

Identification of fallopian tube microbiota and its association with ovarian cancer: a prospective study of intraoperative swab collections from 187 patients

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Abstract

Investigating the human fallopian tube (FT) microbiota has significant implications for understanding the pathogenesis of ovarian cancer (OC). In this large prospective study, we collected swabs intraoperatively from the FT and other surgical sites as controls to profile the microbiota in the FT and to assess its relationship with OC. 81 OC and 106 non-cancer patients were enrolled and 1001 swabs were processed for 16S rRNA gene PCR and sequencing. We identified 84 bacterial species that may represent the FT microbiota and found a clear shift in the microbiota of the OC patients when compared to the non-cancer patients. Of the top 20 species that were most prevalent in the FT of OC patients, 60% were bacteria that predominantly reside in the gastrointestinal tract, while 30% normally reside in the mouth. Serous carcinoma had higher prevalence of almost all 84 FT bacterial species compared to the other OC subtypes. The clear shift in the FT microbiota in OC patients establishes the scientific foundation for future investigation into the role of these bacteria in the pathogenesis of ovarian cancer.

Summary

Introduction

Investigating the human fallopian tube (FT) microbiota has significant implications for understanding the pathogenesis of ovarian cancer (OC), pelvic inflammatory disease, and tubal ectopic pregnancy, as well as normal fertilization. Several studies have provided evidence that the FT may not be sterile, but rigorous controls are needed to assess the microbiota in low biomass samples. In this large prospective study, we collected swabs intraoperatively from the FT and other surgical sites as controls to profile the microbiota in the FT and to assess its relationship with OC.

Methods

We collected swabs from the cervix, FT, ovarian surfaces, and paracolic gutters of patients, and from laparoscopic ports and air in the operating room. Surgical indications included known or suspected ovarian cancers, risk-reducing salpingo-oophorectomies due to genetic risk, and benign gynecological disorders. DNA was extracted from the swabs and the bacterial concentrations were quantified using broad-range bacterial quantitative PCR. Bacterial composition was characterized using amplicon PCR targeting the V3–V4 hypervariable region of the 16S rRNA gene combined with next generation sequencing. Multiple negative controls and filtering approaches were used to differentiate FT microbiota from likely contaminant sequences. Presence of the bacterial taxa in both the cervical and FT sample set was required to identify ascending genital tract bacteria.

Results

A total of 81 ovarian cancer patients and 106 non-cancer patients were enrolled and 1001 swabs were processed. The bacterial concentrations of FT and ovarian surfaces averaged 2.5 copies of 16S rRNA genes/ μ l of DNA (standard deviation, SD 4.6), similar to the paracolic gutter and higher than the controls (p-value < 0.001). We identified 84 bacterial species that may represent the FT microbiota. After ranking the FT bacteria based on the prevalence difference, we found a clear shift in the microbiota of the OC patients when compared to the non-cancer patients. Of the top 20 species that were most prevalent in the FT of OC patients, 60% were bacteria that predominantly reside in the gastrointestinal tract, such as *Klebsiella*, *Faecalibacterium prausnitzii*, *Ruminiclostridium*, and *Roseburia*, while 30% normally reside in the mouth, such as *Streptococcus mitis*, *Corynebacterium simulans/striatum*, and *Dialister invisus*. On the contrary, vaginal bacterial species are more prevalent in the FT from non-cancer patients, representing 75% of the top 20 bacterial species that are most prevalent in non-cancer patients. Serous carcinoma had higher prevalence of almost all 84 FT bacterial species compared to the other OC subtypes.

Conclusion

In this large low biomass microbiota study using intraoperatively collected swabs, we identified a group of bacterial species that appear to reside in the FT across multiple participants. A higher prevalence of some of these bacterial species, especially those that normally reside outside the female genital tract, was noted in the FT from patients with OC, laying the scientific foundation to explore whether these bacteria may have a role in enhancing ovarian cancer risk.

eLife assessment

Little is known about the role of the microbiome alterations in epithelial ovarian cancer. This **important** and rigorous study carefully examined the microbiome composition of 1001 samples from close to 200 ovarian cancer cases and controls, and presents **compelling** evidence that the fallopian tube microbiota are perturbed in ovarian cancer patients. These insights are expected to fuel further exploration into translational opportunities stemming from these findings.

Introduction

The American Cancer Society estimates that in 2023, about 19,710 new cases of ovarian cancer (OC) will be diagnosed and about 13,270 individuals will die from OC in the United States(1). Unlike most other cancers, the mortality rate for OC has declined only slightly in the last 40 years due to lack of early diagnostic tests and effective treatments. The 5-year survival rate for all types of OC averages 49.7%, much lower than many other cancers(2). The high mortality rate of OC is linked to a lack of understanding of ovarian carcinogenesis and precursor lesions, contributing to the failure of early detection and late-stage diagnosis. Even the cell origins of various OC subtypes have not been fully appreciated until recently, with ovarian carcinoma representing a heterogeneous collection of cancer subtypes, each with distinct origins and molecular drivers(3). The Fallopian tube epithelium is the likely origin of most high-grade serous carcinomas, the most common subtype of ovarian carcinoma. There is a critical need for innovative research to illuminate the origins and pathogenesis of this deadly cancer, thus enabling targeted approaches for early detection, treatment and prevention.

Based on the epidemiological data, we propose a novel hypothesis for ovarian carcinogenesis: ascending infection with some genital tract bacteria leads to inflammation in the fallopian tubes and DNA damage to cells contributing to neoplastic transformation. The evidence supporting this hypothesis includes: 1) The fallopian tubes form a conduit between the lower genital tract and the pelvic cavity(4, 5). 2) Blocking the communication between the fallopian tubes and the environment, such as through tubal ligation or hysterectomy, results in lower ovarian cancer incidences(6-8). 3) Increased cellular and bacterial transit between the lower genital tract and the peritoneal cavity, as in endometriosis or pelvic inflammatory disease, is associated with increased ovarian cancer risks(9, 10). 4) Inhibiting ovulation through oral contraceptives may decrease ovarian cancer risks(11-13) by reducing the opportunities of incorporating ascending pathogens into the ovarian surface epithelium during ovulation-induced microtrauma. 5) The fallopian tubal epithelium has been implicated in recent studies as the site of origin for at least a substantial proportion of high-grade serous carcinomas(14-16). Furthermore, recent studies have demonstrated the presence of genital tract bacteria in the upper reproductive tract of women without known infections(17-20). Therefore, bacteria ascending from the lower genital tract may reside in the fallopian tubes and could induce a pro-inflammatory environment, which could influence neoplasia of the tubal or ovarian epithelium, with outcome dependent on species of bacteria, concentration, host factors, and duration of exposure.

Even though the above epidemiological studies have hinted to the possible connection between an altered microbiota in the fallopian tube and OC, few studies have examined this plausible hypothesis. Even the existence of a microbiota in the fallopian tube has not been convincingly demonstrated, in part due to the technical challenges of accessing this site in a sterile fashion. Several studies have provided evidence that the FT may not be sterile(21-23), but rigorous controls and large sample sizes are needed to assess the microbiota in low biomass samples. Here, we examine the fallopian tube microbiota from a large study population with collection of swabs in a sterile fashion in the operating room from both patients with ovarian cancer and those without. The fallopian tube bacterial concentrations and compositions were compared between two groups: women who are cancer free, and women who have any type of OC. We built in numerous controls in our study design to help differentiate likely contaminants from signal of a fallopian tube microbiome.

Results

Patient overview

A total of 187 patients who met the inclusion and exclusion criteria (see Methods) were enrolled in this study. The patient characteristics were different between the cancer and non-cancer patients in their age and menopausal status as summarized in **Table 1** [↗](#), with a higher percentage of the ovarian cancer patients in the older and postmenopausal groups. Most ovarian cancers were diagnosed in stage III and IV, with high grade serous carcinoma being the most common histology subtype (**Table 1** [↗](#)). Laparotomy was more commonly performed in ovarian cancer patients (**Table 1** [↗](#)).

