Shifts in gut and vaginal microbiomes are associated with cancer recurrence time in women with ovarian cancer

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Many studies investigating the human microbiome-cancer interface have focused on the gut microbiome and gastrointestinal cancers. Outside of human papillomavirus driving cervical cancer, little is known about the relationship between the vaginal microbiome and other gynecological cancers, such as ovarian cancer. In this retrospective study, we investigated the relationship between ovarian cancer, platinum-free interval (PFI) length, and vaginal and gut microbiomes. We observed that *Lactobacillus*-dominated vaginal communities were less common in women with ovarian cancer, as compared to existing datasets of similarly aged women without cancer. Primary platinum-resistance (PPR) disease is strongly associated with survivability under one year, and we found over one-third of patients with PPR (PFI<6 months, n = 17) to have a vaginal microbiome dominated by *Escherichia* (> 20% relative abundance), while only one platinum super-sensitive (PFI>24 months, n = 23) patient had an *Escherichia*-dominated microbiome. Additionally, *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. In the gut microbiome, we found patients with PPR disease to have lower phylogenetic diversity than platinum-sensitive patients. The trends we observe in women with ovarian cancer and PPR disease, such as the absence of *Lactobacillus* and presence of *Escherichia* in the vaginal microbiome as well as low gut microbiome phylogenetic diversity have all been linked to other diseases and/or pro-inflammatory states, including bacterial vaginosis and autoimmune disorders. Future prospective studies are necessary to explore the

translational potential and underlying mechanisms driving these associations.

Shifts in Gut and Vaginal Microbiomes are Associated with Cancer Recurrence Time in Women with Ovarian Cancer

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Abstract

Many studies investigating the human microbiome-cancer interface have focused on the gut microbiome and gastrointestinal cancers. Outside of human papillomavirus driving cervical cancer, little is known about the relationship between the vaginal microbiome and other gynecological cancers, such as ovarian cancer. In this retrospective study, we investigated the relationship between ovarian cancer, platinum-free interval (PFI) length, and vaginal and gut microbiomes. We observed that *Lactobacillus*-dominated vaginal communities were less common in women with ovarian cancer, as compared to existing datasets of similarly aged women without cancer. Primary platinum-resistance (PPR) disease is strongly associated with survivability under one year, and we found over one-third of patients with PPR (PFI<6 months, n = 17) to have a vaginal microbiome dominated by *Escherichia* (> 20% relative abundance), while only one platinum super-sensitive (PFI>24 months, n = 23) patient had an *Escherichia*-dominated microbiome. Additionally, *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. In the gut microbiome, we found patients with PPR disease to have lower phylogenetic diversity than platinum-sensitive patients. The trends we observe in women with ovarian cancer and PPR disease, such as the absence of *Lactobacillus* and presence of *Escherichia* in the vaginal microbiome as well as low gut microbiome phylogenetic diversity have all been linked to other diseases and/or pro-inflammatory states, including bacterial vaginosis and autoimmune disorders. Future prospective studies are necessary to explore the translational potential and underlying mechanisms driving these associations.

Introduction

Ovarian cancer is the most deadly gynecological cancer (1); it kills approximately 14,000 women in the United States annually, accounting for 4.9% of all cancer-related deaths in females in the United States. In the majority of cases (> 80%), ovarian cancer is not detected until stage III or later, primarily due to the nonspecific nature of ovarian cancer symptoms and lack of informative biomarkers (2, 3). Early-stage (I or II) detection results in substantially greater five-year survivability compared to late-stage diagnosis (III or IV): 70% versus 36% survival rate, respectively (4), highlighting the importance of discovering early-disease biomarkers.

The standard course of primary treatment in ovarian cancer is cytoreductive surgery (CRS) in combination with platinum-based chemotherapy (5), which causes cytotoxicity through the formation of intra- and inter- strand adducts on DNA in cancer cells (6). The diameter of the remaining tumor after CRS, referred to as gross residual disease, is an important predictor of patient outcome, as individuals with no residual disease or residual disease < 1 cm have improved survivability compared to those with tumors > 1 cm after CRS (7). The combination of CRS and platinum-based chemotherapy is highly effective with approximately 80% of all patients showing no evidence of disease at the conclusion of initial therapy; however, recurrences occur in 70-80% of advanced stage patients and 20-25% of early-stage patients (8). Primary platinum resistance (recurrence of cancerous growth within six months of primary treatment cessation) develops in about 20% of patients, and is highly problematic because it is associated with a survivability of under one year and fewer effective treatment options (9). Other patients may remain free of cancerous growth for more than two years, but the risk of recurrence and eventual development of treatment-resistant cancer is still unacceptably high (10-13). This

merits a focus on discovering biomarkers of ovarian cancer and drivers of platinum-resistance to facilitate early cancer detection as well as better understand variation in treatment outcomes.

Recent evidence suggests that the human microbiome is an important factor in tumorigenesis, carcinogenesis, and effectiveness of chemotherapy (14-17). Gut microbiome dysbiosis can influence colorectal carcinogenesis via production of genotoxic metabolites, such as colibactin, and through the promotion of a pro-inflammatory state, which contributes to cancer cell proliferation, angiogenesis, and metastasis (14, 17-20). While most studies on the microbiome-cancer relationship have focused on the gut microbiome, there is growing evidence in support of the relationship between the vaginal microbiome and gynecological cancers. For example, human papillomavirus (HPV) is a known causative agent of cervical cancer (21-23), while pelvic inflammatory disease, which is associated with shifts in vaginal microbiome composition (24), has been linked to ovarian cancer development (25). Yet, there are still many unknowns about the relationship between the vaginal microbiome and gynecological cancers (21-23, 26). Likewise, links between the vaginal microbiome and platinum-sensitivity remain elusive; however, a previous study demonstrated that cancerous growths in mice with antibiotic-depleted gut microbiomes were less susceptible to platinum-chemotherapies compared to those with high gut microbiome diversity (15). The microbiome has a strong, bi-directional relationship with the host immune system (27, 28) and immune cells in the mice with a depleted gut microbiome produced fewer reactive oxygen species (ROS) than control mice (15). Decreased ROS production and platinum-resistance in mice with low gut microbiome diversity suggests a role for the microbiome in response to platinum-chemotherapy because ROS play a part in cell apoptosis after exposure to platinum chemotherapeutic agents (29).

