathol* 2000;13(9):962-72. doi: 10.1038/modpathol.3880175 [published Online First: 2000/09/28]
511. Casaletto JB, Saotome I, Curto M, et al. Ezrin-mediated apical integrity is required for intestinal homeostasis. *Proc Natl Acad Sci U S A* 2011;108(29):11924-9. doi: 10.1073/pnas.1103418108 [published Online First: 2011/07/07]
512. Krebsbach PH, Villa-Diaz LG. The Role of Integrin alpha6 (CD49f) in Stem Cells: More than a Conserved Biomarker. *Stem Cells Dev* 2017;26(15):1090-99. doi: 10.1089/scd.2016.0319 [published Online First: 2017/05/13]
513. Ying J, Yang L, Yin JC, et al. Additive effects of variants of unknown significance in replication repair-associated DNA polymerase genes on mutational burden and prognosis across diverse cancers. *J Immunother Cancer* 2021;9(9) doi: 10.1136/jitc-2021-002336 [published Online First: 2021/09/05]
514. Fitzgerald HC, Dhakal P, Behura SK, et al. Self-renewing endometrial epithelial organoids of the human uterus. *Proc Natl Acad Sci U S A* 2019;116(46):23132-42. doi: 10.1073/pnas.1915389116 [published Online First: 2019/11/02]
515. Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol* 2000;1(1):72-6. doi: 10.1038/35036093 [published Online First: 2001/06/20]
516. Hayflick L. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res* 1965;37:614-36. doi: 10.1016/0014-4827(65)90211-9 [published Online First: 1965/03/01]
517. Hoffmann K, Berger H, Kulbe H, et al. Stable expansion of high-grade serous ovarian cancer organoids requires a low-Wnt environment. *EMBO J* 2020;39(6):e104013. doi: 10.15252/embj.2019104013 [published Online First: 2020/02/06]
518. Gordon DE, Watson A, Roguev A, et al. A Quantitative Genetic Interaction Map of HIV Infection. *Mol Cell* 2020;78(2):197-209 e7. doi: 10.1016/j.molcel.2020.02.004 [published Online First: 2020/02/23]
519. Condello S, Morgan CA, Nagdas S, et al. beta-Catenin-regulated ALDH1A1 is a target in ovarian cancer spheroids. *Oncogene* 2015;34(18):2297-308. doi: 10.1038/onc.2014.178 [published Online First: 2014/06/24]
520. Salem M, Shan Y, Bernaudo S, et al. miR-590-3p Targets Cyclin G2 and FOXO3 to Promote Ovarian Cancer Cell Proliferation, Invasion, and Spheroid Formation. *Int J Mol Sci* 2019;20(8) doi: 10.3390/ijms20081810 [published Online First: 2019/04/25]
521. Conlon N, Leitao MM, Jr., Abu-Rustum NR, et al. Grading uterine endometrioid carcinoma: a proposal that binary is best. *Am J Surg Pathol* 2014;38(12):1583-7. doi: 10.1097/PAS.0000000000000327 [published Online First: 2014/09/18]
522. Halaoui R, McCaffrey L. Rewiring cell polarity signaling in cancer. *Oncogene* 2015;34(8):939-50. doi: 10.1038/onc.2014.59 [published Online First: 2014/03/19]
523. Kraiczy J, Nayak KM, Howell KJ, et al. DNA methylation defines regional identity of human intestinal epithelial organoids and undergoes dynamic changes

during development. *Gut* 2019;68(1):49-61. doi: 10.1136/gutjnl-2017-314817 [published Online First: 2017/11/17]
524. Joshi R, Castro De Moura M, Pineyro D, et al. The DNA methylation landscape of human cancer organoids available at the American type culture collection. *Epigenetics* 2020;15(11):1167-77. doi: 10.1080/15592294.2020.1762398 [published Online First: 2020/05/13]
525. O'Keefe SJ. Diet, microorganisms and their metabolites, and colon cancer. *Nat Rev Gastroenterol Hepatol* 2016;13(12):691-706. doi: 10.1038/nrgastro.2016.165 [published Online First: 2016/11/17]
526. Laniewski P, Cui H, Roe DJ, et al. Vaginal microbiota, genital inflammation, and neoplasia impact immune checkpoint protein profiles in the cervicovaginal microenvironment. *NPJ Precis Oncol* 2020;4:22. doi: 10.1038/s41698-020-0126-x [published Online First: 2020/08/18]
527. Ilhan ZE, Laniewski P, Thomas N, et al. Deciphering the complex interplay between microbiota, HPV, inflammation and cancer through cervicovaginal metabolic profiling. *EBioMedicine* 2019;44:675-90. doi: 10.1016/j.ebiom.2019.04.028 [published Online First: 2019/04/28]
528. Laniewski P, Barnes D, Goulder A, et al. Linking cervicovaginal immune signatures, HPV and microbiota composition in cervical carcinogenesis in non-Hispanic and Hispanic women. *Sci Rep* 2018;8(1):7593. doi: 10.1038/s41598-018-25879-7 [published Online First: 2018/05/17]
529. Laniewski P, Cui H, Roe DJ, et al. Features of the cervicovaginal microenvironment drive cancer biomarker signatures in patients across cervical carcinogenesis. *Sci Rep* 2019;9(1):7333. doi: 10.1038/s41598-019-43849-5 [published Online First: 2019/05/16]
530. Audirac-Chalifour A, Torres-Poveda K, Bahena-Roman M, et al. Cervical Microbiome and Cytokine Profile at Various Stages of Cervical Cancer: A Pilot Study. *PLoS One* 2016;11(4):e0153274. doi: 10.1371/journal.pone.0153274 [published Online First: 2016/04/27]
531. Fortner RT, Husing A, Kuhn T, et al. Endometrial cancer risk prediction including serum-based biomarkers: results from the EPIC cohort. *Int J Cancer* 2017;140(6):1317-23. doi: 10.1002/ijc.30560 [published Online First: 2016/12/10]
532. Esposito K, Pontillo A, Di Palo C, et al. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. *JAMA* 2003;289(14):1799-804. doi: 10.1001/jama.289.14.1799 [published Online First: 2003/04/10]
533. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. *Nat Rev Cancer* 2004;4(1):11-22. doi: 10.1038/nrc1252 [published Online First: 2004/01/07]
534. Steinbach G, Lynch PM, Phillips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000;342(26):1946-52. doi: 10.1056/NEJM200006293422603 [published Online First: 2000/06/30]
535. Jacoby RF, Seibert K, Cole CE, et al. The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of

adenomatous polyposis. *Cancer Res* 2000;60(18):5040-4. [published Online First: 2000/10/04]
536. Hu J, Wang C, Ye L, et al. Anti-tumour immune effect of oral administration of Lactobacillus plantarum to CT26 tumour-bearing mice. *J Biosci* 2015;40(2):269-79. doi: 10.1007/s12038-015-9518-4 [published Online First: 2015/05/13]
537. Cai S, Kandasamy M, Rahmat JN, et al. Lactobacillus rhamnosus GG Activation of Dendritic Cells and Neutrophils Depends on the Dose and Time of Exposure. *J Immunol Res* 2016;2016:7402760. doi: 10.1155/2016/7402760 [published Online First: 2016/08/16]
538. Khazaie K, Zadeh M, Khan MW, et al. Abating colon cancer polyposis by Lactobacillus acidophilus deficient in lipoteichoic acid. *Proc Natl Acad Sci U S A* 2012;109(26):10462-7. doi: 10.1073/pnas.1207230109 [published Online First: 2012/06/13]
539. Konishi H, Fujiya M, Tanaka H, et al. Probiotic-derived ferrichrome inhibits colon cancer progression via JNK-mediated apoptosis. *Nat Commun* 2016;7:12365. doi: 10.1038/ncomms12365 [published Online First: 2016/08/11]
540. Lee JW, Shin JG, Kim EH, et al. Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. *J Vet Sci* 2004;5(1):41-8. [published Online First: 2004/03/19]
541. Smith HO, Stephens ND, Qualls CR, et al. The clinical significance of inflammatory cytokines in primary cell culture in endometrial carcinoma. *Mol Oncol* 2013;7(1):41-54. doi: 10.1016/j.molonc.2012.07.002 [published Online First: 20120815]
542. Caselli E, Soffritti I, D'Accolti M, et al. Atopobium vaginae And Porphyromonas somerae Induce Proinflammatory Cytokines Expression In Endometrial Cells: A Possible Implication For Endometrial Cancer? *Cancer Manag Res* 2019;11:8571-75. doi: 10.2147/CMAR.S217362 [published Online First: 2019/10/03]
543. Gartner MA, Bondzio A, Braun N, et al. Detection and characterisation of Lactobacillus spp. in the bovine uterus and their influence on bovine endometrial epithelial cells in vitro. *PLoS One* 2015;10(3):e0119793. doi: 10.1371/journal.pone.0119793 [published Online First: 2015/03/25]
544. Schirmer M, Smeekens SP, Vlamakis H, et al. Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. *Cell* 2016;167(7):1897. doi: 10.1016/j.cell.2016.11.046 [published Online First: 2016/12/17]
545. Peter S, Gartner MA, Michel G, et al. Influence of intrauterine administration of Lactobacillus buchneri on reproductive performance and pro-inflammatory endometrial mRNA expression of cows with subclinical endometritis. *Sci Rep* 2018;8(1):5473. doi: 10.1038/s41598-018-22856-y [published Online First: 2018/04/05]
546. Ugolini M, Sander LE. Dead or alive: how the immune system detects microbial viability. *Curr Opin Immunol* 2019;56:60-66. doi: 10.1016/j.coi.2018.09.018 [published Online First: 2018/10/27]
547. Skorup P, Maudsdotter L, Lipcsey M, et al. Mode of bacterial killing affects the inflammatory response and associated organ dysfunctions in a porcine E. coli

intensive care sepsis model. *Crit Care* 2020;24(1):646. doi: 10.1186/s13054-020-03303-9 [published Online First: 2020/11/16]
548. Wang B, Chen R, Gao H, et al. A comparative study unraveling the effects of TNF-alpha stimulation on endothelial cells between 2D and 3D culture. *Biomed Mater* 2020;15(6):065018. doi: 10.1088/1748-605X/ab95e3 [published Online First: 2020/05/23]
549. Amant F, Moerman P, Neven P, et al. Endometrial cancer. *Lancet* 2005;366(9484):491-505. doi: 10.1016/S0140-6736(05)67063-8 [published Online First: 2005/08/09]
550. Morice P, Leary A, Creutzberg C, et al. Endometrial cancer. *Lancet* 2016;387(10023):1094-108. doi: 10.1016/S0140-6736(15)00130-0 [published Online First: 2015/09/12]
551. Spurdle AB, Bowman MA, Shamsani J, et al. Endometrial cancer gene panels: clinical diagnostic vs research germline DNA testing. *Mod Pathol* 2017;30(8):1048-68. doi: 10.1038/modpathol.2017.20 [published Online First: 2017/04/30]
552. O'Mara TA, Glubb DM, Kho PF, et al. Genome-Wide Association Studies of Endometrial Cancer: Latest Developments and Future Directions. *Cancer Epidemiol Biomarkers Prev* 2019;28(7):1095-102. doi: 10.1158/1055-9965.EPI-18-1031 [published Online First: 2019/05/02]
553. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004;4(8):579-91. doi: 10.1038/nrc1408 [published Online First: 2004/08/03]
554. Yin J, Peng Y, Wu J, et al. Toll-like receptor 2/4 links to free fatty acid-induced inflammation and beta-cell dysfunction. *J Leukoc Biol* 2014;95(1):47-52. doi: 10.1189/jlb.0313143 [published Online First: 2013/09/11]
555. Muzio M, Ni J, Feng P, et al. Pillars article: IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997. 278: 1612-1615. *J Immunol* 2013;190(1):16-9. [published Online First: 2012/12/25]
556. Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Cell Biol* 2007;19(2):142-9. doi: 10.1016/j.ceb.2007.02.001 [published Online First: 2007/02/17]
557. Gao Z, Hwang D, Bataille F, et al. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J Biol Chem* 2002;277(50):48115-21. doi: 10.1074/jbc.M209459200 [published Online First: 2002/09/28]
558. Liu YF, Herschkovitz A, Boura-Halfon S, et al. Serine phosphorylation proximal to its phosphotyrosine binding domain inhibits insulin receptor substrate 1 function and promotes insulin resistance. *Mol Cell Biol* 2004;24(21):9668-81. doi: 10.1128/MCB.24.21.9668-9681.2004 [published Online First: 2004/10/16]
559. Poore GD, Kopylova E, Zhu Q, et al. Microbiome analyses of blood and tissues suggest cancer diagnostic approach. *Nature* 2020;579(7800):567-74. doi: 10.1038/s41586-020-2095-1 [published Online First: 2020/03/28]
560. Terrisse S, Derosa L, Iebba V, et al. Intestinal microbiota influences clinical outcome and side effects of early breast cancer treatment. *Cell Death Differ* 2021;28(9):2778-96. doi: 10.1038/s41418-021-00784-1 [published Online First: 2021/05/09]