Sample overview and low biomass study design

854 biological samples and 147 non-biological laboratory controls were analyzed for microbiota composition (**Supplemental Table 1** [↗](#)). 369 swabs were collected from the fallopian tube and ovarian surfaces. We also designed additional layers of controls and collected from multiple sites for comparisons. To assess the microbial environment in the operating room, 130 “air swabs” were collected from the patient’s room. To assess the cervical microbiota, 152 cervical swabs were collected before surgical area preparation and vaginal sterilization. To assess the skin microbiota that may be introduced into the pelvic cavity during laparoscopic port insertion, 81 “laparoscopic port swabs” were collected after the port insertion and before the start of operation. To assess the microbiota in the abdominal cavity away from the surgical or cancer sites, 122 “paracolic gutter swabs” were collected from the right paracolic gutter before the start of operation. To assess potential contamination of DNA extraction reagents, during each batch of DNA extractions we included “buffer controls” that contained only DNA extraction buffer without any swab or other DNA. These 36 “buffer controls” were processed and then sequenced together with the swab samples. To assess potential contamination during PCR and sequencing, we also included multiple no-template PCR controls that contained only PCR reagents and water without extracted DNA or buffer (n=111 total) during each sequencing run (**Supplemental Table 1** [↗](#)).

Bacterial concentration

The bacterial concentrations of FT and ovarian surfaces averaged 2.5 copies of 16S rRNA genes/ μ l of DNA (standard deviation, SD 4.6). Compared to the buffer controls and the OR air swabs, the FT and ovarian surface swabs contained higher concentrations of bacterial DNA than the controls (p-value < 0.001) (**Figure 1** [↗](#)) (**Supplemental Table 2** [↗](#)). As expected, the cervical swabs contained thousands of times higher bacterial concentrations than the fallopian tube and ovarian surface swabs (p-value < 0.001) (**Figure 1** [↗](#)). The bacterial concentrations from the paracolic gutter were similar to those from the FT and ovarian surface swabs (p-value = 0.11) (**Figure 1** [↗](#)) (**Supplemental Table 2** [↗](#)).

Fallopian tube microbiota analysis

In the raw sequencing analysis, 892 bacterial species were present with at least 100 reads in at least one sample. After a series of filtering as described in the Methods, 84 bacterial species were present in at least one cervical and one FT sample with over 100 reads while not present in non-biological and air swabs (**Supplemental Table 3** [↗](#)). These 84 bacterial species may represent the FT microbiota that are present in some women. When analyzing the 340 samples that contained at least one read of these 84 species, the PCA plot showed a high level of similarity between the fallopian tube/ovarian surface and the paracolic gutter swabs, indicating these species represent the shared microbiota community in the abdominal and pelvic cavity within each individual (**Figure 2A** [↗](#)). An overview of these 84 bacterial species and their presence in each fallopian tube/ovarian surface sample shows that some of the bacterial species are prevalent in multiple

	Ovarian Cancer Patients (n = 81)	Non-cancer Patients (n = 106)	P-value
Average Age at diagnosis	59.7	55.3	0.052
Average Age at surgery	59.6	51.6	<.001
Post-menopausal	62 (76.5%)	48 (45.3%)	<.001
Race			0.492
- Asian	7 (8.6%)	4 (3.8%)	
- Black	1 (1.2%)	3 (2.8%)	
- Other	6 (7.4%)	5 (4.7%)	
- White	64 (79.0%)	92 (86.80%)	
- Undisclosed	3 (3.7%)	2 (1.9%)	
Ethnicity			0.218
- Hispanic	3 (3.7%)	1 (0.9%)	
- Non-Hispanic	73 (90.1%)	102 (96.2%)	
- Undisclosed	5 (6.2%)	3 (2.8%)	
Cancer Stage at Surgery			N/A
1	19 (23.5%)	-	
2	6 (7.45%)	-	
3	39 (48.2%)	-	
4	16 (19.8%)	-	
NA	1 (1.2%)	-	
Cancer Grade			N/A
1	6 (7.4%)	-	
2	3 (3.7%)	-	
3	64 (79.0%)	-	
4	1 (1.2%)	-	
Tumor Histology Type			N/A
- adenocarcinoma	3	-	
- carcinosarcoma	1	-	
- clear cell	8	-	
- endometrioid	10	-	
- granulosa cell	1	-	
- mucinous	2	-	
- serous	53	-	
- transitional cell	1	-	
- borderline serous *	-	8	
- other	-	98	
Surgical type			<.001
- Laparotomy	75 (92.6%)	28 (26.4%)	

Table 1.

Demographic characteristics of participants.

* Borderline serous tumors are considered in the non-cancer category in this study due to the non-invasiveness of these tumors.

- Laparoscopy	3 (3.7%)	42 (39.6%)	
- Robotic assisted	3 (3.7%)	36 (34.0%)	
Pelvic washing			<.001
- Positive	43 (53.1%)	3 (2.8%)	
- Negative	22 (27.2%)	99 (93.4%)	
- NA	16 (19.8%)	4 (3.8%)	

Table 1. (continued)

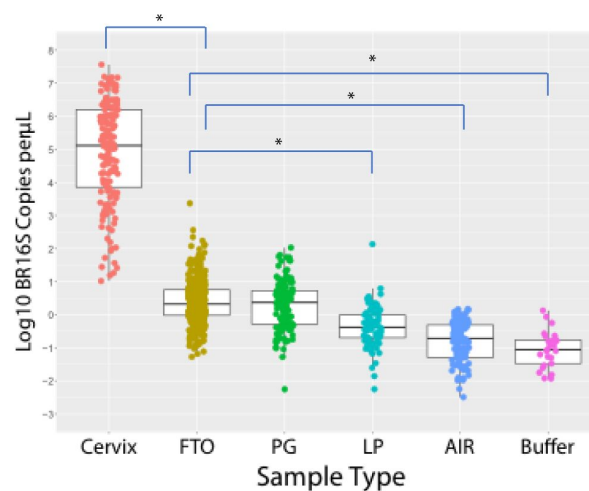


Figure 1.

Bacterial concentration of each swab sample and DNA extraction controls as measured by broad range 16S rRNA gene PCR.
 * p-value < 0.001, paired t-test, comparing each sample type with Fallopian tube/ovarian surface (FTO) samples. PG= Paracolic gutter; LP=Laparoscopic port.

individuals, such as *Klebsiella* or *Anaerococcus*, while others are only present in a few individuals, such as *Casaltella* or *BV-associated bacterium 2 (BVAB2)* (**Figure 3**). Clustering did not identify any obvious groupings by processing batch, cancer status, menopausal status or age for these putative FT bacterial species (**Figure 3**). The Shannon index was calculated for each sample to determine if there were differences in bacterial diversity. There were 726 bacterial species present in the cervix, FT/ovarian surface, and paracolic gutter after filtering out the bacteria present in no-template controls and DNA extraction controls (i.e. after step #5 in **Supplemental Table 3**). The cervical samples had lower Shannon diversity index than the FT/ovarian surface and paracolic gutter samples, while the non-cancer versus OC samples did not show any statistically significant difference in Shannon diversity within each sample type other than the cervical samples (**Figure 2B**).

Comparison between ovarian cancer and non-cancer microbiota

We compared the prevalence of each of these 84 bacterial species in patients with or without OC (**Figure 4**). Some bacteria, such as *Streptococcus parasanguinis* or *Neisseriaceae*, are more common in FT/ovarian surface samples from OC patients, while others (for example, *Ruminiclostridium*, *Dialister invisus*, or *Bacteroides dorei*) are only present in OC patients albeit overall representation was low. After ranking the FT bacteria based on the prevalence difference, we found a clear shift in the microbiota of the OC patients when compared to the non-cancer patients. Interestingly, 90% of the top 20 species that were most prevalent in the FT of OC patients were bacteria that predominantly exist outside the female reproductive tract. Among these, 12 (60%) predominantly reside in the gastrointestinal tract, such as *Klebsiella*, *Faecalibacterium prausnitzii*, *Ruminiclostridium*, and *Roseburia*. Six of the top 20 species (or 30%) normally reside in the mouth, such as *Streptococcus mitis*, *Corynebacterium simulans/striatum*, and *Dialister invisus*. On the contrary, vaginal bacterial species, such as *Corynebacterium amycolatum*, *Gardnerella*, and *Lactobacillus iners*, are more prevalent in the FT from non-cancer patients, representing 75% of the top 20 bacterial species that are most prevalent in non-cancer patients (**Figure 4**). This pattern was also consistently observed in the paracolic gutter samples which share the same low biomass environment of abdominal and pelvic cavity (**Figure 4**), suggesting that the above findings of FT microbiota were not random. When the histology subtypes were considered in the analysis, we found that the most common subtype, serous carcinoma, had higher prevalence of almost all 84 FT bacterial species compared to the other OC subtypes (**Figure 5**), indicating a higher level of perturbation in the FT microbiota in the serous carcinoma cases.