In this study, we assessed how the vaginal and gut microbiomes vary in ovarian cancer patients with different platinum-sensitivities, with the aim of determining whether the human microbiome can be used as a biomarker of platinum-sensitivity.

Materials & Methods

Study Population

Patients, who carried a diagnosis of advanced (Stage III/IV) epithelial ovarian cancer and who were classified as primary platinum-resistant (platinum-free interval [PFI] from completion of primary platinum-based chemotherapy < 6 months) or platinum super-sensitive (PFI > 24 months) and were being treated at the Stephenson Cancer Center at the University of Oklahoma Health Sciences Center, were approached to participate in this study. Patients were approached when they either developed primary platinum-resistant disease or when they were identified as platinum super-sensitive. Patients who had already been diagnosed with primary platinum-resistant disease or as platinum super-responders but had moved on to additional therapy, as well as patients on active anti-cancer therapy or in surveillance, were also included in this study. Patients were excluded if: 1) they were taking antibiotics at the time of sample collection or within 14 days prior to sample collection, or 2) they had active vaginal bleeding or known entero-vaginal fistulae. Samples were also collected from five individuals who were referred for ovarian cancer treatment at the Stephenson Cancer Center but ultimately had benign tumors; these served as a non-chemotherapy exposed control group. Sample collection periods and treatment procedures are outlined in Fig. S1. A brief summary of demographic and medical treatment history for participants in this study (n = 45, median age 62.2, age range 33-83) is

133 provided in Table 1. This study was approved by the University of Oklahoma Health Sciences
134 Center Institutional Review Board (February 22nd, 2016, reference #6458) and all participants
135 gave written informed consent for their participation in the study.
136
137 All patients were treated initially with platinum and taxane chemotherapy for a planned six to
138 eight cycles. These regimens included paclitaxel and carboplatin given every 21 days, paclitaxel
139 given weekly with every 21st day carboplatin, or intraperitoneal administration of either cisplatin
140 or carboplatin with intravenous and intraperitoneal paclitaxel. In patients with platinum resistant
141 disease, standard of care options after recurrence included pegylated liposomal doxorubicin
142 (PLD), weekly paclitaxel, gemcitabine, topotecan or bevacizumab given as monotherapy or in
143 combination therapy. Patients were also screened for eligibility for clinical trials. Patients with a
144 PFI > 24 months had not recurred at the time of study participation and were followed every 6
145 months with surveillance of Ca-125 values and exams. For those who had recurred beyond 24
146 months, treatment options included several platinum-based doublets including carboplatin and
147 PLD given every 28 days, carboplatin and paclitaxel given every 21 days or carboplatin and
148 gemcitabine given on a day one and day eight or day one and day fifteen schedule. Each patient
149 completed a minimum of six cycles of treatment and could undergo more cycles as long as the
150 patient was responding and tolerating therapy.
151
152 ***Sample Collection***
153 Samples were collected during standard of care exams in the gynecologic oncology clinic at the
154 Stephenson Cancer Center in Oklahoma City, OK. Catch-All Sample Collection Swabs
155 (Epicentre) were used to collect vaginal and fecal samples. Vaginal swabs were collected from

three sites per individual: vaginal introitus (VIT), mid-vagina (MDV), and posterior fornix (VPF), and then placed into a dry sample collection tube. Fecal samples were collected via a rectal digital exam, after which any stool collected was placed on a Catch-All swab and placed in a dry collection tube. Two swabs were collected from each site (bilaterally from the vaginal sites and sequentially for the rectal samples). Each participant completed a quality-of-life survey regarding their medical treatment history, antibiotic use within the past year, vitamin consumption, socioeconomic status, and other lifestyle metadata (Table S1).

Laboratory Methods

DNA was extracted from the left-side vaginal swab and first fecal swab from each patient, using the MoBio PowerSoil DNA Isolation Kit (now Qiagen DNeasy PowerSoil Kit), following manufacturer's protocols with the addition of a ten-minute incubation at 65°C prior to the initial bead-beating step, as recommended in the Manual of Protocols for the Human Microbiome Project (30). A quantitative PCR (qPCR), using the SYBR Green PCR Master Mix (Applied Biosystems) and primers targeting the V4 region of the bacterial 16S rRNA gene (31), was conducted; dilutions of *Escherichia coli* DNA corresponding to known 16S rRNA gene copy numbers were used as quantification standards for the DNA extracts. DNA extracts were amplified in triplicate, using Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific) and Illumina-compatible primers 515F and 806R (targeting the V4 region of the16S rRNA gene) with error-correcting Golay barcodes incorporated into the 806R reverse primer (31). PCR products were pooled in equimolar concentrations, purified with the MinElute PCR purification kit (Qiagen), then size-selected between 300 and 450 basepairs using a PippenPrep, quantified

using KAPA Biosystems Illumina library quantification kit, and sequenced across multiple runs of an Illumina MiSeq (500 cycles paired-end sequencing, v2 reagent kit).