561. Reis Ferreira M, Andreyev HJN, Mohammed K, et al. Microbiota- and Radiotherapy-Induced Gastrointestinal Side-Effects (MARS) Study: A Large Pilot Study of the Microbiome in Acute and Late-Radiation Enteropathy. *Clin Cancer Res* 2019;25(21):6487-500. doi: 10.1158/1078-0432.CCR-19-0960 [published Online First: 2019/07/28]
562. Yeung CY, Chan WT, Jiang CB, et al. Amelioration of Chemotherapy-Induced Intestinal Mucositis by Orally Administered Probiotics in a Mouse Model. *PLoS One* 2015;10(9):e0138746. doi: 10.1371/journal.pone.0138746 [published Online First: 2015/09/26]
563. Mi H, Dong Y, Zhang B, et al. Bifidobacterium Infantis Ameliorates Chemotherapy-Induced Intestinal Mucositis Via Regulating T Cell Immunity in Colorectal Cancer Rats. *Cell Physiol Biochem* 2017;42(6):2330-41. doi: 10.1159/000480005 [published Online First: 2017/08/30]
564. Chang CW, Liu CY, Lee HC, et al. Lactobacillus casei Variety rhamnosus Probiotic Preventively Attenuates 5-Fluorouracil/Oxaliplatin-Induced Intestinal Injury in a Syngeneic Colorectal Cancer Model. *Front Microbiol* 2018;9:983. doi: 10.3389/fmicb.2018.00983 [published Online First: 2018/06/06]
565. Southcott E, Tooley KL, Howarth GS, et al. Yoghurts containing probiotics reduce disruption of the small intestinal barrier in methotrexate-treated rats. *Dig Dis Sci* 2008;53(7):1837-41. doi: 10.1007/s10620-008-0275-1 [published Online First: 2008/04/23]
566. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* 1995;125(6):1401-12. doi: 10.1093/jn/125.6.1401 [published Online First: 1995/06/01]
567. Mahdavi M, Laforest-Lapointe I, Masse E. Preventing Colorectal Cancer through Prebiotics. *Microorganisms* 2021;9(6) doi: 10.3390/microorganisms9061325 [published Online First: 2021/07/03]
568. Rafter J, Bennett M, Caderni G, et al. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. *Am J Clin Nutr* 2007;85(2):488-96. doi: 10.1093/ajcn/85.2.488 [published Online First: 2007/02/08]
569. Skiba MB, Kohler LN, Crane TE, et al. The Association between Prebiotic Fiber Supplement Use and Colorectal Cancer Risk and Mortality in the Women's Health Initiative. *Cancer Epidemiol Biomarkers Prev* 2019;28(11):1884-90. doi: 10.1158/1055-9965.EPI-19-0326 [published Online First: 2019/08/29]
570. Hill C, Guarner F, Reid G, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* 2014;11(8):506-14. doi: 10.1038/nrgastro.2014.66 [published Online First: 2014/06/11]
571. Ngugi BM, Hemmerling A, Bukusi EA, et al. Effects of bacterial vaginosis-associated bacteria and sexual intercourse on vaginal colonization with the probiotic Lactobacillus crispatus CTV-05. *Sex Transm Dis* 2011;38(11):1020-7. doi: 10.1097/OLQ.0b013e3182267ac4 [published Online First: 2011/10/14]
572. Hemmerling A, Harrison W, Schroeder A, et al. Phase 2a study assessing colonization efficiency, safety, and acceptability of Lactobacillus crispatus CTV-05 in women with bacterial vaginosis. *Sex Transm Dis* 2010;37(12):745-50. doi: 10.1097/OLQ.0b013e3181e50026 [published Online First: 2010/07/21]

573. Stapleton AE, Au-Yeung M, Hooton TM, et al. Randomized, placebo-controlled phase 2 trial of a Lactobacillus crispatus probiotic given intravaginally for prevention of recurrent urinary tract infection. *Clin Infect Dis* 2011;52(10):1212-7. doi: 10.1093/cid/cir183 [published Online First: 2011/04/19]
574. Sungur T, Aslim B, Karaaslan C, et al. Impact of Exopolysaccharides (EPSs) of Lactobacillus gasseri strains isolated from human vagina on cervical tumor cells (HeLa). *Anaerobe* 2017;47:137-44. doi: 10.1016/j.anaerobe.2017.05.013 [published Online First: 2017/05/31]
575. Li C, Jia L, Yu Y, et al. Lactic acid induced microRNA-744 enhances motility of SiHa cervical cancer cells through targeting ARHGAP5. *Chem Biol Interact* 2019;298:86-95. doi: 10.1016/j.cbi.2018.10.027 [published Online First: 2018/11/14]
576. Verhoeven V, Renard N, Makar A, et al. Probiotics enhance the clearance of human papillomavirus-related cervical lesions: a prospective controlled pilot study. *Eur J Cancer Prev* 2013;22(1):46-51. doi: 10.1097/CEJ.0b013e328355ed23 [published Online First: 2012/06/19]
577. Palma E, Recine N, Domenici L, et al. Long-term Lactobacillus rhamnosus BMX 54 application to restore a balanced vaginal ecosystem: a promising solution against HPV-infection. *BMC Infect Dis* 2018;18(1):13. doi: 10.1186/s12879-017-2938-z [published Online First: 2018/01/07]
578. Wang KD, Xu DJ, Wang BY, et al. Inhibitory Effect of Vaginal Lactobacillus Supernatants on Cervical Cancer Cells. *Probiotics Antimicrob Proteins* 2018;10(2):236-42. doi: 10.1007/s12602-017-9339-x [published Online First: 2017/10/27]
579. Chen ZY, Hsieh YM, Huang CC, et al. Inhibitory Effects of Probiotic Lactobacillus on the Growth of Human Colonic Carcinoma Cell Line HT-29. *Molecules* 2017;22(1) doi: 10.3390/molecules22010107 [published Online First: 2017/01/12]
580. Escamilla J, Lane MA, Maitin V. Cell-free supernatants from probiotic Lactobacillus casei and Lactobacillus rhamnosus GG decrease colon cancer cell invasion in vitro. *Nutr Cancer* 2012;64(6):871-8. doi: 10.1080/01635581.2012.700758 [published Online First: 2012/07/27]
581. Zhao Y, Wang X, Li L, et al. Doxycycline inhibits proliferation and induces apoptosis of both human papillomavirus positive and negative cervical cancer cell lines. *Can J Physiol Pharmacol* 2016;94(5):526-33. doi: 10.1139/cjpp-2015-0481 [published Online First: 2016/02/26]
582. Yang B, Lu Y, Zhang A, et al. Doxycycline Induces Apoptosis and Inhibits Proliferation and Invasion of Human Cervical Carcinoma Stem Cells. *PLoS One* 2015;10(6):e0129138. doi: 10.1371/journal.pone.0129138 [published Online First: 2015/06/26]
583. Li H, Jiao S, Li X, et al. Therapeutic effects of antibiotic drug mefloquine against cervical cancer through impairing mitochondrial function and inhibiting mTOR pathway. *Can J Physiol Pharmacol* 2017;95(1):43-50. doi: 10.1139/cjpp-2016-0124 [published Online First: 2016/11/11]
584. DeStefano Shields CE, Van Meerbeke SW, Housseau F, et al. Reduction of Murine Colon Tumorigenesis Driven by Enterotoxigenic Bacteroides fragilis

Using Cefoxitin Treatment. *J Infect Dis* 2016;214(1):122-9. doi: 10.1093/infdis/jiw069 [published Online First: 2016/02/26]

585. Bullman S, Pedamallu CS, Sicinska E, et al. Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. *Science* 2017;358(6369):1443-48. doi: 10.1126/science.aal5240 [published Online First: 2017/11/25]
586. Lev-Sagie A, Goldman-Wohl D, Cohen Y, et al. Vaginal microbiome transplantation in women with intractable bacterial vaginosis. *Nat Med* 2019;25(10):1500-04. doi: 10.1038/s41591-019-0600-6 [published Online First: 2019/10/09]
587. Schwartz M, Gluck M, Koon S. Norovirus gastroenteritis after fecal microbiota transplantation for treatment of Clostridium difficile infection despite asymptomatic donors and lack of sick contacts. *Am J Gastroenterol* 2013;108(8):1367. doi: 10.1038/ajg.2013.164 [published Online First: 2013/08/06]
588. Quera R, Espinoza R, Estay C, et al. Bacteremia as an adverse event of fecal microbiota transplantation in a patient with Crohn's disease and recurrent Clostridium difficile infection. *J Crohns Colitis* 2014;8(3):252-3. doi: 10.1016/j.crohns.2013.10.002 [published Online First: 2013/11/05]
589. Hohmann EL, Ananthakrishnan AN, Deshpande V. Case Records of the Massachusetts General Hospital. Case 25-2014. A 37-year-old man with ulcerative colitis and bloody diarrhea. *N Engl J Med* 2014;371(7):668-75. doi: 10.1056/NEJMcpc1400842 [published Online First: 2014/08/15]
590. Alang N, Kelly CR. Weight gain after fecal microbiota transplantation. *Open Forum Infect Dis* 2015;2(1):ofv004. doi: 10.1093/ofid/ofv004 [published Online First: 2015/06/03]
591. Gregory JC, Buffa JA, Org E, et al. Transmission of atherosclerosis susceptibility with gut microbial transplantation. *J Biol Chem* 2015;290(9):5647-60. doi: 10.1074/jbc.M114.618249 [published Online First: 2015/01/01]
592. Skye SM, Zhu W, Romano KA, et al. Microbial Transplantation With Human Gut Commensals Containing CutC Is Sufficient to Transmit Enhanced Platelet Reactivity and Thrombosis Potential. *Circ Res* 2018;123(10):1164-76. doi: 10.1161/CIRCRESAHA.118.313142 [published Online First: 2018/10/26]
593. Costea PI, Coelho LP, Sunagawa S, et al. Subspecies in the global human gut microbiome. *Mol Syst Biol* 2017;13(12):960. doi: 10.15252/msb.20177589 [published Online First: 2017/12/16]
594. Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 2012;490(7418):55-60. doi: 10.1038/nature11450 [published Online First: 2012/10/02]
595. Gevers D, Kugathasan S, Denson LA, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* 2014;15(3):382-92. doi: 10.1016/j.chom.2014.02.005 [published Online First: 2014/03/19]
596. Stewart CJ, Ajami NJ, O'Brien JL, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 2018;562(7728):583-88. doi: 10.1038/s41586-018-0617-x [published Online First: 2018/10/26]

597. Vatanen T, Franzosa EA, Schwager R, et al. The human gut microbiome in early-onset type 1 diabetes from the TEDDY study. *Nature* 2018;562(7728):589-94. doi: 10.1038/s41586-018-0620-2 [published Online First: 2018/10/26]
598. Lloyd-Price J, Arze C, Ananthakrishnan AN, et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 2019;569(7758):655-62. doi: 10.1038/s41586-019-1237-9 [published Online First: 2019/05/31]
599. Zhou W, Sailani MR, Contrepois K, et al. Longitudinal multi-omics of host-microbe dynamics in prediabetes. *Nature* 2019;569(7758):663-71. doi: 10.1038/s41586-019-1236-x [published Online First: 2019/05/31]
600. Thomas AM, Manghi P, Asnicar F, et al. Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation. *Nat Med* 2019;25(4):667-78. doi: 10.1038/s41591-019-0405-7 [published Online First: 2019/04/03]
601. Wirbel J, Pyl PT, Kartal E, et al. Meta-analysis of fecal metagenomes reveals global microbial signatures that are specific for colorectal cancer. *Nat Med* 2019;25(4):679-89. doi: 10.1038/s41591-019-0406-6 [published Online First: 2019/04/03]
602. Pasolli E, Asnicar F, Manara S, et al. Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle. *Cell* 2019;176(3):649-62 e20. doi: 10.1016/j.cell.2019.01.001 [published Online First: 2019/01/22]
603. Li J, Jia H, Cai X, et al. An integrated catalog of reference genes in the human gut microbiome. *Nat Biotechnol* 2014;32(8):834-41. doi: 10.1038/nbt.2942 [published Online First: 2014/07/07]
604. Valles-Colomer M, Darzi Y, Vieira-Silva S, et al. Meta-omics in Inflammatory Bowel Disease Research: Applications, Challenges, and Guidelines. *J Crohns Colitis* 2016;10(6):735-46. doi: 10.1093/ecco-jcc/jjw024 [published Online First: 2016/01/24]
605. Mallick H, Ma S, Franzosa EA, et al. Experimental design and quantitative analysis of microbial community multiomics. *Genome Biol* 2017;18(1):228. doi: 10.1186/s13059-017-1359-z [published Online First: 2017/12/01]
606. Knight R, Vrbanac A, Taylor BC, et al. Best practices for analysing microbiomes. *Nat Rev Microbiol* 2018;16(7):410-22. doi: 10.1038/s41579-018-0029-9 [published Online First: 2018/05/26]
607. Franzosa EA, Hsu T, Sirota-Madi A, et al. Sequencing and beyond: integrating molecular 'omics' for microbial community profiling. *Nat Rev Microbiol* 2015;13(6):360-72. doi: 10.1038/nrmicro3451 [published Online First: 2015/04/29]
608. Blanco-Miguez A, Fdez-Riverola F, Sanchez B, et al. Resources and tools for the high-throughput, multi-omic study of intestinal microbiota. *Brief Bioinform* 2019;20(3):1032-56. doi: 10.1093/bib/bbx156 [published Online First: 2017/12/01]
609. Huang S, Chaudhary K, Garmire LX. More Is Better: Recent Progress in Multi-Omics Data Integration Methods. *Front Genet* 2017;8:84. doi: 10.3389/fgene.2017.00084 [published Online First: 2017/07/04]

610. Norman JM, Handley SA, Baldridge MT, et al. Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell* 2015;160(3):447-60. doi: 10.1016/j.cell.2015.01.002 [published Online First: 2015/01/27]
611. Arnold JT, Kaufman DG, Seppala M, et al. Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. *Hum Reprod* 2001;16(5):836-45. doi: 10.1093/humrep/16.5.836 [published Online First: 2001/05/02]
612. Arnold JT, Lessey BA, Seppala M, et al. Effect of normal endometrial stroma on growth and differentiation in Ishikawa endometrial adenocarcinoma cells. *Cancer Res* 2002;62(1):79-88. [published Online First: 2002/01/10]
613. Chakrabarti J, Holokai L, Syu L, et al. Mouse-Derived Gastric Organoid and Immune Cell Co-culture for the Study of the Tumor Microenvironment. *Methods Mol Biol* 2018;1817:157-68. doi: 10.1007/978-1-4939-8600-2_16 [published Online First: 2018/07/01]
614. Dijkstra KK, Cattaneo CM, Weeber F, et al. Generation of Tumor-Reactive T Cells by Co-culture of Peripheral Blood Lymphocytes and Tumor Organoids. *Cell* 2018;174(6):1586-98 e12. doi: 10.1016/j.cell.2018.07.009 [published Online First: 2018/08/14]
615. Tsai S, McOlash L, Palen K, et al. Development of primary human pancreatic cancer organoids, matched stromal and immune cells and 3D tumor microenvironment models. *BMC Cancer* 2018;18(1):335. doi: 10.1186/s12885-018-4238-4 [published Online First: 2018/03/29]
616. Sebrell TA, Hashimi M, Sidar B, et al. A Novel Gastric Spheroid Co-culture Model Reveals Chemokine-Dependent Recruitment of Human Dendritic Cells to the Gastric Epithelium. *Cell Mol Gastroenterol Hepatol* 2019;8(1):157-71 e3. doi: 10.1016/j.jcmgh.2019.02.010 [published Online First: 2019/03/18]
617. Leeman KT, Pessina P, Lee JH, et al. Mesenchymal Stem Cells Increase Alveolar Differentiation in Lung Progenitor Organoid Cultures. *Sci Rep* 2019;9(1):6479. doi: 10.1038/s41598-019-42819-1 [published Online First: 2019/04/25]
618. Watanabe M, Buth JE, Vishlaghi N, et al. Self-Organized Cerebral Organoids with Human-Specific Features Predict Effective Drugs to Combat Zika Virus Infection. *Cell Rep* 2017;21(2):517-32. doi: 10.1016/j.celrep.2017.09.047 [published Online First: 2017/10/12]
619. Nickerson CA, Goodwin TJ, Terlonge J, et al. Three-dimensional tissue assemblies: novel models for the study of Salmonella enterica serovar Typhimurium pathogenesis. *Infect Immun* 2001;69(11):7106-20. doi: 10.1128/IAI.69.11.7106-7120.2001 [published Online First: 2001/10/13]
620. Sainz B, Jr., TenCate V, Uprichard SL. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. *Virol J* 2009;6:103. doi: 10.1186/1743-422X-6-103 [published Online First: 2009/07/17]
621. Laniewski P, Gomez A, Hire G, et al. Human Three-Dimensional Endometrial Epithelial Cell Model To Study Host Interactions with Vaginal Bacteria and Neisseria gonorrhoeae. *Infect Immun* 2017;85(3) doi: 10.1128/IAI.01049-16 [published Online First: 2017/01/06]
622. Barrila J, Radtke AL, Crabbe A, et al. Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions. *Nat Rev Microbiol*