Since the majority of laparotomy cases were performed in OC patients and the non-OC cases were mostly performed with laparoscopy or robotic-assisted laparoscopy, we evaluated the influence of the surgical type on the FT microbiota profiles. When only laparotomy cases were considered in the comparison of OC versus non-cancer patients, the pattern of prevalence of majority of the 84 FT bacterial species was nearly identical as the overall comparison including all surgical types (**Supplemental Figure 1**). When laparotomy cases were compared to laparoscopic or robotic cases within the non-cancer patients, the prevalence of most of the 84 FT bacterial species was similar between the surgical types (**Supplemental Figure 2**). These analyses demonstrate that the differences we observed in the putative FT microbiota between OC and non-OC patients were not caused by the differences in surgical approaches.

Discussion

In this large prospective study, we analyzed the microbiota on swabs collected in a sterile fashion in the operating room during surgery, including from the FT and ovarian surface in 187 patients with or without OC, with the objective of determining a FT microbiome and identifying microbiome differences associated with OC. After detailed analyses and filtering out likely

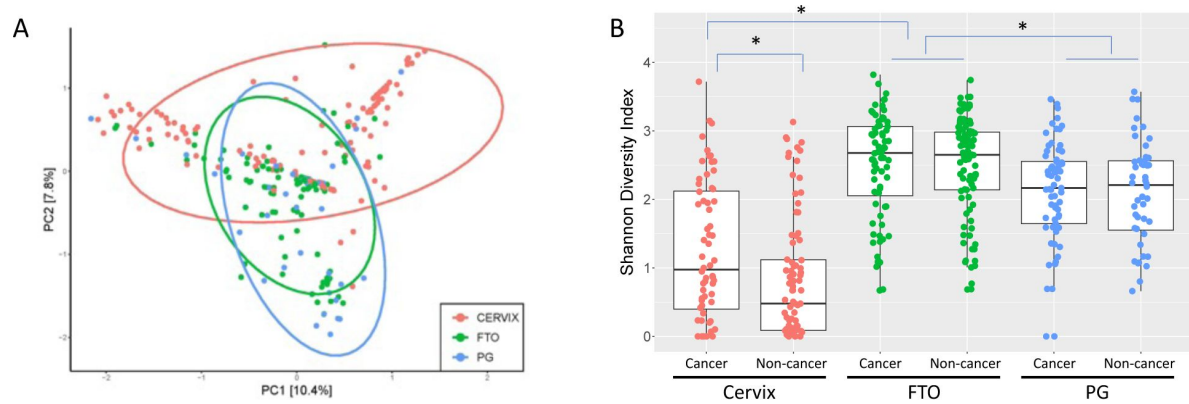


Figure 2.

Diversity of identified bacterial species.

A. PCA plot of candidate FT microbiota (84 bacterial species). FT (green) samples have more similarities with paracolic gutter (blue) than with cervical (red) samples. **B.** Shannon diversity plot of 726 bacterial species identified after filtering step #5 in **Supplemental Table 3** [\[link\]](#). * p-value < 0.001, paired t-test, comparing between sample types and between cancer versus non-cancer samples within the same sample type. FTO= Fallopian tube/ovarian surface; PG= Paracolic gutter.

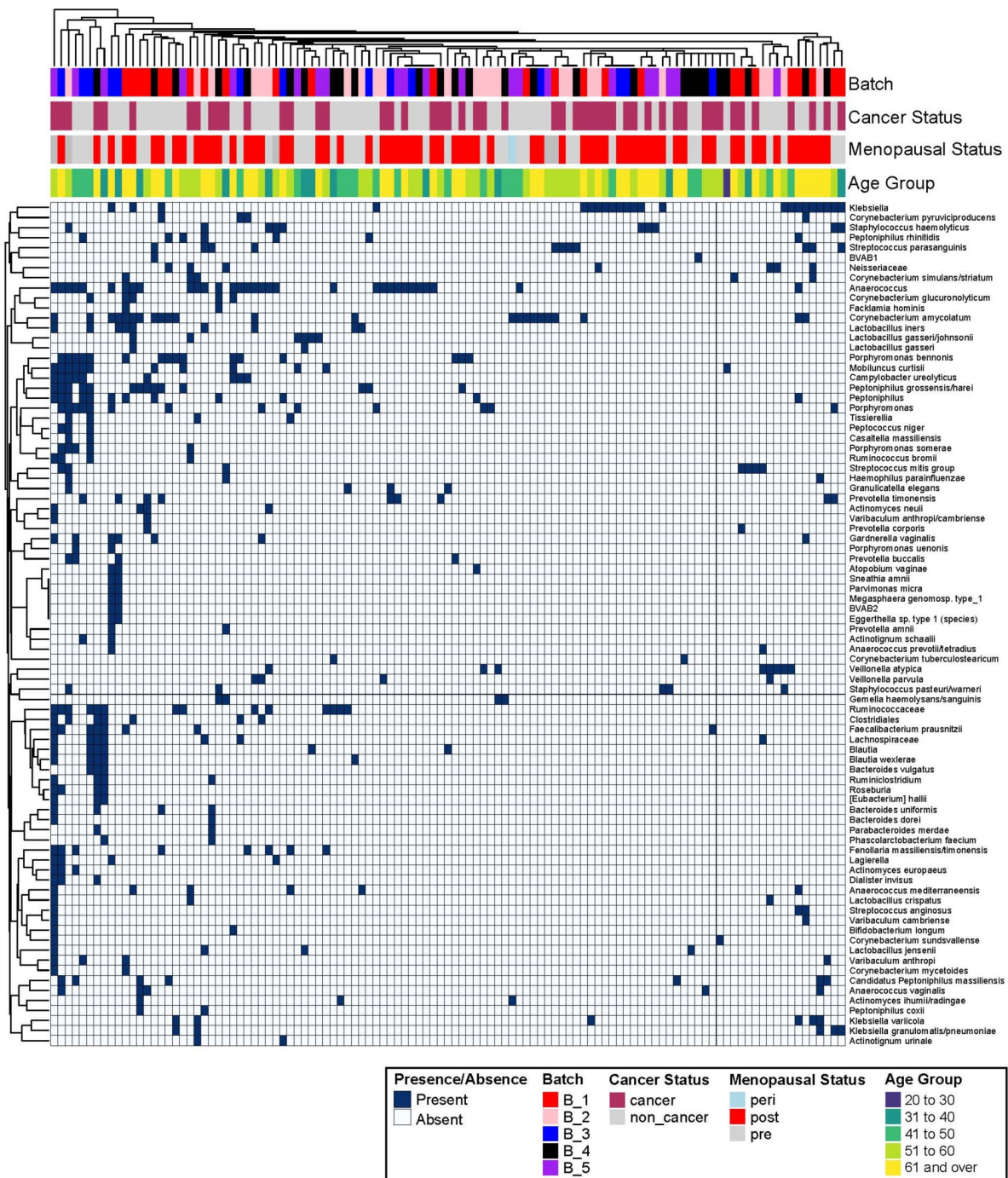


Figure 3.

Overview of all participants with processing batch, cancer status, menopausal status, and age in relation to FT microbiome taxa. Each column is a patient, and each row is a bacterial species. The top rows indicate the metadata of each sample as denoted by the color coding of batch, cancer status, menopausal status, and age group.