Bioinformatic Methods

AdapterRemoval (v2) (32) was used to filter out reads with uncalled bases, reads with Phred quality threshold < 30, and reads less than 150 bp in length. Quality filtered paired-end reads were merged using AdapterRemoval (v2) (32) and then demultiplexed with QIIME (v1.9), followed by removal of chimeric sequences and low-abundance (<5 total sequences) reads (33). The remaining sequences were used for *de novo* Operational Taxonomic Unit (OTU) clustering with USEARCH (v10) at 97% sequence similarity (34). Taxonomy was assigned to each OTU representative using the EzBioCloud 16S rRNA gene database (35). The resulting OTU table was rarefied to 9000 reads and downstream analysis was performed in QIIME (v1.9) (33). The post-rarefaction sample breakdown was: PFI>24 (n = 23), PFI<6 (n = 17), and benign (n = 5). Further details of the bioinformatic methods are given in the Supplementary Material.

Statistical Methods

Phylogenetic diversity and weighted/unweighted UniFrac (36) metrics were generated in QIIME (v1.9) with FastTree2 (37). Tests for significance between study groups for alpha and beta diversity were performed using Kruskal-Wallis and PERMANOVA tests, respectively, in R (38). Vaginal samples were classified into clusters by the dominant bacterial taxon found in each sample, as determined by Ward hierarchical clustering (39), and visualized as a heatmap using the gplots package in R (40). If there were no dominant bacteria, the sample was classified as diverse. Median-unbiased estimated odds ratios were calculated to determine whether study

groups had significantly different odds of dominant bacteria; reported odds ratios and 95% confidence intervals were log-transformed. Kruskal-Wallis tests with a Benjamini and Hochberg false-discovery rate adjustment were used to evaluate differential abundance of individual taxa between study groups. Odds ratios were calculated using epitools (41) in R. Plots were generated using the ggplot2 (42) and ColorBrewer (43) packages in R.

Results

Vaginal microbiome

Samples from the different vaginal sites (VIT, MDV, VIT) that originated from the same individual showed similar taxonomic beta-diversity profiles (Fig. S2A-C). Sequencing failed for at least one of the three vaginal sites of eleven individuals (Supplementary Material), which presented difficulty in analyzing each vaginal site individually as the failed specimens were not limited to only one vaginal site. Due to the similarity in composition between vaginal sites in each individual and the relatively high number of samples which failed sequencing, we concatenated sequencing reads from each individual's three vaginal samples into a single representative vaginal microbiome sample per individual, and then performed downstream analysis with this single representative sample, unless otherwise noted. Combining vaginal sites for each individual allowed us to retain a sample size of 45. Firmicutes was the most dominant phylum in the vaginal microbiome and it was found at over 50% relative abundance in 40% of individuals (Fig. S3A), while Proteobacteria, Bacteroidetes, and Actinobacteria were the next most abundant phyla and found at >50% relative abundance in the vaginal microbiome of 13.3%, 13.3%, and 6.7% of individuals, respectively. At the genus level, *Lactobacillus, Prevotella,*

223 *Escherichia, Gardnerella,* and *Streptococcus* were the most dominant bacteria and accounted for
224 57.2% of all reads (Fig. S3B).
225
226 Individuals were grouped into the following study groups as outlined in the methods section
227 (Fig. S1): PFI < 6 Months (n = 17), PFI > 24 Months (n = 23), and benign (n =5). Table 1
228 presents summary statistics for these study groups. Iterative cytoreductive surgery (iCRS) was
229 more common in PFI < 6 months while primary cytoreductive surgery (pCRS) was more
230 common in PFI > 24 months individuals (log Odds Ratio [OR] = 3.02, 95% CI 1.37 – 4.68, p-
231 value = 0.0003). As expected, PFI < 6 months individuals underwent platinum chemotherapy
232 treatment more recently than PFI > 24 months individuals (p-value = 4.32×10^{-7}) but no other
233 metadata variable was significantly associated with PFI status (Table S2).
234
235 Vaginal microbiome communities were clustered into five community-dominance groups using
236 Ward hierarchical clustering: *Lactobacillus* cluster, *Escherichia* cluster, *Gardnerella* cluster,
237 *Prevotella* cluster, and a high diversity cluster (Fig. 1). Results below are presented
238 demonstrating how each vaginal community (excluding *Gardnerella* due to small cluster size)
239 relate to clinical variables, p-values for each vaginal community and clinical variable are given
240 in Table S3.
241
242 Dominance groups were evenly distributed between patients with PFI < 6 months, PFI > 24
243 months, and benign, with the exception of higher-than-expected dominance of *Escherichia* in
244 patients with PFI < 6 months; five of the six Escherichia-dominated vaginal communities
245 identified with hierarchical clustering belonged to patients with PFI < 6 months (Fig. 1). Vaginal