2010;8(11):791-801. doi: 10.1038/nrmicro2423 [published Online First: 2010/10/16]
623. Cho A, Howell VM, Colvin EK. The Extracellular Matrix in Epithelial Ovarian Cancer - A Piece of a Puzzle. *Front Oncol* 2015;5:245. doi: 10.3389/fonc.2015.00245 [published Online First: 2015/11/19]
624. Fullar A, Dudas J, Olah L, et al. Remodeling of extracellular matrix by normal and tumor-associated fibroblasts promotes cervical cancer progression. *BMC Cancer* 2015;15:256. doi: 10.1186/s12885-015-1272-3 [published Online First: 2015/04/18]
625. Spurgeon ME, den Boon JA, Horswill M, et al. Human papillomavirus oncogenes reprogram the cervical cancer microenvironment independently of and synergistically with estrogen. *Proc Natl Acad Sci U S A* 2017;114(43):E9076-E85. doi: 10.1073/pnas.1712018114 [published Online First: 2017/10/27]
626. Sahoo SS, Quah MY, Nielsen S, et al. Inhibition of extracellular matrix mediated TGF-beta signalling suppresses endometrial cancer metastasis. *Oncotarget* 2017;8(42):71400-17. doi: 10.18632/oncotarget.18069 [published Online First: 2017/10/27]
627. Ford CE, Henry C, Llamosas E, et al. Wnt signalling in gynaecological cancers: A future target for personalised medicine? *Gynecol Oncol* 2016;140(2):345-51. doi: 10.1016/j.ygyno.2015.09.085 [published Online First: 2015/10/04]
628. Gera S, Kumar SS, Swamy SN, et al. Follicle-Stimulating Hormone Is an Autocrine Regulator of the Ovarian Cancer Metastatic Niche Through Notch Signaling. *J Endocr Soc* 2019;3(2):340-57. doi: 10.1210/js.2018-00272 [published Online First: 2019/01/27]
629. Peart TM, Correa RJ, Valdes YR, et al. BMP signalling controls the malignant potential of ascites-derived human epithelial ovarian cancer spheroids via AKT kinase activation. *Clin Exp Metastasis* 2012;29(4):293-313. doi: 10.1007/s10585-011-9451-3 [published Online First: 2012/01/18]
630. Yucer N, Holzapfel M, Jenkins Vogel T, et al. Directed Differentiation of Human Induced Pluripotent Stem Cells into Fallopian Tube Epithelium. *Sci Rep* 2017;7(1):10741. doi: 10.1038/s41598-017-05519-2 [published Online First: 2017/09/08]
631. Al-Ani A, Toms D, Kondro D, et al. Oxygenation in cell culture: Critical parameters for reproducibility are routinely not reported. *PLoS One* 2018;13(10):e0204269. doi: 10.1371/journal.pone.0204269 [published Online First: 2018/10/17]
632. Michl J, Park KC, Swietach P. Evidence-based guidelines for controlling pH in mammalian live-cell culture systems. *Commun Biol* 2019;2:144. doi: 10.1038/s42003-019-0393-7 [published Online First: 2019/05/03]
633. Petrova TV, Koh GY. Organ-specific lymphatic vasculature: From development to pathophysiology. *J Exp Med* 2018;215(1):35-49. doi: 10.1084/jem.20171868 [published Online First: 2017/12/16]

Appendix

Patient Consent Form

The consent form used to recruit all participants into this project is shown below.

Imperial College Healthcare NHS
NHS Trust

AFFIX PATIENT STICKER HERE

Hammersmith Hospital
Du Cane Road
London
W12 0HS
Tel: 020 8383 1000
www.imperial.nhs.uk

Patient Consent Form

Version 8.0 (01/01/2017)

Metabonomics and Integrative Biology for Gynaecological Cancer

1 I confirm that I have read and understood the patient information sheet for he above study and have had the opportunity to ask questions. ☐

2 I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3 I understand that sections of any of my medical notes and the completed questionnaire may be looked at by responsible individuals or by regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4 I agree to allow a sample of tissue obtained from my surgery / biopsy to be used in this research project. I understand that giving this sample is voluntary and that I am free to withdraw my approval for its use at any time without giving a reason and without my medical care or legal rights being affected. ☐

5 I agree to give blood, swabs, stool, and urine samples for research in this project. I understand that giving these samples is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical care or legal rights being affected. ☐

6 I agree that the samples may be used to identify the presence of bacteria. ☐

7 I agree that the samples I have given and the information gathered about me can be stored at Imperial College for possible use in future studies to further the understanding of gynaecological cancers. ☐

8 I understand that some of these projects may be carried out by researchers other than Imperial College. I understand that some of these projects may involve international collaborations. ☐

9 I understand that I shall not benefit financially if future research leads to the development of new treatments or medical tests. ☐

10 I understand that confidentiality will be maintained as all information about me will be anonymous. ☐

11 I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

Patient Information Leaflet

Patient information leaflet provided to all participants recruited at Hammersmith Hospital, Imperial College Healthcare NHS Trust.

Imperial College Healthcare NHS
NHS Trust

Patient Information Sheet

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part. If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part your future care will not be affected by your decision. You are free to withdraw at any time without explanation and your future care will not be affected. Thank you for reading this.

What is the purpose of the study?
We at Imperial College London are constantly trying to improve our knowledge about gynaecological cancer and other gynaecological conditions. This project is designed to see the differences that can be detected in people with different gynaecological conditions in order to achieve a comprehensive understanding of these underlying causes of the diseases. Obesity is associated with hormonal and metabolic abnormalities such as increasing oestrogen levels and insulin resistance (the state when an individual's body tissues have a decreased response to insulin), which may be relevant to the development of endometrial cancer. This study aims to further knowledge the role of obesity and insulin resistance in the natural history of endometrial cancer. Detailed and more technical information about the projects is available from clinic staff. Please note that you are not taking part in a clinical treatment or drug trial.

Why have I been chosen?
You have been chosen as you may be having investigations for a gynaecological condition.

Do I have to take part?
No. Taking part in this study is voluntary. Whatever your decision, it will not affect your current or future medical care or rights in any way. If you do decide to take part you may withdraw from the study at any time without having to give a reason, and it will make no difference to your medical care or treatment.

If you decide to withdraw from the study, tissue samples or data may already be retained and used for the purposes for which consent has already been given, provided they are effectively anonymised and no longer identifiable to the research team or any other persons to whom access will be given. I understand that if I lose capacity to consent whilst I am a participant in the study, all previous data and tissue collected with consent will be retained and used, but no further data collection or procedures will be carried out.

What will happen to me if I take part?
When you come into the hospital for your appointment or procedure, we will ask you if you would agree to complete a questionnaire with details from your medical, personal and family history and donate blood, urine, and tissue (or swabs) from the lining of your womb (endometrium) for research purposes. We would like to study these samples. It will take you approximately 20 minutes to fill in the questionnaire. We will collect an extra 20-40 mls (4-8 teaspoons) of blood along with a urine sample. We will collect these on the day of your appointment/procedure, while you are an inpatient (if you are having surgery) and repeat blood and urine samples after 6 months. We will take those at the same time if you are having your routine blood tests as part of your treatment. Tissue (and/or endocervical swabs or vaginal tampons) from the lining of your womb will be taken on the day of your appointment or procedure. If you are having a general anaesthesia, the samples will be taken while you will be asleep. An optional repeat sample will be taken 6 weeks later and then 6 months later with a small biopsy (called pipelle biopsy) that is inserted in the cavity of your womb and/or an endocervical swab or tampon. You may decide at any time not to have some of these tests. The clinical information we have and will continue to collect in your hospital records may be analysed.

We at Imperial College London, often collaborate with international institutions in order to gain as much information as possible about gynaecological conditions. If you consent to this study, it is possible that some of your samples may be sent to other international centres for further research. All information and samples will be coded in such a way as to make it impossible for you to be identified personally.
You will not be paid for participating in this study. We may be able to reimburse you for reasonable expenses (parking and travel) related to your study visits, over and above visits required if you were not participating in this study.

What are the possible benefits of taking part?
There are no direct benefits to you if you take part in this study. The projects underway now and in the future may shed light on what is going on in gynaecological conditions and cancers. If you do decide to participate, we hope that the knowledge gained from your participation may benefit patients with gynaecological conditions in the future.

What are the possible disadvantages and risks of taking part?
There are no major risks from taking part in this study. Although we will try to perform most of the blood samples at the time of your routine bloods/visits, some additional blood test and visits to the hospital will be required. The blood tests can cause some discomfort, pain, or a bruise. The endometrial pipelle biopsies (and/or endo-cervical swabs) will require an internal vaginal examination. Although most of the time the pipelle biopsy only causes mild discomfort, it can be painful and may also cause spotting/bleeding. Alternatively, the vaginal swabs can be inserted by yourself and cause minimal discomfort.

Will my taking part in this study be kept confidential?
All of your personal details will be kept confidential. Your samples will be held at Imperial College and will only be identified by a unique number. If your samples are sent to a centre outside of Imperial College, they will also be identified only by a unique code.

Who is organising the research?
This study is organised by the staff of Imperial College NHS Trust and Imperial College London. This research may be part of a PhD study proposal of one of the investigators.

Who has reviewed the study?
The West of Scotland REC 5 Research Ethics Committee has reviewed this study as well as it being independently peer reviewed.

What if something goes wrong?

Imperial College London holds insurance policies, which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator **Dr Maria Kyrgiou** on 020 8383 3268. The normal NHS complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial PALS (Patient Advice Liaison Service) on 0208 3130088.

Contact for Further Information

If you want more information, before or after you return your form, you can phone Dr Maria Kyrgiou on 020 8383 3268.

Gynaecological Questionnaire

Imperial College London

Imperial College Healthcare NHS Trust

Participant's Identifiable information:

Today's date: ____/____/_____

Height (cm): _____

Weight (kg): _____

BMI: _____

Section A: Demographic Data

A1. Which of these, best describes your ethnic group? *(Please mark only one.)*

White

☐ 1. British

☐ 2. Irish

☐ 3. Gypsy or Irish Traveller

☐ 4. Any other White background, please specify _____

Mixed/multiple ethnic groups

☐ 5. White and Black Caribbean

☐ 6. White and Black African

☐ 7. White and Asian

☐ 8. Any other Mixed/Multiple ethnic background, please specify _____

Asian/Asian British

☐ 9. Indian

☐ 10. Pakistani

☐ 11. Bangladeshi

☐ 12. Chinese

☐ 13. Any other Asian background, please specify _____

Black/African/Caribbean/Black British

☐ 14. African

☐ 15. Caribbean

☐ 16. Any other background, please specify_____

Other ethnic groups

☐ 17. Arab

☐ 18. Any other ethnic group, please specify

A2. What is the highest level of education that you have completed?

☐ No formal education ☐ GCSE's or equivalent ☐ A-levels or equivalent

☐ Degree/Higher degree ☐ Other, please specify _____

A3. What is your marital status?

☐ Single ☐ Married/civil partnership/living together ☐ Divorced/separated ☐ Widowed

Section B: Reproductive history

This section asks about pregnancies. Please answer, even if you have never been pregnant.

B1. Have you ever been pregnant?

☐ No

☐ Yes – Total number of pregnancies _____ (Include live births, stillbirths, terminations, miscarriages, ectopic pregnancies)

Number of live births _____ (Include live births & stillbirths only)

B2. Have you ever tried to become pregnant for one full year, without becoming pregnant?

☐ No ☐ Yes ☐ Not sure

B3. If you had difficulty getting pregnant, which of these best describes the reason?

☐ Problem with ovaries or hormones ☐ Problem with uterus or cervix ☐ Male partner fertility problem

☐ Problems with fallopian tubes ☐ No problem found ☐ Not sure

B4. Did your doctor ever prescribe medication to help you become pregnant?

☐ No ☐ Yes ☐ Not sure

Section C: Menstrual history

This section asks questions about your menstrual cycle, menstrual history and menopause.

C1. Have you ever had a menstrual period?

☐ No

☐ Yes → At what age did you have your first menstrual period? _____ years

C2. Which best describes the pattern of your menstrual cycle over most of your life?

☐ Regular (could usually predict when I would occur) ☐ Irregular (not predictable)

C3. Which best describes your current menstrual status?

☐ I still have regular periods (Go to **C5**)

☐ I still have irregular periods (Go to **C5**)

☐ Yes: No menstrual periods → at what age was your last period? _____ years

C4. <u>If your menstrual periods ceased PERMANENTLY</u>, which best describes why your periods stopped?

☐ Natural menopause ☐ Surgery (uterus+/-ovaries removed) ☐ Radiation

☐ Medication/drug therapy ☐ Other, please specify _____

C5. Have you ever had a hysterectomy (surgery to remove uterus/womb)?