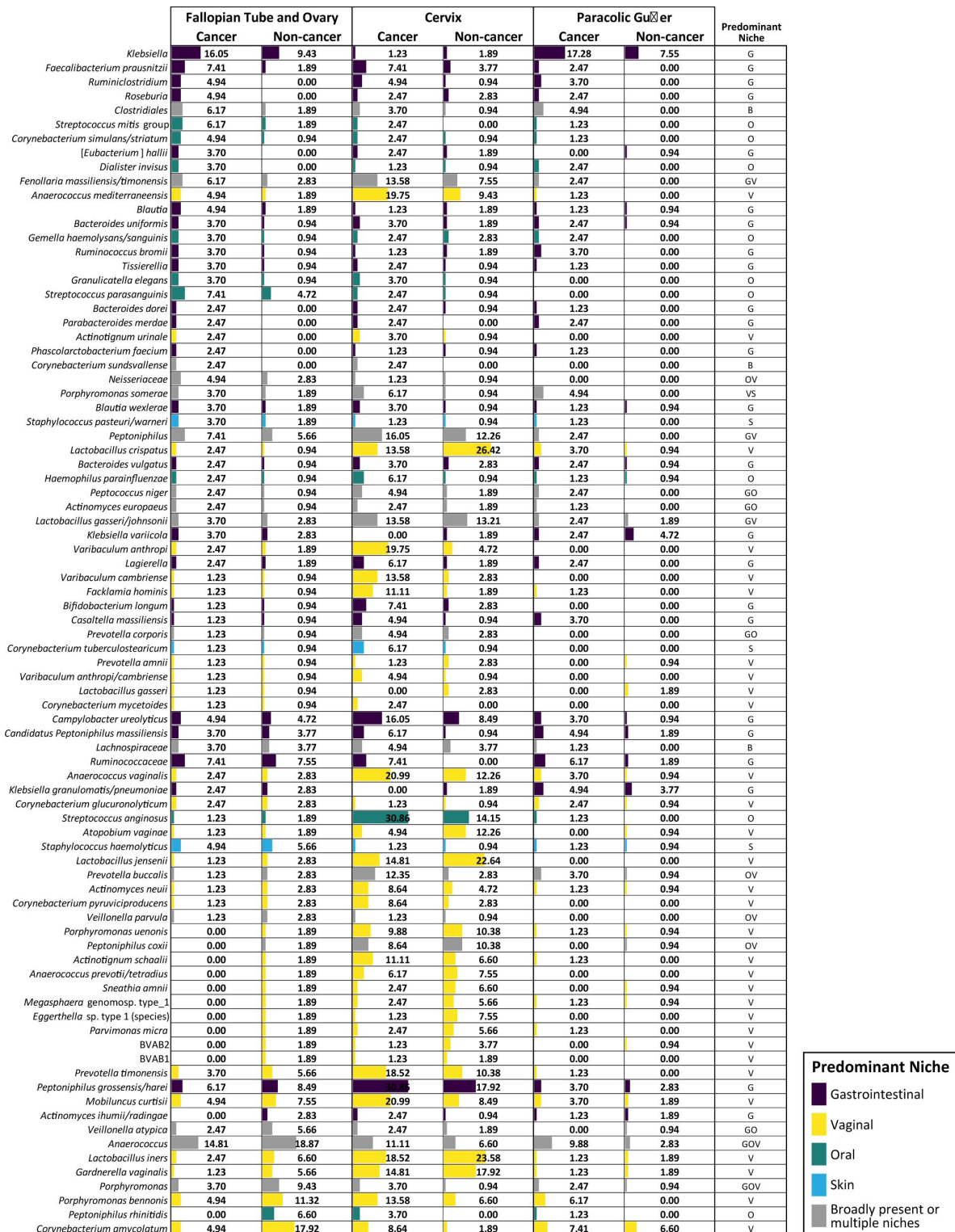


Figure 4.

Prevalence of the 84 bacterial species in ovarian cancer versus non-cancer cases. Each number is the percentage of individuals in each category with the presence of each bacterial species. G= Gastrointestinal; O= Oral; V= Vaginal; S= Skin; B= Broadly present.

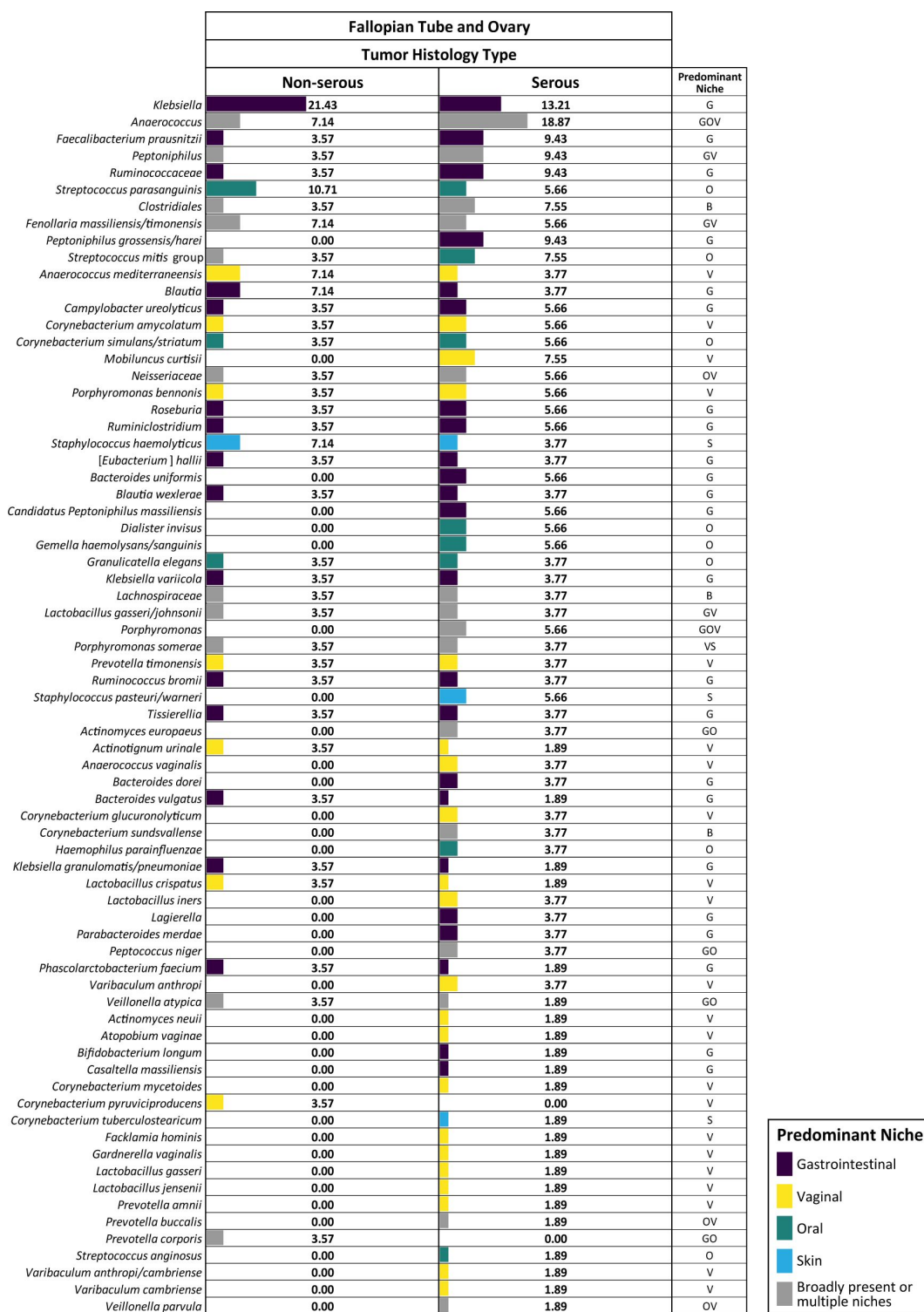


Figure 5.

The bacterial prevalence in FT samples from ovarian cancer patients by histology subtypes (Non-serous versus serous carcinoma). Each number is the percentage of individuals in each category with the presence of each bacterial species.

contaminants, we found 84 bacterial species that are present in FT or ovarian surface, which may represent a FT microbiota in some women. Comparison between the OC and non-cancer cases revealed a significantly higher prevalence of bacterial species that predominantly do not reside in the reproductive tract in patients with OC, while the FT from non-cancer patients favored vaginal bacterial species. It is unclear whether OC leads to this microbiota shift or if the microbiota shift precedes OC.