246 microbiomes dominated by *Escherichia* had higher odds of occurring in PFI < 6 months
247 compared to PFI > 24 months (log OR = 2.812, 95% CI 0.267 – 5.62, p-value = 0.024, Fig. 2A).
248 Additionally, one of the patients with PFI < 6 months and one of the patients with benign
249 pathology identified with a 'diverse' vaginal microbiome had *Escherichia* at greater than 20%
250 relative abundance, while no other patients with PFI > 24 months had *Escherichia* relative
251 abundance above 5% (Fig. 1). In total, 35.3% of the patients with PFI < 6 months had
252 *Escherichia* at greater than 20% relative abundance in the vaginal microbiome, compared to
253 4.34% of PFI > 24 months and only one of five benign individuals. Even though iterative
254 cytoreductive surgery and *Escherichia* are more common in PFI < 6 months individuals, we
255 found no significant relationship between iCRS and *Escherichia* abundance (Table S3, Fig. 2A,
256 p-value = 0.292). Moreover, outside of the relationship between *Escherichia* and platinum
257 resistance, *Escherichia* showed no significant association with any of the other health or lifestyle
258 factors we examined (Table S3, Fig. 2A). Although *Escherichia* is a common lab-grown
259 bacterium and found in feces, our analysis demonstrates *Escherichia* abundance in the vaginal
260 samples is biological and not a technical artifact (Supplementary Material).
261
262 Approximately 24% (11 of 45) of patients in this study had *Lactobacillus*-dominated
263 communities, which is significantly lower as compared to studies of similarly aged women
264 without ovarian cancer (p-value = 0. 037) (44, 45). Other studies have found *Lactobacillus* to be
265 less abundant in Black and Hispanic women (46). and our study consisted of 39 women who
266 self-reported ethnicity as white, two self-reported as Black, and four self-reported as Native
267 American (Table 1). Each of the Black and Native American women had a non-*Lactobacillus*
268 dominated vaginal microbiome (Table S1) but ethnicity was not a statistically significant

determinant of *Lactobacillus*-dominance (Fig 2B, log OR = -1.63, 95% CI -4.62 – 1.30, p-value = 0.27). High microbial cell density, as gauged through qPCR with a standard curve generated from controls with known cell density, was positively correlated with vaginal *Lactobacillus*-dominance, although somewhat weakly (R^2 = 0.278, Fig. S4). Consumption of antibiotics within the past month was associated with a lack of *Lactobacillus*-dominance (Fig. 2B); however, this relationship was not significant (log OR = -2.12, 95% CI -5.54 – 0.84, p-value = 0.0515). Additionally, *Lactobacillus* abundance was uncommon in individuals likely to be estrogen negative (post-menopause and not on hormone replacement therapy) but this result was also not significant (log OR = -1.32, 95% CI -2.83 – 0.33, p-value = 0.0744). *Lactobacillus*-dominance did not have a strong relationship with any of the other health or lifestyle factors we tested, including PFI length (Table S3, Fig. 2B). Only 20% (1 of 5) of patients with benign pathology had a *Lactobacillus*-dominated microbiome but small sample size prohibits statistical inference.

Previous studies have indicated that different *Lactobacillus* species in the vaginal microbiome may have different roles and differential influence on host biology (47-49). While we identified *L. iners, L. brevis, L. mucosae, L. reuteri, L. zeae,* and *L. delbrueckii* in the vaginal microbiome, 99% of the *Lactobacillus* reads in our study were either unclassified at the species level or mapped to *L. iners* (Table S4); ultimately there was not sufficient data to assess the relationship between most of these *Lactobacillus* species and clinical variables. Nevertheless, in individuals with high *Lactobacillus* abundance (n = 11), we found *L. iners* at significantly higher abundance in patients with either no gross residual disease or residual disease < 1cm (n = 7), compared to patients with residual disease > 1cm (n = 4, p-value = 0.0359, Fig. 3).

We did not observe significant associations between either *Prevotella* and clinical variables (Table S3, Fig. 2C) or the high diversity community and clinical variables (Table S3, Fig. 2D). However, *Prevotella* was less common in those with residual disease > 1cm and those with a history of hormonal disease (Fig. 2C), while a highly diverse vaginal microbiome was more common in women over 60 years old and in patients with a history of hormonal disorders, such as thyroid disease (Fig. 2D); but once again, none of these associations were statistically significant (Table S3, p-value > 0.05).

Gut microbiome

The gut microbiome was colonized by typical members of the gut microbiome at the phylum (Bacteroidetes, Firmicutes, Proteobacteria) and genus (*Bacteroides, Akkermansia, Faecalibacterium, Ruminococcus,* and *Prevotella*) levels (Fig. S5A-B). The PFI > 24 months and PFI < 6 months groups were not significantly different with respect to fecal unweighted and weighted UniFrac beta diversity distances as tested through PERMANOVA (Fig. 4, Fig. S6A-C); however, similar to the vaginal microbiome samples, there was a small subset of individuals (n = 9) with a unique microbiome signature. Patients with PFI < 6 months individuals had higher odds (log OR = 1.85, 95% CI -0.85 - 4.497, p-value = 0.12) of being in this unique/outlier fecal microbiome group. These fecal outliers have significantly lower phylogenetic diversity compared to the other fecal samples (p-value = 0.001, Fig. 5A) and have increased abundance of genera belonging to the order Clostridiales (*Lachnospira* [Kruskal Wallis p-value = 0.00039], unidentified Ruminococceae genus [Kruskal Wallis p-value = 0.001337], and *Subdoligranulum* [Kruskal Wallis p-value = 0.01121]) (Fig. S7). Of the six platinum-resistant patients in this outlier subset, two also had *Escherichia*-dominated vaginal communities. Eight of the nine

patients in this subgroup reported consuming antibiotics within the past 6 months but this was not a significantly greater proportion of individuals with recent antibiotic consumption compared to individuals outside this fecal outlier subgroup (log OR = 0.35, 95% CI -1.44 – 3.05 p-value = 0.48).

Overall, patients with platinum-resistant disease had lower fecal phylogenetic diversity compared to patients with platinum-sensitive disease, but the difference was not significant (Fig. 5B, p-value = 0.18). Regardless of PFI, patients with ovarian cancer had significantly higher relative abundance of *Prevotella* in the gut microbiome compared to benign individuals (p-value = 0.028, Fig. S8). Outside of the above-mentioned associations, there were no other significant associations between the gut microbiome and platinum sensitivity or other health/lifestyle variables.