☐ No

☐ Yes – At what age did you have your hysterectomy? _____ years

C6. Have you ever had surgery to remove one/both ovaries?

☐ No

☐ Yes, one ovary removed → at what age did you have one ovary removed? _____ years

☐ Yes, both ovaries removed → at what age did you have both ovaries removed? _____ years

C7. Have you ever had surgery to tie your fallopian tubes (female sterilization)?

☐ No

☐ Yes → At what age did you have your tubes tied? _____ years

Section D: Oral Contraceptive Use

This section asks questions about current and past use of oral contraceptives ('The Pill').

D1. Have you ever taken oral contraceptives ('The Pill')?

(This includes use for reasons other than contraception)

☐ No

☐ Yes – At what age did you start taking oral contraceptives? _____ years

– Total duration of oral contraceptive use (minus any breaks) _____ years

– Are you currently taking oral contraceptives ☐ Yes ☐ No → age when stopped use ___ years

Section E: Hormone Replacement Therapy Use

This section asks questions about current and past use of hormone replacement therapy ('HRT').

E1. Have you ever taken hormone replacement therapy, or medication containing female hormones for relief of menopausal symptoms, irregular periods or to prevent osteoporosis (bone thinning)?

☐ No (Go to section F)

☐ Yes

Hormone replacement therapy may contain oestrogen alone, progesterone alone, or a combination of the two. Please provide information, based on the type/types of HRT that you are taking/have taken.

E2. Have you ever taken hormone replacement therapy containing oestrogen/oestradiol *alone*?

(eg. Climaval®, Elleste-Solo®, Evorel®, Premarin®, Progynova®)

☐ No

☐ Yes – At what age did you start taking these tablets? _____ years

– Total duration of this type of HRT use _____ years

– Are you currently taking this type of HRT ☐ Yes ☐ No → age when stopped use ___ years

E3. Have you ever taken hormone replacement therapy containing progesterone/progestogen *alone*?

(eg. Climanor®, Utrogestan®, Provera, Norethisetrone)

☐ No

☐ Yes – At what age did you start taking these tablets? _____ years

– Total duration of this type of HRT use _____ years

– Are you currently taking this type of HRT ☐ Yes ☐ No → age when stopped use ___ years

– How many days per month do/did you take these tablets? _____ days

E4. Have you ever taken hormone replacement therapy containing oestrogen *and* progesterone?

(eg. Climagest®, Elleste-Duet®, Femoston®, Kliofem®, Kliovance®, Premique®, Prempak-C®)

☐ No

☐ Yes – At what age did you start taking these tablets? _____ years

– Total duration of this type of HRT use _____ months/ _____ years

– Are you currently taking this type of HRT ☐ Yes ☐ No → age when stopped use ___ years

– What is the name of the brand you took for the longest? _____

E5. Have you ever worn patches containing hormone replacement therapy?

(e.g. Elleste conti®, Estraderm®, Evorel®, FemSeven®)

☐ No

☐ Yes – At what age did you start wearing these patches? _____ years

– Total duration of this type of HRT use _____ months/ _____ years

– Are you currently using HRT patches ☐ Yes ☐ No → age when stopped use ___ years

E6. Have you ever used any other form of hormone replacement therapy? (Mark all that apply)

☐ Vaginal cream/gel ☐ Vaginal ring ☐ Progesterone IUD

☐ Other, please specify _____

Section F: Body Mass

This section asks questions about height and weight. If you are not sure, please estimate.

F1. What is your height? _____ feet, _____ inches **or** _____ centimetres

F2. What was your weight at the following times? (If you were pregnant at the time, please state weight immediately prior to pregnancy)

12 months ago? _____ stone or _____kg or _____lbs

10 years ago? _____ stone or _____kg or _____lbs

at 18 years of age? _____ stone or _____kg or _____lbs

F3. On which area of your body do you tend to gain weight?

☐ Chest/shoulders ☐ Waist/stomach ☐ Hips/thighs

☐ Equally all over ☐ Don't gain weight ☐ Other, please specify

Section G: Social History

This section asks questions about cigarette smoking.

G1. Have you ever smoked more than 100 cigarettes or more, in your lifetime?

☐ No

☐ Yes – Have you ever smoked cigarettes regularly? (i.e., at least one per day, for 6 months or more)

☐ No (Go to section H)

☐ Yes – At what age did you start smoking regularly? _____ years

– Do you smoke currently? ☐ No → at what age did you stop? _____ years

☐ Yes

G2. How many cigarettes/day do/did you smoke on average? _____ cigarettes/day

G3. Do you drink coffee regularly (daily basis)? ☐ Yes ☐ No

On average, how many cups of ***caffeinated*** coffee do you drink each day? ☐ 1 ☐ 2 ☐ 3 ☐ >4

On average, how many cups of ***decaffeinated*** coffee do you drink each day? ☐ 1 ☐ 2 ☐ 3 ☐ >4

Section H: Medical History

Please tick 'yes' if a doctor has ever diagnosed you with any of these medical problems, and state your approximate age at diagnosis.

Condition	
H1a. Hyperthyroidism (overactive thyroid)?	☐ No ☐ Yes → Age at diagnosis _____ years

H1b. Hypothyroidism (underactive thyroid)?	☐ No ☐ Yes → Age at diagnosis _____ years
H1c. Adult onset diabetes (Type 2 diabetes mellitus)?	☐ No ☐ Yes → Age at diagnosis _____ years
H1d. Diabetes (Type 1 diabetes mellitus)?	☐ No ☐ Yes → Age at diagnosis _____ years
H1e. Hypertension (high blood pressure)?	☐ No ☐ Yes → Age at diagnosis _____ years
H1f. Endometriosis?	☐ No ☐ Yes → Age at diagnosis _____ years
H1g. Fibroid (non-cancerous tumors) of the uterus?	☐ No ☐ Yes → Age at diagnosis _____ years
H1h. Polycystic ovarian disease or Stein-Leventhal Syndrome?	☐ No ☐ Yes → Age at diagnosis _____ years
H1i. Other non-cancerous cysts or tumors of the ovary?	☐ No ☐ Yes → Age at diagnosis _____ years
H1j. Pelvic inflammatory disease (PID)?	☐ No ☐ Yes → Age at diagnosis _____ years
H1k. Breast cancer?	☐ No ☐ Yes → Age at diagnosis _____ years
H1l. Borderline ovarian cancer (ovarian tumor of low malignant potential or LMP)?	☐ No ☐ Yes → Age at diagnosis _____ years
H1m. Invasive ovarian cancer?	☐ No ☐ Yes → Age at diagnosis _____ years
H1n. Endometrial cancer (cancer of the lining of the uterus/womb)?	☐ No ☐ Yes → Age at diagnosis _____ years

H1o. Colon (bowel) cancer?	☐ No ☐ Yes → Age at diagnosis _____ years
H1p. Other types of cancer not listed above If yes, please specify _____	☐ No ☐ Yes → Age at diagnosis _____ years

Section I: Family History of Cancer

This section asks questions about the medical history of your family. Please only include *blood relatives*, with whom you share both biological parents and not adopted or step relatives.

I1. How many full sisters and/or full brothers do/did you have? ____ Sisters ____ Brothers

I2. Have many daughters and/or sons have you given birth to? _____ Daughters _____ Sons

I3. Has your mother, any full sisters or daughters ever been diagnosed with any type of cancer?

☐ No

☐ Yes → ☐ Mother ☐ Sister ☐ Daughter ☐ All

Please specify the type of cancer

_____

How old she was when the cancer was first diagnosed

_____

I4. Has your father, any full brother or sons ever been diagnosed with any type of cancer?

☐ No

☐ Yes → ☐ Father ☐ Brother ☐ Son ☐ All

Please specify the type of cancer

_____

How old he was when the cancer was first diagnosed

_____

Section J: Medication History

This section asks questions about over-the-counter medications you take/haven taken both as a once off and regularly. (This means at least once a week for one year, or more than 50 tablets in a one-year period).

J1. Have you ever taken metformin, insulin or other diabetic medication, regularly?

☐ No

☐ Yes - What is the name of the mediation(s) _____

– At what age did you start taking this medication regularly? _____ years

– Total duration of regular use _____ years

– Are you currently taking this medication ☐ Yes ☐ No

If more than one diabetic medication, please specify details for each_____

_____

__

J2. Have you ever taken aspirin, or aspirin-containing medications, regularly?

☐ No

☐ Yes – At what age did you start taking this medication regularly? _____ years

– Total duration of regular use _____ years

– Are you currently taking this medication ☐ Yes ☐ No

J3. Have you ever taken paracetamol, or paracetamol-containing medications, regularly?

☐ No

☐ Yes – At what age did you start taking this medication regularly? _____ years

– Total duration of regular use _____ years

– Are you currently taking this medication ☐ Yes ☐ No

J4. Have you ever taken painkillers or anti-inflammatory drugs *other than* aspirin, or paracetamol-containing medications, regularly? (e.g. Feminax®, Neurofen®(ibuprofen), Naproxen, Indomethacin, Piroxicam)

☐ No

☐ Yes – At what age did you start taking this medication regularly? _____ years

– Total duration of regular use _____ years

– Are you currently taking this medication ☐ Yes ☐ No

J5. Have you ever taken COX-2 inhibitors (usually prescribed for arthritis) regularly?
(e.g. Celebrex® (celecoxib), Vioxx®(rofecoxib)

☐ No

☐ Yes – At what age did you start taking this medication regularly? _____ years

– Total duration of regular use _____ years

– Are you currently taking this medication ☐ Yes ☐ No

J6. Have you ever taken Tamoxifen regularly?

☐ No

☐ Yes – At what age did you start taking this medication regularly? _____ years

– Total duration of regular use _____ years

– Are you currently taking this medication ☐ Yes ☐ No

J7. Have you ever taken Raloxifene (Evista®) regularly?

☐ No

☐ Yes – At what age did you start taking this medication regularly? _____ years
– Total duration of regular use _____ years
– Are you currently taking this medication ☐ Yes ☐ No

J8. Have you ever taken bisphosphonates (drugs to prevent osteoporosis/bone thinning) regularly?
(e.g. Fosamax® (alendronate), Actonel®(Risedronate),

☐ No

☐ Yes – At what age did you start taking this medication regularly? _____ years
– Total duration of regular use _____ years
– Are you currently taking this medication ☐ Yes ☐ No

J9. Have you taken antibiotics, antifungals e.g. Canestan™/ clotrimazole, or probiotics e.g. Yakult™, BioKult™ in the past 4 weeks? Please document all antibiotics etc. if there are more than one.

☐ No

☐ Yes
- What is the name of the antibiotic/antifungal/probiotic (s)? _____
- For how long did you take it? _____days
- What dose did you take? _____mg
- What route was the medication given (circle as appropriate)?
  Oral Vaginal Cream/ointment Via drip Intra-Muscular
- What was the reason for the medication? _____

J10. Have you used any vaginal pessaries/ tablets/ creams or ointments in the past 4 weeks?

☐ No

☐ Yes
- What did you use? _____
- For long long? _____

J11. Do you practice vaginal douching (internal washing of the vagina)?

☐ No

☐ Yes - How often: Once per day ☐ more frequently: _____
Once per week ☐ twice per week ☐ Any other_____

J12. Have you had vaginal intercourse in the past 2 weeks?

☐ No

☐ Yes

THANK YOU FOR YOUR TIME

OTUs removed as contaminants

OTU	Phylum	Class	Order	Family	Genera	Species
Otu00013	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	Prevotella_copri
Otu00020	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus_fornicalis
Otu00024	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	Prevotella_copri
Otu00036	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Psychrobacter	Psychrobacter_vallis
Otu00055	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	Propionibacterium_acnes
Otu00066	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Massiliprevotella	Massiliprevotella_massiliensis
Otu00070	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Catenibacterium	Catenibacterium_mitsuokai
Otu00076	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Georgfuchsia	Georgfuchsia_unclassified
Otu00078	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	Methylobacterium_extorquens
Otu00079	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Butyricicoccus	Butyricicoccus_unclassified
Otu00082	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	Prevotella_copri
Otu00092	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus_epidermidis
Otu00098	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_lividum
Otu00103	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_zoogloeoides
Otu00111	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	Coprococcus_eutactus
Otu00113	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_phyllosphaerae
Otu00124	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	Prevotella_unclassified
Otu00134	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Clostridium XVIII	Clostridium XVIII_unclassified
Otu00139	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_faecis
Otu00149	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_radiobacter
Otu00150	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Gemmiger	Gemmiger_formicilis
Otu00152	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_ferrireducens
Otu00154	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_luti

Otu00156	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	Sporosarcina_globispora
Otu00158	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	Coprococcus_unclassified
Otu00160	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	Corynebacterium_faecale
Otu00163	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_ferrireducens
Otu00169	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_jejuensis
Otu00179	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces	Streptomyces_albidoflavus
Otu00187	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum	Ochrobactrum_pseudogrignonense
Otu00188	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia	Delftia_lacustris
Otu00191	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	Bifidobacterium_adolescentis
Otu00196	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu00198	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_pakistanensis
Otu00199	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_asturiensis
Otu00201	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_cryoconitis
Otu00205	Proteobacteria	Betaproteobacteria	Burkholderiales	Sutterellaceae	Sutterella	Sutterella_wadsworthensis
Otu00206	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Megamonas	Megamonas_funiformis
Otu00209	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_antarcticus
Otu00212	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum	Herbaspirillum_rubrisubalbicans
Otu00226	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Rugamonas	Rugamonas_rubra
Otu00229	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	Roseburia_intestinalis
Otu00230	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus	Trichococcus_pasteurii
Otu00235	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_caeni
Otu00239	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides_eggerthii
Otu00240	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVa	Clostridium XlVa_unclassified
Otu00241	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter	Oscillibacter_unclassified
Otu00242	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Cryobacterium	Cryobacterium_arcticum
Otu00247	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Agreia	Agreia_pratensis
Otu00251	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_tagluense