We proposed a novel hypothesis of ovarian carcinogenesis based on epidemiological evidence before initiating our study, namely that ascending infection with some genital tract bacteria leads to inflammation in the FT and causes DNA damage to cells resulting in OC. Our study showed the existence of bacterial species at a low concentration in the FT and ovarian surface in most women without overt signs of infection. Some of these bacterial species overlapped between the fallopian tube and the cervical swabs, indicating potential ascension from the lower to the upper genital tract. Some other bacterial species are not typical vaginal microbiota, and the origins of these bacteria in the fallopian tube samples are less clear. Patients with OC, especially the serous carcinoma subtype, showed significantly higher prevalence of many of the bacterial species we identified as the putative FT microbiota, indicating a potential link between a perturbed FT microbiota and OC. The FT microbiota may be different in different OC histology subtype, which was consistent with a recent study with limited sample size(23 [↗](#)).

One of the most interesting findings from our study is that most of the top bacterial species that were more prevalent in OC patients had predominant niche outside of female reproductive tract, normally residing in the gastrointestinal or oral tract. Some of these bacterial species were also identified in previous small studies examining the bacteria incorporated into the FT and ovarian cancer tissues, such as *Anaerococcus*, *Klebsiella*, *Bacteroides* and *Streptococcus*(23 [↗](#), 24 [↗](#)). The origin and route of ascension of these bacteria to the FT and ovarian surfaces remain unknown. In late-stage cancers, even when there are no overt signs of gastrointestinal invasion, the permeability of the intestinal tract may be increased which can increase the prevalence of these bacterial species in the FT, ovarian surfaces, and paracolic gutters. The causal effect of bacterial or other pathogens on the FT inflammation or carcinogenesis will need to be investigated in future studies using preclinical models. However, our study provides a list of top bacterial candidates that can be further investigated.

Our approach in collecting swabs in a sterile fashion in the operating room before the surgical case starts is unique and has not been used in previous studies(21 [↗](#)-23 [↗](#)). This approach enables us to evaluate the microbiota in each individual patient at multiple sites to infer transfer among sites. Previous studies mostly used the tissues collected from the pathology department after gross examination, which can introduce contamination. These studies infrequently included negative controls to assess for environmental or laboratory contamination, which is critically important when assessing a low biomass microbiome(25 [↗](#)). We collected swabs not only from the site of interest, i.e., fallopian tube and ovarian surface, but also from key control sites linked to cases, such as the air in the OR, the laparoscopic port, the paracolic gutter, and the cervix. These biological controls, along with the DNA extraction and PCR controls during the sample processing and sequencing processes, provide a fuller picture of the patient-specific microbiota and differentiate the biologically meaningful bacterial species from likely contaminants. The approach taken here may serve as a guide for others undertaking microbiome studies of low biomass environments, particularly when considering study design, types of controls, and analytical approaches(26 [↗](#)). The sample size is among the largest in microbiome studies of low biomass sites, which is helpful in differentiating signal from the noise in this setting. All previous microbiome studies that included FT as a study site had less than 40 patients in each group(21 [↗](#)-23 [↗](#)).

There are some limitations to our study. Due to the cross-sectional nature of the study, we have limited ability to link specific bacteria to ovarian carcinogenesis, as we would need to demonstrate that exposure to bacteria precedes the cancer. However, identifying associations between FT microbiota and OC is a critical first step. Further investigations, especially backed by *in vitro* studies, are needed to test our initial hypotheses. Due to the large sample size, we processed the samples in five batches and sequenced the libraries on four plates. Even though we separated the cervical samples with the low-biomass samples on different sequencing plates to prevent cross-contamination, we sequenced the low-biomass samples on three different sequencing plates, which could introduce bias due to batch effects. However, all samples were processed by a single experienced technician, and each sequencing plate contained samples from different processing batches, which helped to diminish potential batch effects. By requiring the bacterial species be present in both fallopian tube/ovarian surface swabs and the cervical swabs, we may have missed part of the fallopian tube microbiota that may have originated from sources other than the lower genital tract.

In conclusion, in this large prospective study with extensive controls, we analyzed the FT swabs from 187 patients together with controls that were collected intraoperatively in a sterile fashion, and identified a putative FT microbiota that exists in many women without any overt signs of infection. Patients with OC had a high prevalence of some key bacterial species, indicating a potentially perturbed microbiota co-existing with OC. Further studies are needed to investigate whether there is a causal relationship between these bacterial species and ovarian carcinogenesis.

Materials and Methods

Study population

Specimens and clinical information were obtained from patients who provided informed consent under a protocol approved by the institutional review board at University of Washington (Protocol # 2872). Surgical indications included ovarian cancers of various histological types, risk-reducing salpingo-oophorectomies due to germline *BRCA* or other mutations, and benign gynecological disorders such as ovarian cysts or endometriosis. Exclusion criteria include pelvic inflammatory disease, presence of an intrauterine device, use of antibiotics, endometrial biopsy, intrauterine device removal or hysteroscopy in the 30 days prior to the intended enrollment.

Sample collection

During the surgeries that involve salpingectomies, we collected fallopian tube swabs in the operating room in a sterile fashion. From most enrolled patients, we collected from the following sites: 1) a swab from the air in the operating room, 2) a cervical swab before vaginal sterile prep, 3) a swab from the laparoscopy port after the port insertion and before introduction of any instrument in laparoscopic cases, 4) a swab from the unaffected peritoneum in the paracolic gutter above the liver, 5) a swab from the fallopian tube and ovarian surface on each side. In some patients, the swabs may not have been collected from all sites due to surgical accessibility, time constraint, or unintentional omission. All biological samples were assigned anonymous participant identification numbers, which were matched with participants' names in a securely stored link file.

DNA extraction and quantitative PCR

All swabs were vortexed and washed in 200 μ l 0.9% saline, in which saline was filtered by MilliporeSigma™ Amicon™ Ultra Centrifugal 100k Da Filter Units (Thermo Fisher Scientific, Waltham, MA). DNA from swab prep mixture was extracted using the QIAamp BiOstic Bacteremia DNA KIT (QIAGEN, Germantown, MD) and eluted in a mixture of 25 μ l EB buffer provided in the kit plus 25 μ l 0.2X Tris-EDTA. In each quality control qPCR assay, 5 μ l DNA was loaded per reaction.

Digestion buffer control was DNA extracted from sham swabs to assess presence of contaminant in kit or during extraction procedure. Absence of PCR inhibitor was confirmed by an internal amplification control (IAC) quantitative polymerase chain reaction (qPCR)([27](#)). IAC qPCR was designed to compare amplification of spiked-in jellyfish gDNA between DNA samples and water. Total bacterial concentration was measured by a broad range qPCR targeting V3-V4 region of the bacterial 16S rRNA gene([28](#)).

Broad-Range PCR and Deep Sequencing of 16S rRNA Gene Amplicons

Broad-range amplicon PCR targeting V3–V4 hypervariable region of the 16S rRNA gene was performed using an adapter attached 338F and 806R primer formulation([28](#), [29](#)). The optimal quantity of DNA per reaction is 2.4×10^6 copies of the 16S rRNA gene. Maximum allowed volume was added in amplicon PCR (20 μ l DNA/ 30 μ l reaction) for samples from collection sites other than the cervix, which were defined as lower biomass samples. Filtered water was added to PCRs as a no-template (negative) control. A sham DNA extraction was performed to assess contamination of extraction reagents and the resulting DNA added to PCRs as another form of negative control with each batch of samples processed. Amplicon PCR products were cleaned using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN) to remove primer dimer and reaction buffers. Cleaned amplicons were applied to barcoded Index PCR using NexteraXT index kits v2 (Illumina, San Diego, CA). Per index PCR, 15 μ l of lower biomass amplicon or 5 μ l of higher biomass amplicon was loaded. Index PCR product went through a second-round bead clean and was eluted in 30 μ l $1 \times$ TE buffer. DNA concentration of each sample was measured by the Quant-iT dsDNA assay kit-HS (Thermo Fisher Scientific, Waltham, MA). For the low biomass samples, the entire volume of cleaned index PCR product was pooled into sub pools, which then were concentrated roughly fifty times by MilliporeSigma™ Amicon™ Ultra Centrifugal 10kDa Filter Units (Thermo Fisher Scientific, Waltham, MA). Equal quantities (8 nM or lower) of sub pools were pooled into a master pool, which was then subjected to deep sequencing.