Discussion

One of the major findings of this study is the inverse relationship between a *Lactobacillus*-dominant vaginal microbiome and ovarian cancer. In our study, fewer women than expected (24.4%) have *Lactobacillus*-dominated vaginal communities compared to similarly aged, healthy women (47.2%) from other studies (p-value = 0.037) (44, 45). *Lactobacillus* dominance is not found in any of the Black (n = 2) or Native American (n = 4) women in our study but only eleven of the 39 white women have *Lactobacillus*-dominated vaginal microbiomes; therefore, ethnicity was not a driving factor in *Lactobacillus* abundance in this study (p-value = 0.27). These results suggest the possibility that the low abundance of *Lactobacillus* may be indicative of a broader relationship between ovarian cancer and the vaginal microbiome. This finding corroborates a

previous study that also observed a reduced frequency of the *Lactobacillus*-dominated vaginal microbiome in women with ovarian cancer, particularly in women under 50 (45). A partial explanation may be that *Lactobacillus*-dominance, while typically viewed as a healthy state in the female genital tract, is a non-resilient ecology prone to disruption by variable factors, including changes in glycogen availability (50, 51), antibiotic exposure (52), and shifts in hormone abundance induced during stress responses (53). While we observed no statistical relationships between *Lactobacillus* dominance and platinum-sensitivity or other lifestyle/medical variables, there was a nearly significant decrease in *Lactobacillus*-dominance associated with taking antibiotics within the past month (p-value = 0.0515), as well as a positive association between *Lactobacillus*-dominance and microbial cell density ($R^2 = 0.278$).

Lactobacillus maintains a low pH in the vaginal environment by producing lactic acid as a byproduct of glycogen metabolism and this low pH inhibits growth of pro-inflammatory bacteria (21, 22, 54). Vaginal *Lactobacillus* may protect from gynecological cancers by inhibiting pro-inflammatory bacteria, such as those implicated in pelvic inflammatory disease, and by reducing inflammatory cytokines IL-1β and IL-6 (55). The low *Lactobacillus* levels we observed may be related to glycogen availability – nearly 75% of women in this study were post-menopause, and vaginally produced glycogen is known to decrease after menopause; likewise, chemotherapy can inhibit ovarian estrogen production and result in lower glycogen levels (44, 54, 56, 57). More importantly, many women in this study have had at least one ovary surgically removed during initial cancer treatment. Ovary removal leads to decreased estrogen production and thus, a likely decrease in vaginal glycogen levels; however, more research is needed to fully explain the ovarian-estrogen-glycogen dynamic (50). Glycogen abundance may also help explain the

relationship between vaginal microbiome cell density and *Lactobacillus,* as widely available glycogen may encourage a densely colonized *Lactobacillus* vaginal community due to high nutrient availability (57). Hormone replacement therapy (HRT) can be used to replace estrogen production that is stopped after menopause, and four women (two pre-menopause and two post-menopause) were on HRT. Women on HRT or pre-menopause were more likely to have *Lactobacillus*-dominated communities, but this was not significant (p-value = 0.0744). While we did not document glycogen levels in our study, the positive relationship between likely estrogen presence and *Lactobacillus* abundance lends credence to the idea that the low proportion of women with *Lactobacillus* abundance in our study is due to low estrogen, and thus glycogen. The retrospective nature of our study means that we were unable to assess *Lactobacillus* levels in women with ovarian cancer before they progressed to stage III/IV, or prior to chemotherapy. Therefore, we could not investigate anti-gynecological cancer properties of vaginal *Lactobacillus*; nevertheless, by comparing patients in this study to similarly aged women without ovarian cancer, we present further evidence that low *Lactobacillus* levels are more common in women with ovarian cancer (p-value = 0.037).

The presence, and size, of residual disease is strongly correlated with decreased survivability in ovarian cancer (7) and therefore our finding that *L. iners* was at significantly higher abundance ($p = 0.0359$) in patients with either no gross residual disease or residual disease < 1cm after treatment may point to *L. iners* as a potential path toward a biomarker. *L. iners* is a common vaginal bacterium (46, 58, 59) but its role in health and disease is sometimes contradictory (21); *L. iners* has been found at high relative abundance in low-grade squamous intraepithelial lesions in the cervix but at low abundance in high-grade squamous intraepithelial lesions (26). Yet,

384 another study found *L. iners* at high abundance in women with normal cytology when compared
385 to women with squamous intraepithelial lesions (60). Other studies have found *L. iners* to be
386 positively associated with cervical cancer (61, 62) but *L. iners* is also linked to clearance of HPV
387 (63), which is a causative agent of cervical cancer. Further research is necessary to better
388 understand the role of *L. iners* in gynecological cancers in general, and in the potential inhibition
389 of gross residual disease in ovarian cancer.
390
391 *Lactobacillus* is known to inhibit colonization and growth of *Escherichia* in the vaginal
392 microbiome (64, 65). The low abundance of *Lactobacillus* found in our study may present ample
393 opportunity for typically low abundance vaginal bacteria, such as *Escherichia*, to thrive and
394 proliferate in the absence of competition. Overgrowth of *Escherichia* only occurred in 17.8% of
395 patients in our study, yet 75% of those patients were in the PFI < 6 months group; this finding
396 was statistically significant (p-value = 0.024). Put another way, 35.3% of patients with PFI < 6
397 months showed greater than 20% relative abundance of *Escherichia*, as compared to 4.34% of
398 PFI > 24 months and only one of 5 benign cases. The explanation for why *Escherichia* was
399 significantly more common in patients with platinum-resistant tumors is unclear, and because
400 this study was retrospective, we were unable to track *Escherichia* abundance before we knew
401 each patient's platinum-sensitivity. One possible pathway is via interactions between the
402 microbiome, immune system, and how platinum-based chemotherapies induce cancer cell death.
403 Platinum chemotherapies partially rely on ROS produced by host myeloid cells (15). Microbes
404 strongly influence immune system function, and hence, alteration in ROS production may be
405 more common in *Escherichia*-dominant vaginal microbiomes, which may render platinum-based
406 chemotherapies less effective, leading to platinum-resistance. Vaginal *Escherichia* may also