Otu00255	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	Acidovorax_facilis
Otu00258	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	Corynebacterium_efficiens
Otu00260	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVa	Clostridium XlVa_unclassified
Otu00265	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Klebsiella	Kosakonia_sacchari
Otu00275	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pseudorhodoferax	Pseudorhodoferax_soli
Otu00283	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planococcus	Planococcus_maitriensis
Otu00294	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Giesbergeria	Giesbergeria_giesbergeri
Otu00299	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Simplicispira	Simplicispira_psychrophila
Otu00304	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter	Oscillibacter_unclassified
Otu00305	Actinobacteria	Actinobacteria	Actinomycetales	Demequinaceae	Lysinimicrobium	Lysinimicrobium_unclassified
Otu00306	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	Rhodococcus_qingshengii
Otu00312	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides_faecis
Otu00318	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Sphaerotilus	Sphaerotilus_montanus
Otu00323	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Eisenbergiella	Eisenbergiella_unclassified
Otu00329	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_unclassified
Otu00331	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Erysipelotrichaceae_incertae_sedis	Erysipelotrichaceae_incertae_sedis_unclassified
Otu00339	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu00341	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planococcus	Planococcus_antarcticus
Otu00343	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium sensu stricto_unclassified
Otu00345	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	Devosia_yakushimensis
Otu00358	Cyanobacteria/ Chloroplast	Chloroplast	Chloroplast	Chloroplast	Bacillariophyta	Bacillariophyta_unclassified
Otu00359	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_noncentrifugens
Otu00363	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Serpens	Serpens_unclassified
Otu00380	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus	Trichococcus_pasteurii
Otu00384	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga	Hydrogenophaga_caeni

Otu00390	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	Brevundimonas_staleyi
Otu00393	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	Variovorax_ginsengisoli
Otu00405	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu00413	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	Bifidobacterium_adolescentis
Otu00414	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_aquatile
Otu00421	Firmicutes	Bacilli	Bacillales	Bacillales_Incertae Sedis XI	Gemella	Gemella_haemolysans
Otu00423	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_saidenbachensis
Otu00425	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_faecis
Otu00429	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	Caulobacter_henricii
Otu00431	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Butyricicoccus	Butyricicoccus_unclassified
Otu00432	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus_gasseri
Otu00433	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	Bradyrhizobium_jicamae
Otu00440	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	Lysinibacillus_fusiformis
Otu00441	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_piscis
Otu00442	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_hartonius
Otu00448	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia	Ralstonia_insidiosa
Otu00454	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00456	Firmicutes	Clostridia	Clostridiales	Peptococcaceae 1	Peptococcus	Peptococcus_unclassified
Otu00458	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	Arcobacter_venerupis
Otu00460	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_saidenbachensis
Otu00462	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	Carnobacterium_jeotgali
Otu00465	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_estertheticum
Otu00466	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_piscis
Otu00467	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	Arcobacter_unclassified
Otu00475	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Rhizobacter	Rhizobacter_dauci
Otu00484	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_mandelii

Otu00485	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Flavonifractor	Flavonifractor_unclassified
Otu00489	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XIII	Anaerovorax	Anaerovorax_unclassified
Otu00490	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	Arcobacter_cryaerophilus
Otu00498	Proteobacteria	Betaproteobacteria	Burkholderiales	Sutterellaceae	Sutterella	Sutterella_stercoricanis
Otu00502	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_lusitanum
Otu00511	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Serpens	Serpens_unclassified
Otu00513	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	Lactococcus_lactis
Otu00519	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Agreia	Agreia_unclassified
Otu00523	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiracea_incertae_sedis	Lachnospiracea_incertae_sedis_unclassified
Otu00530	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Conyzicola	Conyzicola_lurida
Otu00531	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_unclassified
Otu00533	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	Roseburia_unclassified
Otu00538	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_segetis
Otu00540	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	Propionibacterium_acnes
Otu00544	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Jeotgalicoccus	Jeotgalicoccus_psychrophilus
Otu00549	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	Cetobacterium_unclassified
Otu00556	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	Kineosporia	Kineosporia_rhamnosa
Otu00557	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus_paracasei
Otu00559	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_jejuensis
Otu00561	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_aromaticivorans
Otu00564	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_caeni
Otu00567	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XI	Anaerococcus	Anaerococcus_unclassified
Otu00568	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XI	Parvimonas	Parvimonas_micra
Otu00569	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	Enterococcus_aquimarinus
Otu00573	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	Caulobacter_vibrioides

Otu00575	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_tiangeerense
Otu00578	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	Methylobacterium_adhaesivum
Otu00581	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_alluvionis
Otu00589	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_parvum
Otu00592	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Allisonella	Allisonella_histaminiformans
Otu00597	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_aurantiaca
Otu00601	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_phyllosphaerae
Otu00603	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_aerolata
Otu00614	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella	Shinella_curvata
Otu00620	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_duraquae
Otu00621	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Rivicola	Rivicola_unclassified
Otu00622	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_psychrotolerans
Otu00624	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	Bifidobacterium_stercoris
Otu00626	Actinobacteria	Actinobacteria	Actinomycetales	Nakamurellaceae	Nakamurella	Nakamurella_unclassified
Otu00627	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_alluvionis
Otu00629	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_lacusfryxellense
Otu00630	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	Arcobacter_unclassified
Otu00631	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Slackia	Slackia_isoflavoniconvertens
Otu00632	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria	Neisseria_perflava
Otu00634	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_rigui
Otu00636	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_nipponensis
Otu00642	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Nocardia	Nocardia_coeliaca
Otu00644	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu00645	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI	Clostridium_hiranonis
Otu00648	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans	Limnohabitans_planktonicus
Otu00651	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_sulfureus
Otu00653	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Parapusillimonas	Parapusillimonas_unclassified

Otu00654	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_rigui
Otu00656	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00655	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Reyranella	Reyranella	Reyranella_massiliensis
Otu00660	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	Devosia_limi
Otu00664	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_vitis
Otu00663	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_oligophenolica
Otu00665	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Rhizobacter	Rhizobacter_unclassified
Otu00670	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_zoogloeoides
Otu00677	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	Corynebacterium_unclassified
Otu00678	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Vitellibacter	Vitellibacter_unclassified
Otu00679	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_steynii
Otu00681	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Psychrobacter	Psychrobacter_fulvigenes
Otu00688	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_noncentrifugens
Otu00691	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Elizabethkingia	Elizabethkingia_miricola
Otu00692	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_baumannii
Otu00695	Actinobacteria	Actinobacteria	Solirubrobacterales	Patulibacteraceae	Patulibacter	Patulibacter_unclassified
Otu00700	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	Streptococcus_alactolyticus
Otu00702	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_arthrosphaerae
Otu00705	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	Acidovorax_defluvii
Otu00708	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Allofustis	Allofustis_unclassified
Otu00711	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudorhodobacter	Pseudorhodobacter_collinsensis
Otu00713	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_unclassified
Otu00717	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Haliscomenobacter_unclassified
Otu00722	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00723	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_hamtensis
Otu00725	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_branchiarum
Otu00726	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	Carnobacterium_viridans

Otu00727	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Megasphaera	Megasphaera_indica
Otu00728	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimonas	Petrimonas_unclassified
Otu00730	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_segetis
Otu00733	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Hoeflea	Hoeflea_alexandrii
Otu00734	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_soyangensis
Otu00737	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_rigui
Otu00738	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_aerolata
Otu00740	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_duraquae
Otu00743	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	Devosia_chinhatensis
Otu00748	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_sinopsychrotolerans
Otu00752	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_unclassified
Otu00754	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_taiwanensis
Otu00756	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_faeni
Otu00757	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Simplicispira	Simplicispira_unclassified
Otu00765	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	Carnobacterium_gallinarum
Otu00766	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_algicola
Otu00767	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_naphthalenivorans
Otu00771	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_unclassified
Otu00775	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu00776	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVa	Clostridium XlVa_unclassified
Otu00777	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Parapusillimonas	Parapusillimonas_unclassified
Otu00778	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium	Mesorhizobium_amorphae
Otu00779	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Clostridium XVIII	Clostridium_spiroforme
Otu00782	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Salinibacterium	Salinibacterium_unclassified
Otu00786	Candidatus Saccharibacteria	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis_unclassified

Otu00790	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_algicola
Otu00791	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Niastella	Niastella_unclassified
Otu00794	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_alluvionis
Otu00795	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Atopostipes	Atopostipes_unclassified
Otu00799	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Romboutsia	Romboutsia_unclassified
Otu00801	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_chaponense
Otu00800	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00803	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_alluvionis
Otu00805	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Aeromicrobium	Aeromicrobium_fastidiosum
Otu00809	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_unclassified
Otu00811	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_rivuli
Otu00816	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_phocarum
Otu00817	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_taibaishanense
Otu00826	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00828	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Aerococcus	Aerococcus_urinaeequi
Otu00829	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Rivicola	Rivicola_unclassified
Otu00833	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	Planomicrobium_unclassified
Otu00835	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVb	Clostridium XlVb_unclassified
Otu00836	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Malikia	Malikia_spinosa
Otu00837	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudorhodobacter	Pseudorhodobacter_aquimaris
Otu00839	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Paenalcaligenes	Paenalcaligenes_unclassified
Otu00842	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_petrophilus
Otu00845	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Deefgea	Deefgea_rivuli
Otu00847	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	Sphingobacterium_unclassified
Otu00849	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu00856	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_luti
Otu00859	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_skierniewicense

Otu00860	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Galenea	Galenea_unclassified
Otu00869	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_seohonense
Otu00872	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Jahnella	Jahnella_unclassified
Otu00875	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Hephaestia	Hephaestia_caeni
Otu00879	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00877	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_fluoreni
Otu00882	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00887	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis	Asticcacaulis_benevestitus
Otu00886	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_psychrolimnae
Otu00888	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Agreia	Agreia_unclassified
Otu00895	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	Brevundimonas_mediterranea
Otu00896	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea	Zoogloea_unclassified
Otu00897	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Clavibacter	Clavibacter_michiganensis
Otu00900	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Georgfuchsia	Georgfuchsia_unclassified
Otu00902	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_punonensis
Otu00918	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Methylibium	Methylibium_petroleiphilum
Otu00920	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_vietnamense
Otu00922	Candidatus Saccharibacteria	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis_unclassified
Otu00921	Firmicutes	Clostridia	Thermoanaerobacterales	Thermoanaerobacteraceae	Thermanaeromonas	Thermanaeromonas_unclassified
Otu00928	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	Carnobacterium_inhibens
Otu00931	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu00933	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propioniciclava	Propioniciclava_unclassified
Otu00936	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_polytrichastri
Otu00940	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Xylophilus	Xylophilus_ampelinus
Otu00943	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Caenimonas	Caenimonas_koreensis

Otu00941	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_jejuensis
Otu00945	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu00951	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu00954	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_guangdongensis
Otu00952	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Aquihabitans	Aquihabitans_daechungensis
Otu00956	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_litoralis
Otu00958	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Clostridium XVIII	Clostridium XVIII_unclassified
Otu00957	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu00961	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_humicola
Otu00960	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter	Oscillibacter_unclassified
Otu00963	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu00965	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	Methylobacterium_adhaesivum
Otu00966	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pseudosphingobacterium	Pseudosphingobacterium_unclassified
Otu00967	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium	Sphingobium_baderi
Otu00971	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_johnsonii
Otu00973	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_nipponensis
Otu00978	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_stercoris
Otu00980	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	Devosia_glacialis
Otu00983	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_bauzanensis
Otu00986	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Oligella	Oligella_ureolytica
Otu00990	Bacteroidetes	Cytophagia	Cytophagales	Chryseolinea	Chryseolinea	Chryseolinea_unclassified
Otu00996	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Giesbergeria	Giesbergeria_kuznetsovii
Otu00997	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum	Ochrobactrum_unclassified
Otu01000	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Psychrobacter	Psychrobacter_lutiphocae
Otu00998	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_unclassified
Otu01002	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Ferruginibacter	Ferruginibacter_lapsinanis
Otu01001	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_fryxellicola

Otu01007	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Pseudolabrys	Pseudolabrys_unclassified
Otu01006	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Thermomonas	Thermomonas_brevis
Otu01014	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	Comamonas_guangdongensis
Otu01018	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Plantibacter	Plantibacter_flavus
Otu01017	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Serpens	Serpens_unclassified
Otu01023	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Butyricimonas	Butyricimonas_virosa
Otu01022	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	Planomicrobium_unclassified
Otu01027	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_limicola
Otu01026	Actinobacteria	Actinobacteria	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	Solirubrobacter_unclassified
Otu01028	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Pelosinus	Pelosinus_unclassified
Otu01030	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Lysinimonas	Lysinimonas_kribbensis
Otu01033	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_resinovorum
Otu01036	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	Sporosarcina_unclassified
Otu01038	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_aahli
Otu01045	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium	Sphingobium_yanoikuyae
Otu01047	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_branchiarum
Otu01049	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_svalbardensis
Otu01050	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01057	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Arenitalea	Arenitalea_unclassified
Otu01060	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium sensu stricto_unclassified
Otu01052	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Levilinea	Levilinea_unclassified
Otu01055	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_hominis
Otu01063	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Mucinivorans	Mucinivorans_unclassified
Otu01067	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_hercynium
Otu01069	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter	Oscillibacter_unclassified
Otu01072	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_terrigena
Otu01071	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_unclassified

Otu01074	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Polymorphobacter	Polymorphobacter_multimanifer
Otu01077	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	Devosia_soli
Otu01078	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiracea_incertae_sedis	Lachnospiracea_incertae_sedis_unclassified
Otu01081	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	Faecalibacterium_prausnitzii
Otu01083	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_echinoides
Otu01086	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_unclassified
Otu01089	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	Sporosarcina_siberiensis
Otu01090	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_araucananum
Otu01094	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Alkanindiges	Alkanindiges_unclassified
Otu01097	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium sensu stricto_unclassified
Otu01096	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_desiccabilis
Otu01098	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter	Leucobacter_exalbidus
Otu01099	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_unclassified
Otu01103	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_jejuense
Otu01108	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	Arcobacter_defluvii
Otu01109	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	Caulobacter_fusiformis
Otu01104	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Salinibacterium	Salinibacterium_unclassified
Otu01113	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus	Paracoccus_marinus
Otu01115	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_unclassified
Otu01117	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Cellvibrio	Cellvibrio_gandavensis
Otu01121	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Chitinophaga	Chitinophaga_oryziterrae
Otu01122	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_zoogloeoides
Otu01125	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Arcicella	Arcicella_unclassified
Otu01124	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_resistens
Otu01129	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Cellvibrio	Cellvibrio_diazotrophicus
Otu01128	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Sulfurisoma	Sulfurisoma_unclassified