Next Generation Sequencing (NGS) of the bacterial 16S rRNA gene PCR product was performed on the Illumina MiSeq instrument (Illumina). PhiX Control Library v3 (Illumina) was combined with the amplicon library at 15% for high biomass samples and at 20% for low biomass samples.

NGS Data Analysis

Sequence reads were processed using the DADA2 package([30](#)) for error correction, dereplication, paired-end assembly, and chimera removal and a list of unique sequence variants (SVs) were generated. A custom vaginal reference set was used to assign taxonomy to individual SVs([28](#)). Taxonomic assignments were made as described previously([31](#)). Briefly, a multiple sequence alignment of both query and reference sequences was created using *cmalign*([32](#)) and query sequences were placed on the phylogenetic tree using *pplacer*([33](#)). Taxonomy was assigned to each unique SV based on location on the tree. Bacterial taxa represented by fewer than 100 reads in a sample were excluded from that sample to minimize environmental contaminant sequences from being included in the final dataset. The samples from the same site and the same patient were merged as one sample for downstream analysis. The NGS data was filtered using a processing pipeline that we developed in this study to track samples and sequence types (**Supplemental Table 3**). R packages were used for the statistical analyses and graphics. Sequences have been submitted to the NCBI Short Read Archive (Accession number pending).

Data Availability

All data produced in the present study are available upon reasonable request to the authors.

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Conflict of interest disclosures

The authors declare that there are no conflicts of interests.

Authors contribution statement

BY, EMS, DNF conceived and designed the study, obtained funding. CL processed the samples and prepared sequencing libraries. SP, SS processed and analyzed the data. EM, SL, EMS collected the samples. BY drafted the manuscript. CL, SP, SS, EMS, DNF edited the manuscript. All authors have read and approved the final manuscript.

Sample Type	Total Samples	Ovarian cancer		Non-cancer	
		Samples	Patients	Samples	Patients
No Template PCR Control – Water	111	N/A	N/A	N/A	N/A
DNA Extraction Control – Buffer	36	N/A	N/A	N/A	N/A
Operating Room Air	130	53	53	77	77
Laparoscopic Port	81	8	4	73	45
Paracolic Gutter	122	73	70	49	48
Fallopian Tube and Ovarian Surface	369	160	72	209	99
Cervix	152	65	65	87	87
TOTAL	1001	359	81	495	106

Supplemental Table 1.

Summary of samples sequenced.

Sample type	Mean	Standard Deviation	P-value (compared to FT samples)
Cervix	4.912207	1.553575	<0.001
Fallopian tube	0.3975917	0.6684811	N/A
Paracolic gutter	0.2764742	0.7500778	0.11
Laparoscopic port	-0.3592626	0.6236524	<0.001
Air	-0.8201336	0.6245525	<0.001
Buffer	-1.044277	0.5530808	<0.001

Supplemental Table 2.

Bacterial concentration (log₁₀[16S rRNA genes/μl of DNA]) of each sample type and the p-value of each comparison.

	Processing Summary	Samples and taxa after each step
1	Starting set	1001 Samples
2	Merged Sample by Participant and Sample Type	767 Samples X 1337 Taxa
3	Filter #1. Remove Taxa present in only 1 Sample	767 Samples X 892 Taxa
4	Filter #2. Remove Taxa present in > 1 No Template controls	645 Samples X 881 Taxa
5	Filter #3. Remove Taxa present in DNA extraction controls	619 Samples X 836 Taxa
6	Filter #4. Remove Taxa in > 1 AIR samples	575 Samples X 753 Taxa
7	Remove No Template Controls, DNA Extraction Controls, and AIR Controls	479 Samples X 753 Taxa
8	Filter #5. Filter Taxa for presence in at least 1 Fallopian Tube and Ovarian surface (FTO) sample	457 Samples X 715 Taxa
9	Filter #6. Filter Taxa for presence in at least 1 cervical sample	457 Samples X 84 Taxa
10	Filter #7. Filter for final sample set. At least 1 read for a given sample	340 Samples X 84 Taxa

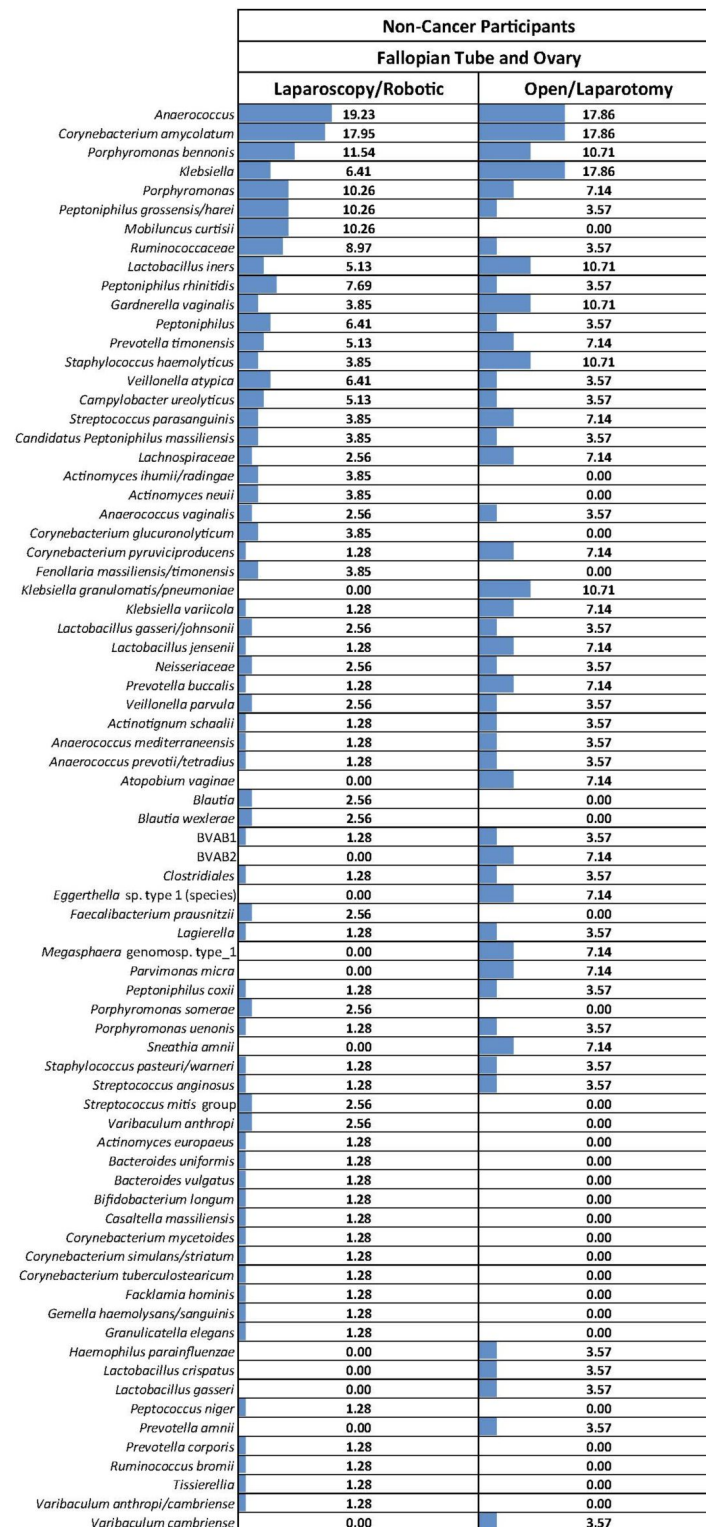
Supplemental Table 3.

Processing steps and result summary after each step.