cause an increased inflammatory response, such as during pelvic inflammatory disease (66), and promote cancerous growth, resulting in a shortened PFI. The effect of *Escherichia* on platinum-sensitivity warrants further investigation.

We also observed differences in the gut microbiome of women with platinum-resistant tumors, compared to benign and platinum-sensitive. Similar to the prevalence of *Escherichia*-dominant vaginal microbiomes, 35.3% of patients with PFI < 6 months were fecal beta-diversity outliers compared to the remainder of the fecal samples, while only 13.0% of PFI > 24 and none of the benign cases fell into this cluster (p-value = 0.12). Genera belonging to the Clostridiales order (*Subdoligranulum* (p-value = 0.01121) and *Lachnospira* (p-value = 0.00039*)* were at higher abundance in this group of outlier samples. *Subdoligranulum,* has been found at high abundance in the stool of individuals with gastrointestinal neoplasms (67) and has a positive association with blood-based markers of inflammation (68). *Lachnospira* has been found to be positively correlated with a plant-based diet (69) and at high abundance in healthy controls in a study investigating chronic kidney disease (70); yet, *Lachnospira* is also found at high abundance in women with metabolic disorder and obesity (69). Additionally, we note lower phylogenetic diversity in the gut microbiome of platinum-resistant tumors compared to both platinum-sensitive and benign tumors (p-value = 0.18). Chemotherapy is a well-documented driver of decreased gut microbiome alpha diversity (71-73); however, we did not observe a shift in alpha diversity with time since last cycle of chemotherapy. High gut microbiome alpha diversity is typically associated with improved human health (74, 75), although recent studies have started to question this paradigm (76). Nevertheless, the unique gut microbiome beta diversity profile in a subset of PFI < 6 months, and the decreased alpha diversity in the full PFI < 6 months population

indicates that there may be long term relationship between platinum-resistance and the gut microbiome.

Prevotella is enriched in both the platinum-resistant and platinum-sensitive study groups, compared to the benign group (p-value = 0.028). *Prevotella* is typically only found at high abundance in the gut microbiomes of non-industrial, traditional populations (77) and it is associated with consumption of a plant-rich diet (77). Yet, some strains of *Prevotella* are found in the gut microbiomes of industrial populations and are linked to pro-inflammatory states in the gut microbiome (78, 79). Similar to decreased *Lactobacillus* in the vaginal microbiome of patients with ovarian cancer, the relatively high abundance of *Prevotella* in the gut microbiome of women with ovarian cancer indicates a notable shift in microbial composition. Studies with larger control groups are necessary to address this relationship.

Conclusions

Our results demonstrate an association between the vaginal and gut microbiomes and platinum-sensitivity in women with ovarian cancer. *Escherichia*-dominant vaginal communities are significantly more likely to be present in patients with platinum-resistant tumors but the explanatory mechanism for this relationship is currently unclear. Lab contamination and/or collection methodology does not appear to play a role in vaginal *Escherichia* abundance, which indicates that finding *Escherichia* at high relative abundance in patients with PFI < 6 months is a biological trend.

452 We also observed shared vaginal and gut microbiome profiles in women with ovarian cancer,
453 with decreased dominance of *Lactobacillus* and increased relative abundance of *Prevotella,*
454 respectively, regardless of platinum-sensitivity. These results suggest shifts in microbiome
455 composition that are related to the ovarian cancer disease state, which may possibly be related to
456 chemotherapy, but the retrospective nature of our study does not allow us to distinguish the exact
457 mechanism of action.
458
459 Our results call for deeper investigation into the relationship between the vaginal and gut
460 microbiomes and ovarian cancer. A future avenue for research is a prospective, longitudinal
461 study that tracks how the vaginal and gut microbiomes change throughout the course of ovarian
462 cancer therapy, with an aim to disentangle how *Escherichia*-abundance impacts response to
463 chemotherapy. Similarly, a study tracking *Lactobacillus* abundance in an aged-matched,
464 lifestyle-matched cohort of women with and without ovarian cancer may provide insights into
465 how microbial risk factors impact occurrence and outcomes of ovarian cancer. This work must
466 also investigate why high *L. iners* abundance is found nearly exclusively in cases with < 1 cm or
467 no gross residual disease, while other *Lactobacillus* species are found in cases with > 1 cm
468 residual disease. Finally, ovarian cancer microbiome research also presents an opportunity for
469 microbial metagenomics and metabolomics to provide a fuller picture of the vaginal and gut
470 microbiome ecosystems in health and disease.
471

472 ## Acknowledgements

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Data Availability

The 16S rRNA V4 sequencing data for each sample are available on NCBI under project ID PRJNA662091. Bioinformatic scripts for processing the 16S rRNA V4 data are given in the Supplementary Material.

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Table 1(on next page)

Table 1: Study Group Demographics

Number of individuals belonging to each study group per clinical/lifestyle variable. The percent of individuals within each study category belonging to each metadata variable are presented in parentheses. Metadata variables that are significantly different between study groups are represented by [&] and Table S1 presents the p-value for each metadata variable.