Otu01133	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Dongia	Dongia_unclassified
Otu01132	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Frigoribacterium	Frigoribacterium_unclassified
Otu01131	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	Planomicrobium_unclassified
Otu01134	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Pseudarcicella	Pseudarcicella_unclassified
Otu01130	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Zhouia	Zhouia_unclassified
Otu01136	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	Diplorickettsia	Diplorickettsia_unclassified
Otu01137	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Micromonospora	Micromonospora_olivasterospora
Otu01143	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu01149	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Coprobacillus	Coprobacillus_unclassified
Otu01148	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Faecalicoccus	Faecalicoccus_unclassified
Otu01152	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Bacteriovorax	Bacteriovorax_unclassified
Otu01151	Acidobacteria	Acidobacteria_Gp4	Gp4	Gp4	Gp4	Gp4_unclassified
Otu01157	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_unclassified
Otu01154	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu01161	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01162	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	Kineococcus	Kineococcus_unclassified
Otu01159	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera	Thauera_terpenica
Otu01163	Candidatus Saccharibacteria	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis_unclassified
Otu01166	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Marmoricola	Marmoricola_aequoreus
Otu01169	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	Lactococcus_lactis
Otu01181	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Frigoribacterium	Frigoribacterium_unclassified
Otu01179	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	Sporosarcina_unclassified
Otu01184	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	Aeromonas_dhakensis
Otu01185	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_myungsuense
Otu01183	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01189	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Atopostipes	Atopostipes_unclassified

Otu01191	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_luteum
Otu01193	Proteobacteria	Deltaproteobacteria	Myxococcales	Labilitrichaceae	Labilithrix	Labilithrix_unclassified
Otu01203	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Aquabacterium	Aquabacterium_citratiphilum
Otu01201	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus_unclassified
Otu01207	Actinobacteria	Actinobacteria	Actinomycetales	Demequinaceae	Demequina	Demequina_aurantiaca
Otu01204	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Herbiconiux	Herbiconiux_unclassified
Otu01213	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	Enterococcus_cecorum
Otu01215	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_unclassified
Otu01218	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus_argenteus
Otu01216	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus	Trichococcus_palustris
Otu01221	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_keumense
Otu01219	Acidobacteria	Acidobacteria_Gp6	Gp6	Gp6	Gp6	Gp6_unclassified
Otu01222	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Rhodopila	Rhodopila_unclassified
Otu01226	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Sulfurimonas	Sulfurimonas_unclassified
Otu01228	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	Prevotella_stercorea
Otu01232	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Lapillicoccus	Lapillicoccus_unclassified
Otu01236	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_panacis
Otu01241	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01239	Actinobacteria	Actinobacteria	Solirubrobacterales	Patulibacteraceae	Patulibacter	Patulibacter_unclassified
Otu01242	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu01243	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_indicus
Otu01244	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_crusticola
Otu01248	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_tagluense
Otu01247	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_aquatilis
Otu01249	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01250	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	Methylobacterium_bullatum

Otu01252	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Mariniflexile	Mariniflexile_unclassified
Otu01256	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Piscinibacter	Piscinibacter_aquaticus
Otu01255	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu01258	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu01254	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	Weissella_hellenica
Otu01260	Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	Aureimonas	Aureimonas_unclassified
Otu01266	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVa	Clostridium_asparagiforme
Otu01265	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_noncentrifugens
Otu01259	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Leuconostoc	Leuconostoc_mesenteroides
Otu01268	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Anoxybacillus	Anoxybacillus_tepidamans
Otu01267	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_russicus
Otu01269	Cyanobacteria/ Chloroplast	Chloroplast	Chloroplast	Chloroplast	Cryptomonadaceae	Cryptomonadaceae_unclassified
Otu01271	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_hercynium
Otu01281	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Arcicella	Arcicella_unclassified
Otu01272	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Giesbergeria	Giesbergeria_unclassified
Otu01275	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_paludis
Otu01280	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Oryzihumus	Oryzihumus_unclassified
Otu01274	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu01279	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Salinirepens	Salinirepens_unclassified
Otu01276	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	Stenotrophomonas_rhizophila
Otu01282	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Alkanindiges	Alkanindiges_unclassified
Otu01284	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01289	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Taibaiella	Taibaiella_unclassified
Otu01294	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Marinospirillum	Marinospirillum_unclassified
Otu01297	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Advenella	Advenella_alkanexedens
Otu01299	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_ginsengisoli

Otu01296	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_agri
Otu01308	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	Nocardioides_unclassified
Otu01312	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Collimonas	Collimonas_pratensis
Otu01315	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis	Asticcacaulis_biprosthecium
Otu01320	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_antarcticus
Otu01319	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Holdemania	Holdemania_massiliensis
Otu01325	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_unclassified
Otu01323	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingorhabdus	Sphingorhabdus_arenilitoris
Otu01330	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_liquefaciens
Otu01329	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XI	Tissierella	Tissierella_unclassified
Otu01332	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_nipponensis
Otu01335	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_westerhofensis
Otu01339	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea	Bosea_massiliensis
Otu01348	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Arcticibacter	Arcticibacter_unclassified
Otu01345	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Erythrobacter	Erythrobacter_atlanticus
Otu01347	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacterium	Phyllobacterium_trifolii
Otu01346	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_cauense
Otu01353	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01354	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01355	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_deceptionensis
Otu01356	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Psychrobacter	Psychrobacter_sanguinis
Otu01357	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_unclassified
Otu01359	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_araucananum
Otu01360	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu01361	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola	Fluviicola_unclassified
Otu01362	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	Sporosarcina_unclassified
Otu01369	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_zoogloeoides

Otu01371	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_nutrimenti
Otu01370	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Rhizorhapis	Sphingomonas_changbaiensis
Otu01378	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_hydrogenotrophica
Otu01387	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium sensu stricto_unclassified
Otu01376	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_unclassified
Otu01385	Proteobacteria	Betaproteobacteria	Sulfuricellales	Sulfuricellaceae	Sulfuricella	Sulfuricella_unclassified
Otu01392	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu01388	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Ferruginibacter	Ferruginibacter_unclassified
Otu01390	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Simplicispira	Simplicispira_metamorpha
Otu01398	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria_incertae_sedis	Eionea	Eionea	Eionea_unclassified
Otu01397	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	Planomicrobium_unclassified
Otu01406	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_arilaitensis
Otu01407	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas	Dysgonomonas_unclassified
Otu01415	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Aquihabitans	Aquihabitans_unclassified
Otu01416	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	Diplorickettsia	Diplorickettsia_unclassified
Otu01410	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01411	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes	Rhodoplanes_unclassified
Otu01417	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_zoogloeoides
Otu01418	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_flevense
Otu01424	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Povalibacter	Povalibacter_unclassified
Otu01426	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Haematobacter	Haematobacter_unclassified
Otu01440	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_hominis
Otu01436	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_unclassified
Otu01439	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_lividum
Otu01437	Firmicutes	Bacilli	Bacillales	Thermoactinomycetaceae 1	Lihuaxuella	Lihuaxuella_unclassified
Otu01431	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_qilianensis

Otu01442	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	Aquaspirillum_arcticum
Otu01446	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01441	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_unclassified
Otu01445	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea	Zoogloea_oleivorans
Otu01452	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Byssovorax	Byssovorax_unclassified
Otu01457	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	Paludibacter_unclassified
Otu01463	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Ferribacterium	Ferribacterium_unclassified
Otu01461	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_algicola
Otu01462	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_petrophilus
Otu01459	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_flavum
Otu01460	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_unclassified
Otu01468	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter	Leucobacter_unclassified
Otu01467	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_naphthalenivorans
Otu01469	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium	Sphingobium_qiguonii
Otu01476	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	Devriesea	Devriesea_unclassified
Otu01478	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Emticicia	Emticicia_oligotrophica
Otu01477	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Ferribacterium	Ferribacterium_unclassified
Otu01472	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Haliscomenobacter_unclassified
Otu01475	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Sphaerotilus	Sphaerotilus_montanus
Otu01487	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_unclassified
Otu01481	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pseudorhodoferax	Pseudorhodoferax_unclassified
Otu01494	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Enterorhabdus	Enterorhabdus_unclassified
Otu01501	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides_unclassified
Otu01500	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_unclassified
Otu01502	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	Methylobacterium_longum
Otu01495	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	Nitritalea	Nitritalea_unclassified
Otu01497	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma	Spirosoma_unclassified

Otu01510	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_lwoffii
Otu01506	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01511	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_unclassified
Otu01504	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	Phenylobacterium_lituiforme
Otu01505	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Porphyrobacter	Porphyrobacter_donghaensis
Otu01517	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Mitsuokella	Mitsuokella_multacida
Otu01513	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_soyangensis
Otu01526	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01525	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	Hyphomicrobium_denitrificans
Otu01527	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_unclassified
Otu01528	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	Dechloromonas_unclassified
Otu01535	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_harbinensis
Otu01536	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Salinibacterium	Salinibacterium_unclassified
Otu01548	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_unclassified
Otu01544	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Simplicispira	Simplicispira_psychrophila
Otu01540	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_hengshuiensis
Otu01559	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_albensis
Otu01554	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_unclassified
Otu01558	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu01556	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Flectobacillus	Flectobacillus_lacus
Otu01552	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_lacticum
Otu01555	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Naasia	Naasia_unclassified
Otu01551	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ramlibacter	Ramlibacter_ginsenosidimutans
Otu01557	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Runella	Runella_unclassified
Otu01561	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Bellilinea	Bellilinea_unclassified
Otu01563	Bacteroidetes	Cytophagia	Cytophagales	Chryseolinea	Chryseolinea	Chryseolinea_unclassified
Otu01564	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas	Dysgonomonas_unclassified

Otu01568	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	Brevundimonas_unclassified
Otu01566	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter	Geobacter_psychrophilus
Otu01565	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_unclassified
Otu01569	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas_caeni
Otu01582	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium sensu stricto_unclassified
Otu01579	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_bowmanii
Otu01577	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_hamtensis
Otu01584	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01576	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Sulfurisoma	Sulfurisoma_unclassified
Otu01589	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_jeonii
Otu01588	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu01592	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Pseudenhygromyxa	Pseudenhygromyxa_unclassified
Otu01598	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01594	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Galbibacter	Galbibacter_unclassified
Otu01596	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Xanthobacter	Xanthobacter_autotrophicus
Otu01614	Actinobacteria	Actinobacteria	Solirubrobacterales	Conexibacteraceae	Conexibacter	Conexibacter_unclassified
Otu01615	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_rivuli
Otu01624	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_utahensis
Otu01634	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Yersiniaceae	Ewingella	Ewingella_americana
Otu01629	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_swingsii
Otu01637	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pseudorhodoferax	Pseudorhodoferax_unclassified
Otu01642	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Cytophaga	Cytophaga_hutchinsonii
Otu01639	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_unclassified
Otu01641	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Eoetvoesia	Eoetvoesia_caeni
Otu01647	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_cheniae
Otu01646	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified

Otu01638	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Piscinibacter	Piscinibacter_aquaticus
Otu01655	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	Corynebacterium_glutamicum
Otu01649	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Crenotalea	Crenotalea_unclassified
Otu01653	Firmicutes	Bacilli	Bacillales	Planococcaceae	Jeotgalibacillus	Jeotgalibacillus_unclassified
Otu01658	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteibacter	Luteibacter_yeojuensis
Otu01654	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_incertae_sedis	Rhizomicrobium	Rhizomicrobium	Rhizomicrobium_unclassified
Otu01666	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_unclassified
Otu01669	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01677	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Flectobacillus	Flectobacillus_unclassified
Otu01672	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_kyungheensis
Otu01674	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Reyranella	Reyranella	Reyranella_terrae
Otu01671	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis	Sphingopyxis_italica
Otu01682	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Subtercola	Subtercola_unclassified
Otu01687	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Cytophaga	Cytophaga_unclassified
Otu01692	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Haliscomenobacter_unclassified
Otu01684	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Herbiconiux	Herbiconiux_unclassified
Otu01690	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_panipatense
Otu01688	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	Weissella_paramesenteroides
Otu01701	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Ferribacterium	Ferribacterium_unclassified
Otu01702	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01700	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylotenera	Methylotenera_versatilis
Otu01706	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Pelosinus	Pelosinus_fermentans
Otu01725	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Agreia	Agreia_unclassified
Otu01722	Firmicutes	Bacilli	Bacillales	Planococcaceae	Caryophanon	Caryophanon_tenue
Otu01730	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_aerolatum
Otu01728	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Oxalobacter	Oxalobacter_unclassified

Otu01733	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionicimonas	Propionicimonas_unclassified
Otu01737	Armatimonadetes	Chthonomonadetes	Chthonomonadales	Chthonomonadaceae	Chthonomonas/Armatimonadetes_gp3	Chthonomonas/Armatimonadetes_gp3_unclassified
Otu01741	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01738	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	Phenylobacterium_zucineum
Otu01748	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_hercynium
Otu01747	Actinobacteria	Actinobacteria	Actinomycetales	Jonesiaceae	Jonesia	Jonesia_unclassified
Otu01751	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_calamicampi
Otu01758	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	Paludibacter_unclassified
Otu01746	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus	Paracoccus_alcaliphilus
Otu01759	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Salinispira	Salinispira_unclassified
Otu01775	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_succinicans
Otu01770	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01773	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Georgfuchsia	Georgfuchsia_unclassified
Otu01767	Acidobacteria	Acidobacteria_Gp5	Gp5	Gp5	Gp5	Gp5_unclassified
Otu01768	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Zhouia	Zhouia_unclassified
Otu01782	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	Cetobacterium_unclassified
Otu01779	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Flaviflexus	Flaviflexus_unclassified
Otu01784	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_parvum
Otu01794	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	Aeromonas_bestiarum
Otu01796	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	Devosia_unclassified
Otu01788	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylotenera	Methylotenera_versatilis
Otu01789	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_jejuensis
Otu01807	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Atopostipes	Atopostipes_suicloacalis
Otu01798	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_chaponense
Otu01805	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Peredibacter	Peredibacter_unclassified
Otu01804	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Sphaerochaeta	Sphaerochaeta_unclassified