	All Surgical Types		Only Laparotomy Cases	
	Fallopian Tube and Ovary		Fallopian Tube and Ovary	
	Cancer	Non-cancer	Cancer	Non-cancer
<i>Klebsiella</i>	16.05	9.43	18.31	18.52
<i>Faecalibacterium prausnitzii</i>	7.41	1.89	8.45	0.00
<i>Ruminiclostridium</i>	4.94	0.00	5.63	0.00
<i>Roseburia</i>	4.94	0.00	5.63	0.00
<i>Clostridiales</i>	6.17	1.89	7.04	3.70
<i>Streptococcus mitis</i> group	6.17	1.89	5.63	0.00
<i>Corynebacterium simulans</i> /striatum	4.94	0.94	5.63	0.00
[<i>Eubacterium</i>] <i>hallii</i>	3.70	0.00	4.23	0.00
<i>Dialister invisus</i>	3.70	0.00	4.23	0.00
<i>Fenollaria massiliensis</i> /timonensis	6.17	2.83	7.04	0.00
<i>Anaerococcus mediterraneensis</i>	4.94	1.89	5.63	3.70
<i>Blautia</i>	4.94	1.89	5.63	0.00
<i>Bacteroides uniformis</i>	3.70	0.94	4.23	0.00
<i>Gemella haemolysans</i> /sanguinis	3.70	0.94	4.23	0.00
<i>Ruminococcus bromii</i>	3.70	0.94	4.23	0.00
<i>Tissierella</i>	3.70	0.94	4.23	0.00
<i>Granulicatella elegans</i>	3.70	0.94	4.23	0.00
<i>Streptococcus parasanguinis</i>	7.41	4.72	8.45	7.41
<i>Bacteroides dorei</i>	2.47	0.00	2.82	0.00
<i>Parabacteroides merdae</i>	2.47	0.00	2.82	0.00
<i>Actinotignum urinale</i>	2.47	0.00	2.82	0.00
<i>Phascolarctobacterium faecium</i>	2.47	0.00	2.82	0.00
<i>Corynebacterium sundsvallense</i>	2.47	0.00	2.82	0.00
<i>Neisseriaceae</i>	4.94	2.83	5.63	3.70
<i>Porphyromonas somerae</i>	3.70	1.89	4.23	0.00
<i>Blautia wexlerae</i>	3.70	1.89	4.23	0.00
<i>Staphylococcus pasteurii</i> /warneri	3.70	1.89	4.23	3.70
<i>Peptoniphilus</i>	7.41	5.66	8.45	3.70
<i>Lactobacillus crispatus</i>	2.47	0.94	2.82	3.70
<i>Bacteroides vulgatus</i>	2.47	0.94	2.82	0.00
<i>Haemophilus parainfluenzae</i>	2.47	0.94	2.82	3.70
<i>Peptococcus niger</i>	2.47	0.94	2.82	0.00
<i>Actinomyces europaeus</i>	2.47	0.94	2.82	0.00
<i>Lactobacillus gasseri</i> /johnsonii	3.70	2.83	4.23	3.70
<i>Klebsiella varicola</i>	3.70	2.83	4.23	7.41
<i>Varibaculum anthrapi</i>	2.47	1.89	2.82	0.00
<i>Logierella</i>	2.47	1.89	2.82	3.70
<i>Varibaculum cambriense</i>	1.23	0.94	1.41	3.70
<i>Facklamia hominis</i>	1.23	0.94	1.41	0.00
<i>Bifidobacterium longum</i>	1.23	0.94	1.41	0.00
<i>Casaltella massiliensis</i>	1.23	0.94	1.41	0.00
<i>Prevotella corporis</i>	1.23	0.94	0.00	0.00
<i>Corynebacterium tuberculostrictum</i>	1.23	0.94	1.41	0.00
<i>Prevotella amnii</i>	1.23	0.94	1.41	3.70
<i>Varibaculum anthrapi</i> /cambriense	1.23	0.94	1.41	0.00
<i>Lactobacillus gasseri</i>	1.23	0.94	1.41	3.70
<i>Corynebacterium mycetoides</i>	1.23	0.94	1.41	0.00
<i>Campylobacter ureolyticus</i>	4.94	4.72	5.63	3.70
<i>Candidatus Peptoniphilus massiliensis</i>	3.70	3.77	4.23	3.70
<i>Lachnospiraceae</i>	3.70	3.77	4.23	7.41
<i>Ruminococcaceae</i>	7.41	7.55	8.45	3.70
<i>Anaerococcus vaginalis</i>	2.47	2.83	2.82	3.70
<i>Klebsiella granulomatis</i> /pneumoniae	2.47	2.83	2.82	11.11
<i>Corynebacterium glucuronolyticum</i>	2.47	2.83	2.82	0.00
<i>Streptococcus anginosus</i>	1.23	1.89	1.41	3.70
<i>Atopobium vaginae</i>	1.23	1.89	1.41	7.41
<i>Staphylococcus haemolyticus</i>	4.94	5.66	5.63	11.11
<i>Lactobacillus jensenii</i>	1.23	2.83	1.41	7.41
<i>Prevotella buccalis</i>	1.23	2.83	1.41	7.41
<i>Actinomyces neui</i>	1.23	2.83	1.41	0.00
<i>Corynebacterium pyruviciproducens</i>	1.23	2.83	1.41	7.41
<i>Veillonella parvula</i>	1.23	2.83	1.41	3.70
<i>Porphyromonas uenonis</i>	0.00	1.89	0.00	3.70
<i>Peptoniphilus coxii</i>	0.00	1.89	0.00	3.70
<i>Actinotignum schaalii</i>	0.00	1.89	0.00	3.70
<i>Anaerococcus prevotii</i> /tetradius	0.00	1.89	0.00	3.70
<i>Sneathia amnii</i>	0.00	1.89	0.00	7.41
<i>Megasphaera genomosp. type 1</i>	0.00	1.89	0.00	7.41
<i>Eggerthella sp. type 1</i> (species)	0.00	1.89	0.00	7.41
<i>Parvimonas micra</i>	0.00	1.89	0.00	7.41
BVAB2	0.00	1.89	0.00	7.41
BVAB1	0.00	1.89	0.00	3.70
<i>Prevotella timonensis</i>	3.70	5.66	4.23	7.41
<i>Peptoniphilus grossensis</i> /harei	6.17	8.49	7.04	3.70
<i>Mobiluncus curtisii</i>	4.94	7.55	5.63	0.00
<i>Actinomyces ihumii</i> /radingae	0.00	2.83	0.00	0.00
<i>Veillonella atypica</i>	2.47	5.66	1.41	3.70
<i>Anaerococcus</i>	14.81	18.87	16.90	18.52
<i>Lactobacillus iners</i>	2.47	6.60	2.82	11.11
<i>Gardnerella vaginalis</i>	1.23	5.66	1.41	11.11
<i>Porphyromonas</i>	3.70	9.43	2.82	7.41
<i>Porphyromonas bennoni</i>	4.94	11.32	5.63	11.11
<i>Peptoniphilus rhinitidis</i>	0.00	6.60	0.00	3.70
<i>Corynebacterium amycolatum</i>	4.94	17.92	5.63	18.52

Supplemental Figure 1.

Comparison of overall and laparotomy cases in the bacterial prevalence in FT samples from ovarian cancer versus non-cancer patients. Each number is the percentage of individuals in each category with the presence of each bacterial species.



Supplemental Figure 2.

The bacterial prevalence in FT samples from non-cancer patients: comparison of laparoscopic/robotic and laparotomy cases. Each number is the percentage of individuals in each category with the presence of each bacterial species.

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Reviewer #2 (Public Review):

The authors aimed to investigate the microbiota present in the fallopian tubes (FT) and its potential association with ovarian cancer (OC). They collected swabs intraoperatively from the FT and other surgical sites as controls to profile the FT microbiota and assess its relationship with OC.

They observed a clear shift in the FT microbiota of OC patients compared to non-cancer patients. Specifically, the FT of OC patients had more types of bacteria typically found in the gastrointestinal tract and the mouth. In contrast, vaginal bacterial species were more prevalent in non-cancer patients. Serous carcinoma, the most common OC subtype, showed a higher prevalence of almost all FT bacterial species compared to other OC subtypes.

The strengths of the study include its large sample size, rigorous collection methods, and use of controls to identify the possible contaminants. Additionally, the study employed advanced sequencing techniques for microbiota analysis. However, there are some weaknesses to consider. The study relied on swabs collected intraoperatively, which may not fully represent the microbiota in the FT during normal physiological conditions. The study also did not establish causality between the identified bacteria and OC but rather demonstrated an association. Regardless, the findings are important and these questions need to be addressed by future studies. A few additions in data representation and analysis are instead recommended.