	Study Group			
	Clinical/Lifestyle Variable	**PFI < 6 Months (n = 17)**	**PFI > 24 Months (n = 23)**	**Benign (n = 5)**
	Median Age (Range)	64.6 (38.5 - 82.7)	63.6 (45.5 - 77.9)	57.8 (33.7 - 79)
Self-Reported Ethnicity	**White (% of study group)**	15 (88.2%)	21 (91.3%)	3 (60%)
	African American (%)	1 (5.9%)	1 (4.3 %)	0 (0%)
	Native American (%)	1 (5.9%)	1 (4.3%)	2 (40%)
Cytoreductive Surgery (CRS)	**No Surgery (% of study group)**	1 (5.9%)	0 (0%)	4 (80%)
	Iterative CRS (%) &	13 (76.5%)	4 (17.4%)	1 (20%)
	Primary CRS (%) &	3 (17.6%)	19 (82.6%)	NA
	Median Months Last Platinum Cycle (range) &	7 (1 - 38)	57 (8 - 150)	NA
	Expected Estrogen Positive (%)	4 (23.5%)	9 (39.1%)	1 (20%)
	Take Probiotics	4 (23.5%)	4 (17.4%)	1 (20%)
	Vitamin supplement	6 (35.3%)	12 (52.2%)	1 (20%)
	Antibiotics within 6 months	14 (82.4%)	13 (56.5%)	3 (80%)
	Residual Disease > 1cm	6 (35.3%)	3 (13.0%)	NA

Figure 1

Figure 1: Heatmap of the 25 most abundant genera in the vaginal microbiome.

Each column represents a single individual's vaginal microbiome, color coded by study group. Colors of each cell are based on a heatmap, ranging from 0 reads of the bacteria in that individual (white) to 9000 reads of the bacteria in that individual (red). Samples were rarefied to 9000 reads, so bright red indicates every read in the sample comes from that bacteria. Samples were clustered together based on similarity of vaginal microbiome using Ward hierarchical clustering. 11 microbiomes were *Lactobacillus*-dominated, six *Escherichia*-dominated, three Gardnerella-dominated, nine *Prevotella*, and 16 highly diverse.