Otu01817	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	Caulobacter_unclassified
Otu01812	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavihumibacter	Flavihumibacter_unclassified
Otu01821	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Marmoricola	Marmoricola_aequoreus
Otu01814	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_lentum
Otu01815	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_oligophenolica
Otu01826	Acidobacteria	Acidobacteria_Gp6	Gp6	Gp6	Gp6	Gp6_unclassified
Otu01831	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ottowia	Ottowia_unclassified
Otu01829	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_boryungensis
Otu01852	Bacteroidetes	Cytophagia	Cytophagales	Chryseolinea	Chryseolinea	Chryseolinea_unclassified
Otu01851	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	Corynebacterium_faecale
Otu01838	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu01849	Actinobacteria	Actinobacteria	Gaiellales	Gaiellaceae	Gaiella	Gaiella_occulta
Otu01836	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	Legionella_unclassified
Otu01864	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_oxydans
Otu01873	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	Bacillus_unclassified
Otu01860	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	Comamonas_jiangduensis
Otu01876	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_unclassified
Otu01874	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_ginsengisoli
Otu01869	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01867	Actinobacteria	Actinobacteria	Gaiellales	Gaiellaceae	Gaiella	Gaiella_unclassified
Otu01871	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Sphaerochaeta	Sphaerochaeta_globosa
Otu01858	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_indica
Otu01870	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Subtercola	Subtercola_boreus
Otu01896	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Emticicia	Emticicia_paludis
Otu01893	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01891	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Jahnella	Jahnella_unclassified
Otu01884	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_unclassified

Otu01886	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Pelagibacterium	Pelagibacterium_unclassified
Otu01895	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_huautlense
Otu01900	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	Hyphomicrobium_unclassified
Otu01911	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Jahnella	Jahnella_unclassified
Otu01902	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter	Leucobacter_unclassified
Otu01905	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylotenera	Methylotenera_versatilis
Otu01899	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_koreensis
Otu01907	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Rhodopseudomonas	Rhodopseudomonas_palustris
Otu01922	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Arcicella	Arcicella_rigui
Otu01925	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Eisenbergiella	Eisenbergiella_unclassified
Otu01932	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Parapusillimonas	Parapusillimonas_unclassified
Otu01921	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Pelolinea	Pelolinea_unclassified
Otu01942	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_hominis
Otu01947	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu01968	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Ferruginibacter	Ferruginibacter_alkalilentus
Otu01979	Actinobacteria	Actinobacteria	Actinomycetales	Jonesiaceae	Jonesia	Jonesia_unclassified
Otu01976	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylotenera	Methylotenera_unclassified
Otu01969	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_psychrotolerans
Otu01957	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	Nitritalea	Nitritalea_unclassified
Otu01964	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu01954	Candidatus Saccharibacteria	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis_unclassified
Otu01958	Firmicutes	Bacilli	Bacillales	Thermoactinomycetaceae 1	Thermoflavimicrobium	Thermoflavimicrobium_unclassified
Otu01972	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_terreum
Otu01975	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Zhouia	Zhouia_unclassified
Otu01995	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerostipes	Anaerostipes_hadrus

Otu01997	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_unclassified
Otu01989	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium sensu stricto_unclassified
Otu01981	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01991	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu01994	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	Legionella_unclassified
Otu01982	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	Nocardioides_alpinus
Otu01986	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Noviherbaspirillum	Noviherbaspirillum_suwonense
Otu02020	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Basilea	Basilea_unclassified
Otu02023	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_tagluense
Otu02009	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Micromonospora	Micromonospora_sonneratiae
Otu02013	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ottowia	Ottowia_unclassified
Otu02008	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Rhizorhabdus	Rhizorhabdus_unclassified
Otu02022	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Sulfuricurvum	Sulfuricurvum_unclassified
Otu02035	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Alkanindiges	Alkanindiges_unclassified
Otu02028	Acidobacteria	Acidobacteria_Gp1	Bryocella	Bryocella	Bryocella	Bryocella_unclassified
Otu02034	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Ornatilinea	Ornatilinea_unclassified
Otu02040	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Schumannella	Schumannella_unclassified
Otu02048	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Humitalea	Humitalea_unclassified
Otu02046	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Jannaschia	Jannaschia_unclassified
Otu02057	Chloroflexi	Chloroflexia	Kallotenuales	Kallotenuaceae	Kallotenue	Kallotenue_unclassified
Otu02052	Candidatus Saccharibacteria	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis_unclassified
Otu02060	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Verrucosispora	Verrucosispora_maris
Otu02078	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis	Asticcacaulis_biprosthecium
Otu02073	Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	Dietzia	Dietzia_alimentaria
Otu02067	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified

Otu02076	Actinobacteria	Actinobacteria	Gaiellales	Gaiellaceae	Gaiella	Gaiella_unclassified
Otu02062	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter	Leucobacter_unclassified
Otu02070	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Marinospirillum	Marinospirillum_unclassified
Otu02064	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_aromaticivorans
Otu02066	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_unclassified
Otu02065	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudorhodobacter	Pseudorhodobacter_unclassified
Otu02074	Actinobacteria	Actinobacteria	Actinomycetales	Streptosporangiaceae	Thermocatellispora	Thermocatellispora_unclassified
Otu02080	Acidobacteria	Acidobacteria_Gp6	Gp6	Gp6	Gp6	Gp6_unclassified
Otu02093	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Longilinea	Longilinea_unclassified
Otu02094	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides	Myroides_unclassified
Otu02092	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_aromaticivorans
Otu02088	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_capsulatum
Otu02112	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Aquabacterium	Aquabacterium_unclassified
Otu02110	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Asanoa	Asanoa_siamensis
Otu02107	Acidobacteria	Acidobacteria_Gp3	Candidatus Solibacter	Candidatus Solibacter	Candidatus Solibacter	Candidatus Solibacter_unclassified
Otu02109	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	Corynebacterium_stationis
Otu02108	Actinobacteria	Actinobacteria	Actinomycetales	Cryptosporangiaceae	Cryptosporangium	Cryptosporangium_unclassified
Otu02102	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	Faecalibacterium_prausnitzii
Otu02116	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Hafnia	Hafnia_unclassified
Otu02123	Bacteroidetes	Cytophagia	Cytophagales	Flammeovirgaceae	Imperialibacter	Imperialibacter_unclassified
Otu02124	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_incertae_sedis	Variibacter	Variibacter_unclassified
Otu02141	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Afipia	Afipia_unclassified
Otu02148	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu02143	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Iamia	Iamia_unclassified
Otu02137	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Methylibium	Methylibium_petroleiphilum

Otu02129	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu02165	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_algicola
Otu02160	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu02170	Acidobacteria	Acidobacteria_Gp6	Gp6	Gp6	Gp6	Gp6_unclassified
Otu02169	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Heliimonas	Heliimonas_unclassified
Otu02154	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_foliorum
Otu02155	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Oceanospirillum	Oceanospirillum_unclassified
Otu02164	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_alluvionis
Otu02159	Candidatus Saccharibacteria	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis_unclassified
Otu02181	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Aeromicrobium	Aeromicrobium_unclassified
Otu02186	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Cnuella	Cnuella_unclassified
Otu02192	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylomarinum	Methylomarinum_unclassified
Otu02182	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Undibacterium	Undibacterium_jejuense
Otu02195	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Zhouia	Zhouia_unclassified
Otu02212	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_indicus
Otu02211	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XIII	Anaerovorax	Anaerovorax_unclassified
Otu02217	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_unclassified
Otu02220	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	Corynebacterium_casei
Otu02216	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Jahnella	Jahnella_unclassified
Otu02209	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Methylibium	Methylibium_unclassified
Otu02215	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	Paludibacter_unclassified
Otu02218	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_giardinii
Otu02219	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Salinispira	Salinispira_unclassified
Otu02206	Actinobacteria	Thermoleophilia	Thermoleophilales	Thermoleophilaceae	Thermoleophilum	Thermoleophilum_unclassified
Otu02228	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_kyonggiensis

Otu02223	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_bowmanii
Otu02240	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_fluvii
Otu02229	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Ilumatobacter	Ilumatobacter_unclassified
Otu02227	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu02243	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Youngiibacter	Youngiibacter_unclassified
Otu02233	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Zhouia	Zhouia_unclassified
Otu02251	Acetothermia	Acetothermia_genera_incertae_sedis	Acetothermia_genera_incertae_sedis	Acetothermia_genera_incertae_sedis	Acetothermia_genera_incertae_sedis	Acetothermia_genera_incertae_sedis_unclassified
Otu02263	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	Arthrobacter_antarcticus
Otu02260	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Cnuella	Cnuella_unclassified
Otu02271	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Leptobacterium	Leptobacterium_unclassified
Otu02257	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter	Leucobacter_unclassified
Otu02265	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Rhizorhabdus	Rhizorhabdus_argentea
Otu02254	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	Sporosarcina_unclassified
Otu02276	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Aminobacter	Aminobacter_anthyllidis
Otu02287	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_psychrolimnae
Otu02301	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu02293	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans	Limnohabitans_parvus
Otu02289	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Marinospirillum	Marinospirillum_megaterium
Otu02282	Actinobacteria	Actinobacteria	Actinomycetales	Bogoriellaceae	Oceanitalea	Oceanitalea_unclassified
Otu02288	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu02330	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Arenimonas	Arenimonas_unclassified
Otu02321	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Cnuella	Cnuella_unclassified
Otu02315	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Friedmanniella	Friedmanniella_spumicola
Otu02310	Actinobacteria	Actinobacteria	Solirubrobacterales	Patulibacteraceae	Patulibacter	Patulibacter_unclassified
Otu02316	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Psychrosinus	Psychrosinus_unclassified
Otu02328	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Rivicola	Rivicola_unclassified

Otu02335	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Ferruginibacter	Ferruginibacter_unclassified
Otu02348	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Mobilitalea	Mobilitalea_unclassified
Otu02338	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	Nitrospira_unclassified
Otu02359	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu02354	Actinobacteria	Actinobacteria	Actinomycetales	Streptosporangiaceae	Thermocatellispora	Thermocatellispora_unclassified
Otu02350	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Youngiibacter	Youngiibacter_unclassified
Otu02367	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Agrococcus	Agrococcus_unclassified
Otu02366	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	Desulfatirhabdium	Desulfatirhabdium_unclassified
Otu02374	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_rivuli
Otu02373	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	Halomonas_desiderata
Otu02363	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans	Limnohabitans_unclassified
Otu02376	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_angelicae
Otu02385	Actinobacteria	Actinobacteria	Solirubrobacterales	Patulibacteraceae	Patulibacter	Patulibacter_unclassified
Otu02379	Firmicutes	Bacilli	Bacillales	Planococcaceae	Psychrobacillus	Psychrobacillus_unclassified
Otu02398	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Aeromicrobium	Aeromicrobium_flavum
Otu02407	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Aquihabitans	Aquihabitans_unclassified
Otu02404	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Catonella	Catonella_unclassified
Otu02415	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Tardiphaga	Tardiphaga_unclassified
Otu02421	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_octamycinicus
Otu02435	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chishuiella	Chishuiella_unclassified
Otu02433	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	Methylobacterium_mesophilicum
Otu02418	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Olsenella	Olsenella_unclassified
Otu02442	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Abiotrophia	Abiotrophia_defectiva
Otu02455	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Emticicia	Emticicia_fontis
Otu02463	Actinobacteria	Actinobacteria	Actinomycetales	Nakamurellaceae	Nakamurella	Nakamurella_unclassified
Otu02449	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Oerskovia	Oerskovia_unclassified
Otu02457	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Pleomorphomonas	Pleomorphomonas_oryzae

Otu02444	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu02441	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propioniciclava	Propioniciclava_unclassified
Otu02462	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_unclassified
Otu02476	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	Devosia_chinhatensis
Otu02503	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_unclassified
Otu02498	Actinobacteria	Actinobacteria	Actinomycetales	Beutenbergiaceae	Serinibacter	Serinibacter_unclassified
Otu02508	Proteobacteria	Alphaproteobacteria	Caulobacterales	Hyphomonadaceae	Marinicauda	Marinicauda_unclassified
Otu02531	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	Nocardioides_unclassified
Otu02510	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus	Paracoccus_alcaliphilus
Otu02559	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Corallibacter	Corallibacter_unclassified
Otu02589	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Cytophaga	Cytophaga_unclassified
Otu02570	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Gemmobacter	Gemmobacter_unclassified
Otu02563	Acidobacteria	Acidobacteria_Gp6	Gp6	Gp6	Gp6	Gp6_unclassified
Otu02554	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Longilinea	Longilinea_unclassified
Otu02588	Actinobacteria	Actinobacteria	Actinomycetales	Demequinaceae	Lysinimicrobium	Lysinimicrobium_unclassified
Otu02556	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium	Mycobacterium_hodleri
Otu02561	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Orientia	Orientia_unclassified
Otu02553	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_unclassified
Otu02562	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingorhabdus	Sphingomonas_jatrophae
Otu02592	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Bacteriovorax	Bacteriovorax_unclassified
Otu02611	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	Facklamia_unclassified
Otu02622	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Holdemanella	Holdemanella_biformis
Otu02596	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Iamia	Iamia_unclassified
Otu02597	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Magnetospira	Magnetospira_unclassified
Otu02608	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Methylibium	Methylibium_unclassified
Otu02594	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylocapsa	Methylocapsa_unclassified
Otu02617	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Mucinivorans	Mucinivorans_unclassified

Otu02602	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Polyangium	Polyangium_unclassified
Otu02614	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_asaccharolytica
Otu02610	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_koreensis
Otu02595	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XI	Tissierella	Tissierella_unclassified
Otu02642	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	Chryseobacterium_gregarium
Otu02645	Acidobacteria	Acidobacteria_Gp6	Gp6	Gp6	Gp6	Gp6_unclassified
Otu02666	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	Nocardioides_iriomotensis
Otu02636	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudorhodobacter	Pseudorhodobacter_unclassified
Otu02673	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_aerolata
Otu02659	Proteobacteria	Deltaproteobacteria	Myxococcales	Vulgatibacteraceae	Vulgatibacter	Vulgatibacter_unclassified
Otu02702	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Advenella	Advenella_faeciporci
Otu02722	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_unclassified
Otu02687	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium sensu stricto_unclassified
Otu02682	Actinobacteria	Actinobacteria	Actinomycetales	Demequinaceae	Demequina	Demequina_unclassified
Otu02711	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola	Fluviicola_unclassified
Otu02729	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiliaceae	Geofilum	Geofilum_unclassified
Otu02703	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Haliscomenobacter_unclassified
Otu02708	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_unclassified
Otu02681	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Psychrosinus	Psychrosinus_unclassified
Otu02679	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Rathayibacter	Rathayibacter_tritici
Otu02691	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Segetibacter	Segetibacter_unclassified
Otu02690	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Serpens	Serpens_unclassified
Otu02747	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_rivuli
Otu02753	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_swingsii
Otu02746	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu02763	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified

Otu02776	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Ilumatobacter	Ilumatobacter_unclassified
Otu02777	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_lacus
Otu02779	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_unclassified
Otu02756	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter	Oscillibacter_unclassified
Otu02770	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	Planomicrobium_flavidum
Otu02751	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu02745	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Rhodopila	Rhodopila_unclassified
Otu02789	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_durhamensis
Otu02810	Acidobacteria	Acidobacteria_Gp4	Blastocatella	Blastocatella	Blastocatella	Blastocatella_unclassified
Otu02811	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_unclassified
Otu02803	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	Clostridium_vincentii
Otu02790	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter	Leucobacter_aerolatus
Otu02787	Actinobacteria	Actinobacteria	Actinomycetales	Nakamurellaceae	Nakamurella	Nakamurella_multipartita
Otu02818	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Pseudenhygromyxa	Pseudenhygromyxa_unclassified
Otu02791	Firmicutes	Clostridia	Thermoanaerobacterales	Thermodesulfobiaceae	Thermodesulfobium	Thermodesulfobium_unclassified
Otu02834	Acidobacteria	Acidobacteria_Gp4	Blastocatella	Blastocatella	Blastocatella	Blastocatella_unclassified
Otu02849	Caldiserica	Caldisericia	Caldisericales	Caldisericaceae	Caldisericum	Caldisericum_unclassified
Otu02832	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Frigidibacter	Frigidibacter_unclassified
Otu02826	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Ideonella	Ideonella_unclassified
Otu02840	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Serpens	Serpens_unclassified
Otu02825	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Thiobacimonas	Thiobacimonas_unclassified
Otu02877	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_unclassified
Otu02886	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	Desulfatirhabdium	Desulfatirhabdium_unclassified
Otu02875	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus_coryniformis
Otu02912	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leucobacter	Leucobacter_unclassified
Otu02879	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_saidenbachensis

Otu02917	Actinobacteria	Actinobacteria	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	Solirubrobacter_unclassified
Otu02894	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Spirochaeta	Spirochaeta_unclassified
Otu02970	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu02944	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	Gemmatimonas_unclassified
Otu02928	Proteobacteria	Deltaproteobacteria	Myxococcales	Labilitrichaceae	Labilithrix	Labilithrix_unclassified
Otu02951	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Lacihabitans	Lacihabitans_unclassified
Otu02957	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylorosula	Methylorosula_unclassified
Otu02977	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	Microbacterium_hydrothermale
Otu02969	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	Paludibacter_propionicigenes
Otu02943	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_incertae_sedis	Phreatobacter	Phreatobacter_oligotrophus
Otu02954	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu02987	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Aquabacterium	Aquabacterium_unclassified
Otu03042	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Brumimicrobium	Brumimicrobium_unclassified
Otu02990	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_phyllosphaerae
Otu03017	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Jeotgalibaca	Jeotgalibaca_arthritidis
Otu03036	Chloroflexi	Caldilineae	Caldilineales	Caldilineaceae	Litorilinea	Litorilinea_unclassified
Otu02998	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_aquatica
Otu02978	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu03013	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Sulfuricurvum	Sulfuricurvum_unclassified
Otu03018	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Sulfuritalea	Sulfuritalea_unclassified
Otu03075	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Aspromonas	Aspromonas_unclassified
Otu03096	Firmicutes	Bacilli	Bacillales	Paenibacillaceae 1	Fontibacillus	Fontibacillus_unclassified
Otu03053	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_unclassified
Otu03065	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Lentilitoribacter	Lentilitoribacter_unclassified
Otu03047	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	Opitutus_unclassified

Otu03091	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Oxalobacter	Oxalobacter_unclassified
Otu03094	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Proteiniphilum	Proteiniphilum_unclassified
Otu03101	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_unclassified
Otu03142	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Aequorivita	Aequorivita_unclassified
Otu03158	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Butyricicoccus	Butyricicoccus_unclassified
Otu03149	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	Desulfovibrio_idahonensis
Otu03148	Proteobacteria	Alphaproteobacteria	Magnetococcales	Magnetococcaceae	Magnetococcus	Magnetococcus_unclassified
Otu03105	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Plesiocystis	Plesiocystis_pacifica
Otu03099	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	Sporosarcina_unclassified
Otu03232	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	Arcobacter_cibarius
Otu03172	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Dactylosporangium	Dactylosporangium_vinaceum
Otu03230	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_dankookense
Otu03229	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu03217	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	Gemmatimonas_unclassified
Otu03209	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_unclassified
Otu03210	Proteobacteria	Oligoflexia	Oligoflexales	Oligoflexaceae	Oligoflexus	Oligoflexus_unclassified
Otu03206	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Paucimonas	Paucimonas_unclassified
Otu03174	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Salinispira	Salinispira_unclassified
Otu03199	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Sphaerotilus	Sphaerotilus_montanus
Otu03198	Spirochaetes	Spirochaetia	Spirochaetales	Leptospiraceae	Turneriella	Turneriella_parva
Otu03177	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_incertae_sedis	Vasilyevaea	Vasilyevaea_enhydra
Otu03270	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Duganella	Duganella_phyllosphaerae
Otu03253	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_unclassified
Otu03245	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_alluvionis
Otu03364	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Dyadobacter	Dyadobacter_hamtensis
Otu03343	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Luedemannella	Luedemannella_unclassified

Otu03323	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylococcus	Methylococcus_unclassified
Otu03336	Candidatus Saccharibacteria	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis	Saccharibacteria_genera_incertae_sedis_unclassified
Otu03363	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma	Spirosoma_spitsbergense
Otu03424	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Acetanaerobacterium	Acetanaerobacterium_unclassified
Otu03410	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_wexlerae
Otu03388	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_plurextorum
Otu03437	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Ideonella	Ideonella_sakaiensis
Otu03369	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Jeotgalibaca	Jeotgalibaca_unclassified
Otu03440	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	Nocardioides_conyzicola
Otu03472	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu03491	Acidobacteria	Acidobacteria_Gp16	Gp16	Gp16	Gp16	Gp16_unclassified
Otu03525	Acidobacteria	Acidobacteria_Gp6	Gp6	Gp6	Gp6	Gp6_unclassified
Otu03534	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	Ruminococcus_unclassified
Otu03596	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_junii
Otu03577	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Blautia_unclassified
Otu03566	Bacteroidetes	Cytophagia	Cytophagales	Flammeovirgaceae	Fabibacter	Fabibacter_unclassified
Otu03580	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_unclassified
Otu03602	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Pelosinus	Pelosinus_unclassified
Otu03570	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Proteiniclasticum	Proteiniclasticum_unclassified
Otu03541	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_ferrireducens
Otu03563	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Salinibacterium	Salinibacterium_unclassified
Otu03599	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis	Sphingopyxis_unclassified
Otu03565	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Taibaiella	Taibaiella_unclassified
Otu03638	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus	Anaerotruncus_unclassified
Otu03680	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified

Otu03688	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	Fusobacterium_unclassified
Otu03675	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Gelidibacter	Gelidibacter_unclassified
Otu03674	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Haliscomenobacter_unclassified
Otu03708	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Desulfuromonadaceae	Malonomonas	Malonomonas_unclassified
Otu03670	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Marinospirillum	Marinospirillum_unclassified
Otu03690	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides	Nocardioides_gilvus
Otu03684	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_unclassified
Otu03695	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	Phenylobacterium_unclassified
Otu03615	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Proteiniphilum	Proteiniphilum_unclassified
Otu03627	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Salinispira	Salinispira_unclassified
Otu03782	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Aquihabitans	Aquihabitans_unclassified
Otu03748	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_algicola
Otu03755	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Haliscomenobacter_unclassified
Otu03794	Chloroflexi	Chloroflexia	Kallotenuales	Kallotenuaceae	Kallotenue	Kallotenue_unclassified
Otu03769	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Minicystis	Minicystis_unclassified
Otu03743	Actinobacteria	Actinobacteria	Actinomycetales	Nakamurellaceae	Nakamurella	Nakamurella_unclassified
Otu03721	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Roseicyclus	Roseicyclus_unclassified
Otu03716	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	Shewanella_profunda
Otu03899	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Altererythrobacter	Altererythrobacter_palmitatis
Otu03934	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Anaerocella	Anaerocella_unclassified
Otu03905	Armatimonadetes	Armatimonadia	Armatimonadales	Armatimonadaceae	Armatimonas/Armatimonadetes_gp1	Armatimonas/Armatimonadetes_gp1_unclassified
Otu03916	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Atopostipes	Atopostipes_unclassified
Otu03917	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Gemmobacter	Gemmobacter_unclassified
Otu03825	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Herbiconiux	Herbiconiux_unclassified
Otu03891	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Minicystis	Minicystis_unclassified
Otu03902	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	Mucilaginibacter_unclassified
Otu03820	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	Paludibacter_unclassified

Otu03896	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Parapedobacter	Parapedobacter_unclassified
Otu03894	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	Polaromonas_cryoconiti
Otu03892	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	Rhizobium_cauense
Otu03851	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XI	Tissierella	Tissierella_unclassified
Otu04023	Acidobacteria	Acidobacteria_Gp4	Aridibacter	Aridibacter	Aridibacter	Aridibacter_nitratireducens
Otu04043	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_terrae
Otu04029	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Pelosinus	Pelosinus_unclassified
Otu03937	Actinobacteria	Actinobacteria	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	Solirubrobacter_unclassified
Otu04007	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis	Sphingopyxis_witflariensis
Otu03947	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Sulfuritalea	Sulfuritalea_unclassified
Otu04049	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XI	Tissierella	Tissierella_unclassified
Otu04097	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Lacihabitans	Lacihabitans_unclassified
Otu04095	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Prosthecobacter	Prosthecobacter_unclassified
Otu04119	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Pseudoxanthomonas	Pseudoxanthomonas_spadix
Otu04066	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus_vitulinus
Otu04281	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Aquaspirillum	Aquaspirillum_putridiconchylium
Otu04319	Chloroflexi	Ardenticatenia	Ardenticatenales	Ardenticatenaceae	Ardenticatena	Ardenticatena_unclassified
Otu04253	Cyanobacteria/ Chloroplast	Chloroplast	Chloroplast	Chloroplast	Chlorarachniophyceae	Chlorarachniophyceae_unclassified
Otu04214	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Cnuella	Cnuella_unclassified
Otu04235	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu04268	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Ilumatobacter	Ilumatobacter_unclassified
Otu04256	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	Nitrospira_unclassified
Otu04227	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_unclassified
Otu04237	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Plesiocystis	Plesiocystis_unclassified
Otu04322	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified

Otu04184	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Salinispira	Salinispira_unclassified
Otu04230	Lentisphaerae	Lentisphaeria	Victivallales	Victivallaceae	Victivallis	Victivallis_unclassified
Otu04345	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Arcicella	Arcicella_unclassified
Otu04457	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter	Paludibacter_unclassified
Otu04422	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Phytohabitans	Phytohabitans_unclassified
Otu04328	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Portibacter	Portibacter_unclassified
Otu04385	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Rhizobacter	Rhizobacter_bergeniae
Otu04392	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_incertae_sedis	Vasilyevaea	Vasilyevaea_enhydra
Otu04559	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Byssovorax	Byssovorax_unclassified
Otu04620	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Catonella	Catonella_unclassified
Otu04496	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	Haliscomenobacter_unclassified
Otu04571	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Ilumatobacter	Ilumatobacter_unclassified
Otu04579	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Oxalobacter	Oxalobacter_unclassified
Otu04599	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	Phaeodactylibacter	Phaeodactylibacter_unclassified
Otu04701	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Aquihabitans	Aquihabitans_unclassified
Otu04757	Cyanobacteria/ Chloroplast	Chloroplast	Chloroplast	Chloroplast	Chlorophyta	Chlorophyta_unclassified
Otu04758	Cyanobacteria/ Chloroplast	Chloroplast	Chloroplast	Chloroplast	Chlorophyta	Chlorophyta_unclassified
Otu04651	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Ferruginibacter	Ferruginibacter_alkalilentus
Otu04734	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu04646	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	Legionella_unclassified
Otu04759	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_rosa
Otu04756	Firmicutes	Bacilli	Bacillales	Paenibacillaceae 1	Paenibacillus	Paenibacillus_glacialis
Otu04726	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	Rhodoferax_ferrireducens
Otu04719	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_soli
Otu04864	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Anaerorhabdus	Anaerorhabdus_unclassified
Otu04832	Acidobacteria	Acidobacteria_Gp3	Gp3	Gp3	Gp3	Gp3_unclassified

Otu04948	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	Rhodobacter_unclassified
Otu05002	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Roseomonas	Roseomonas_unclassified
Otu05176	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	Dechloromonas_denitrificans
Otu05161	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibrio	Propionivibrio_unclassified
Otu05103	Proteobacteria	Deltaproteobacteria	Myxococcales	Sandaracinaceae	Sandaracinus	Sandaracinus_unclassified
Otu05156	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	Sorangium_unclassified
Otu05198	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Treponema	Treponema_unclassified
Otu05307	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Alkanindiges	Alkanindiges_unclassified
Otu05287	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavihumibacter	Flavihumibacter_unclassified
Otu05365	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavitalea	Flavitalea_unclassified
Otu05460	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Taibaiella	Taibaiella_unclassified
Otu05733	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Acinetobacter_unclassified
Otu05570	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	Actinoplanes_abujensis
Otu05639	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	Flavobacterium_unclassified
Otu05535	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces	Streptomyces_globisporus
Otu05953	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Byssovorax	Byssovorax_unclassified
Otu05799	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas	Dysgonomonas_unclassified
Otu05899	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	Pedobacter_unclassified
Otu06011	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Salinispira	Salinispira_unclassified
Otu06169	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	Janthinobacterium_svalbardensis
Otu06118	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	Pelomonas_unclassified
Otu06615	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	Phenylobacterium_aquaticum
Otu06785	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Rivicola	Rivicola_unclassified
Otu06606	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Taibaiella	Taibaiella_unclassified