Overall, the authors achieved their aims of identifying the FT microbiota and assessing its relationship with OC. The results support the conclusion that there is a clear shift in the FT microbiota in OC patients, paving the way for further investigations into the role of these bacteria in the pathogenesis of ovarian cancer.

The identification of specific bacterial species associated with OC could contribute to the development of novel diagnostic and therapeutic approaches. The study design and the data generated here can be valuable to the research community studying the microbiota and its impact on cancer development. However, further research is needed to validate these findings and elucidate the underlying mechanisms linking the FT microbiota shift and OC.

<https://doi.org/10.7554/eLife.89830.2.sa1>

Reviewer #3 (Public Review):

The findings of Bo Yu and colleagues titled "Identification of fallopian tube microbiota and its association with ovarian cancer: a prospective study of intraoperative swab collections from 187 patients" describes the identification of the fallopian tube microbiome and relationship with ovarian cancer. The studies are highly rigorous obtaining specimens from the fallopian tube, ovarian surfaces, paracolic gutter of patients of known or suspected ovarian cancer or benign tumor patients. The investigators took great care to insure there was no or limited

contamination including test the surgical suite air, as the test locations are from low abundance microbiota. The findings provide evidence that the microbiota in the fallopian tube, especially in ovarian cancer has similarities to gut microbial communities. This is a potentially novel observation.

The studies investigate the microbiome of >1000 swabs from 81 ovarian cancer and 106 non-cancer patients. The sites collected are low biomass microbiota making the study particularly challenging. The studies provide descriptive evidence that the ovarian cancer fallopian tube microbiota contain species that are similar to the gut microbiota. In contrast the fallopian tube microbiota of non-cancer patients that exhibit more similarity to the uterine/cervical microbiota. This may be a relevant observation but is highly descriptive with limited insights on the functional relevance.

The data indicate the presence of low biomass FT microbiota. The findings support the existence of FT microbiota in ovarian cancer that appears to be related to gut microbial species. While interesting, there is no insights on how and why these microbial species are found in the FT. The studies only identify the species but there is no transcriptomic analysis to provide an indication on whether the bacteria are activating DNA damage pathways. This is an interesting observation that requires more insights to address how these bacteria reach the fallopian tube and a related question is whether these bacteria are found in the peritoneum.

An additional concern is whether these data can be used to develop biomarkers of disease and early detection of disease.

<https://doi.org/10.7554/eLife.89830.2.sa0>

Author Response

Reviewer #1 (Public Review):

The authors propose a hypothesis for ovarian carcinogenesis based on epidemiological data, and more specifically they suggest that the latter relates to ascending genital tract "infection" or "dysbiosis", the resulting fallopian tube inflammation ultimately predisposing to ovarian cancer.

While this hypothesis would ideally be addressed in a longitudinal set-up with repeated female genital tract sampling, such an approach is obviously hard to realize. Rather, the authors present this hypothesis as a rationale for a cross-sectional study involving 81 patients with ovarian cancer (most with the most common subtype of high grade serous ovarian carcinoma, though other subtypes were also included), as well as 106 control patients with various non-infectious conditions including endometriosis and benign ovarian cysts. In all patients was there a comprehensive microbiome sampling of ovarian surface/fallopian tube, cervix and peritoneal cavity as well sampling of a number of potential sources of contamination, including surgery sites, ambient environment, consumables used in the DNA extraction and sequencing pipeline, etc. In line with the hypothesis presented at the outset, species with a threshold of at least 100 reads in both at least one cervical and at least one fallopian tube sample, while absent from environmental swabs, were considered relevant to the postulated pathway.

Remarkably, fallopian tube microbiota in ovarian cancer patients tended to cluster more closely to those retrieved from the paracolic gutter, than fallopian tube microbiota in non-cancer controls, which showed more relative similarity to vaginal/genital tract microbiota.

Although not really addressed by the authors, there also seem to be quite a few differences, at least in terms of abundance, in cervical microbiota between ovarian cancer patients and controls as well, which is an interesting finding, even when accounting for differences in age distribution between ovarian cancer patients and included control patients.

Overall, very few data are available thus far on the upper genital tract/fallopian tube microbiome, while also invariably controversial, as it has proven extremely difficult to obtain pelvic samples in a valid, "sterile" manner, i.e. without affecting a resident low-biomass microbiome to be analyzed. The authors took a number of measures to counter so, and in this respect, this is likely the largest and most valid study on the subject, even though biases and contamination can never be completely excluded in this context.

As such, I believe the strength of this study and paper primarily relates to the rigour of the methodology, thereby giving us a valuable insight in the presumed fallopian tube/ovarian surface microbiome, which may definitely serve as an impetus and a reference to future translational ovarian cancer research, or ovarian microbiome research for that matter.

I believe that the authors should acknowledge in more detail, that the data obtained from their cross-sectional study, valid as these are, do not provide any direct support to the hypothesis - albeit also plausible - set forth, a discussion that I somehow missed to a certain extent. It is important to realize in this and related contexts that neoplasia may well induce microbiome alterations through a variety of mechanisms, hence microbiome alterations not per se being causative. Conclusions should therefore be more reserved. Along the same lines, potential biases introduced through the selection of control patients (some detail here would be insightful) also deserves some discussion, as it is not known, whether other conditions such as benign ovarian cysts or endometriosis have some relationship with the human microbiome, be it causative or 'reversely causative', see for instance very recent work in Science Translational Medicine.

We appreciate the reviewer's detailed review and thoughtful comments. We have added the following sentences in the Discussion to address the reviewer's concern: "Due to the cross-sectional nature of the study, we have limited ability to link specific bacteria to ovarian carcinogenesis, as we would need to demonstrate that exposure to bacteria precedes the cancer. However, identifying associations between FT microbiota and OC is a critical first step. Further investigations, especially backed by in vitro studies, are needed to test our initial hypotheses."

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The authors aimed to investigate the microbiota present in the fallopian tubes (FT) and its potential association with ovarian cancer (OC). They collected swabs intraoperatively from the FT and other surgical sites as controls to profile the FT microbiota and assess its relationship with OC.

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We appreciate the reviewer's detailed review and positive comments.

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An additional concern is whether these data can be used to develop biomarkers of disease and early detection of disease. can the investigators detect the ovarian cancer FT

microbiota in cervical/vaginal secretions? That may yield more significant insights for the field.

We appreciate the reviewer's detailed review and thoughtful comments. We have added the following sentences in the Discussion to acknowledge the reviewer's concern: "Due to the cross-sectional nature of the study, we have limited ability to link specific bacteria to ovarian carcinogenesis, as we would need to demonstrate that exposure to bacteria precedes the cancer. However, identifying associations between FT microbiota and OC is a critical first step. Further investigations, especially backed by in vitro studies, are needed to test our initial hypotheses."

I have no additional comments here.

Reviewer #2 (Recommendations For The Authors):

The data analysis and data representation could be improved by the following points:

- 1. To compare the microbiota and assess the overall microbiota structure difference between the cancer vs non cancer cohort alpha- and beta-diversity of the microbial communities can be conducted.*
- 1. A differential abundance analysis could also be conducted to assess the differences at the genera and taxa level between the cancer vs non cancer cohorts.*
- 1. The analysis suggested above can also be conducted in the serous vs non serous cancer cohorts.*
- 1. In Figure 4 and 5 it would be more intuitive to show the predominant niche of each bacterium by color coding*

We appreciate these helpful suggestions from the reviewer. We have added Figure 2B to address the diversity as well as the differences between cancer versus non-cancer cohorts. We have added in the Results section the description of our findings in Figure 2B. We have added color coding to Figure 4 and 5 as the reviewer suggested.

Reviewer #3 (Recommendations For The Authors):

These studies are interesting but are very descriptive with no obvious approaches for understanding the mechanisms of FT microbiota in ovarian cancer. The identification of these bacteria is not sufficient to draw implications on their impact on ovarian cancer development or progression. This needs to be addressed.

We agree with the reviewer and have added the following sentences in the Discussion to acknowledge the reviewer's concern: "Due to the cross-sectional nature of the study, we have limited ability to link specific bacteria to ovarian carcinogenesis, as we would need to demonstrate that exposure to bacteria precedes the cancer. However, identifying associations between FT microbiota and OC is a critical first step. Further investigations, especially backed by in vitro studies, are needed to test our initial hypotheses."