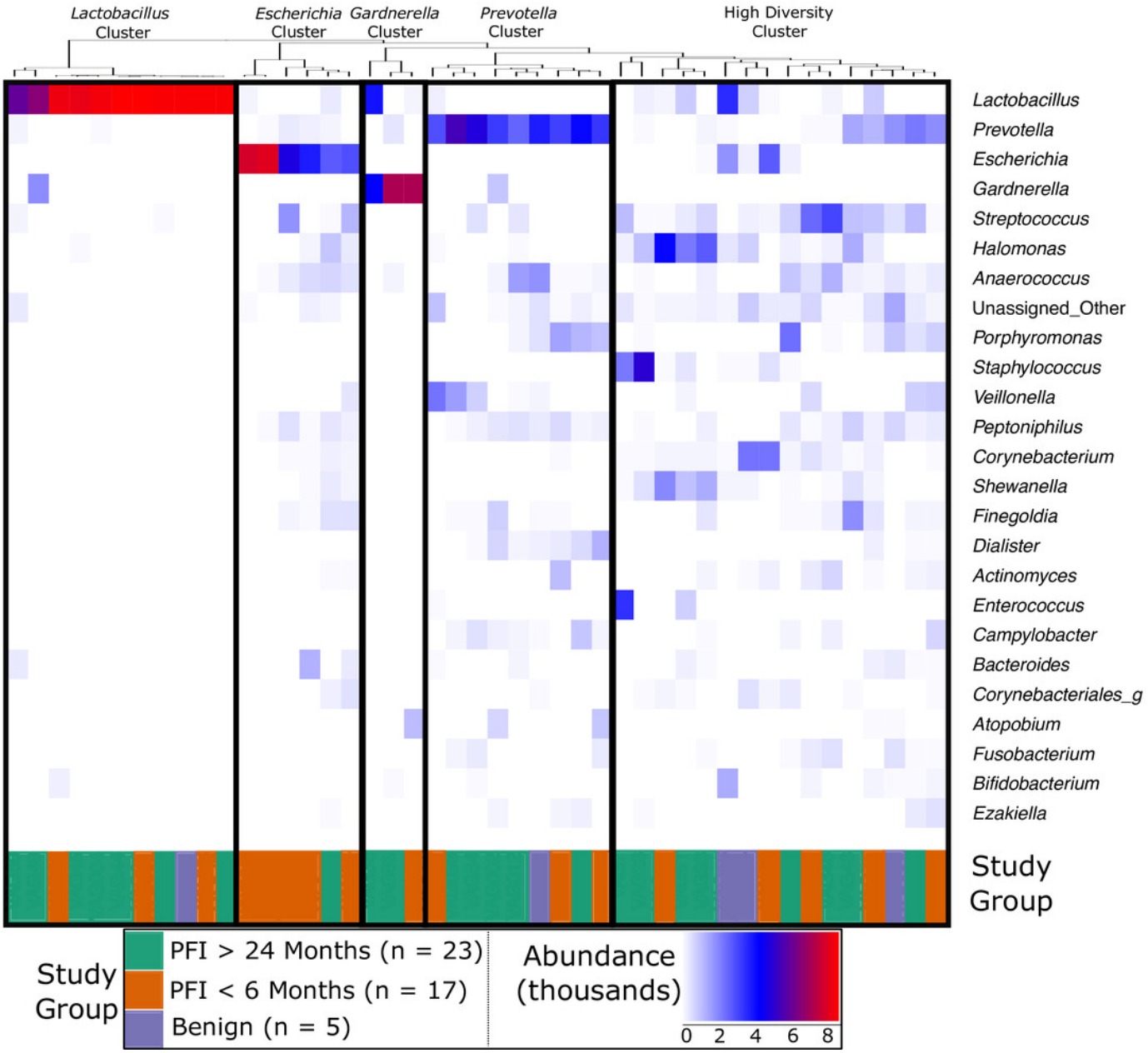
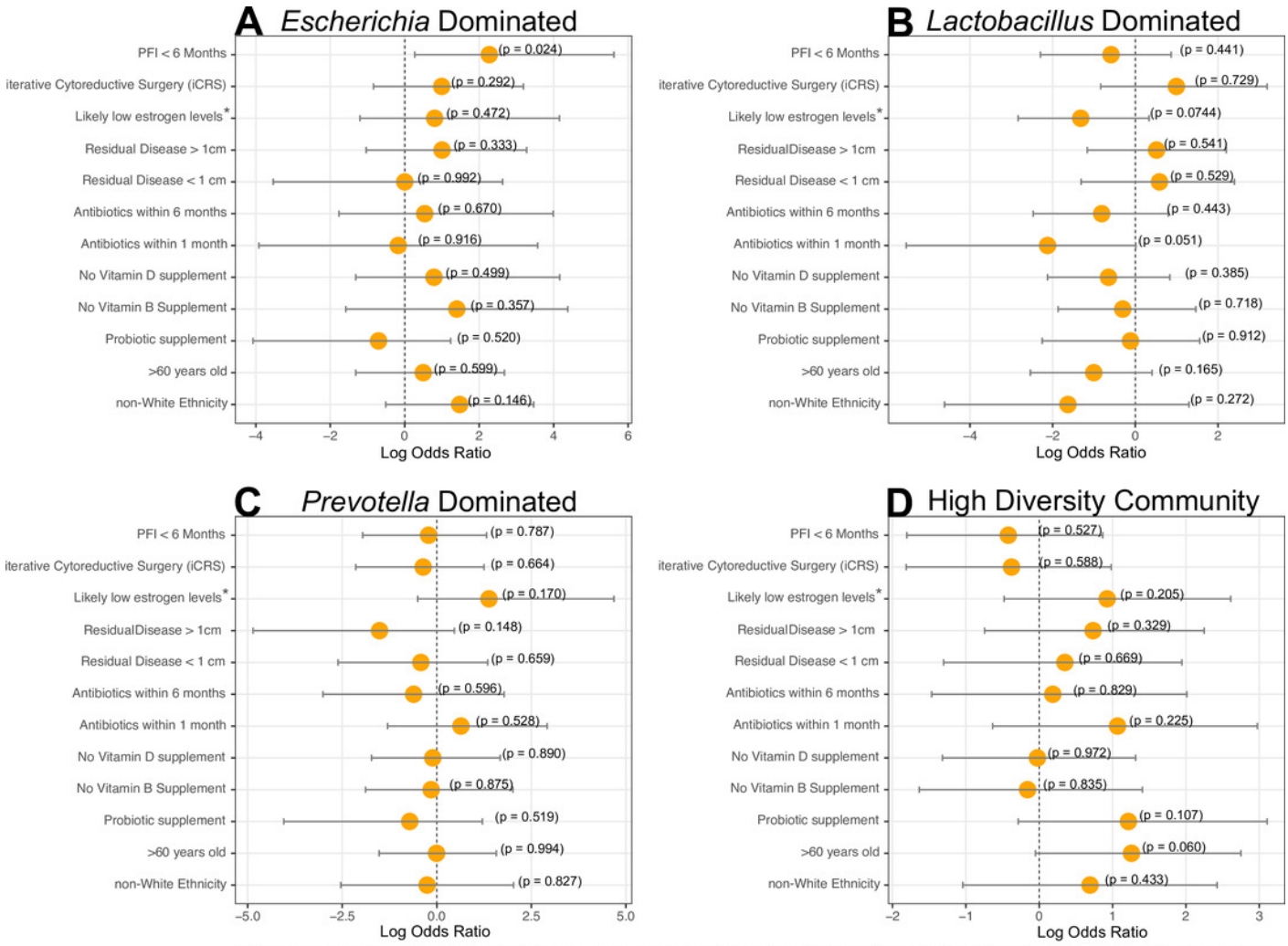

Lactobacillus Cluster
Escherichia Cluster
Gardnerella Cluster
Prevotella Cluster
High Diversity Cluster
Lactobacillus
Prevotella
Escherichia
Gardnerella
Streptococcus
Halomonas
Anaerococcus
Unassigned_Other
Porphyromonas
Staphylococcus
Veillonella
Peptoniphilus
Corynebacterium
Shewanella
Finegoldia
Dialister
Actinomyces
Enterococcus
Campylobacter
Bacteroides
Corynebacteriales_g
Atopobium
Fusobacterium
Bifidobacterium
Ezakiella
Study Group
Study Group
PFI > 24 Months (n = 23)
PFI < 6 Months (n = 17)
Benign (n = 5)
Abundance (thousands)
0 2 4 6 8

Figure 2

Figure 2A-D: Log-transformed odds ratio vaginal microbiome dominance.

Log-transformed odds are represented by the orange circle and the bars represent 95% confidence intervals. P-values are given for each odds ratio and a significant result is indicated when the 95% confidence interval is completely greater than 0 or completely less than 0. A) *Escherichia*-dominance had significantly higher odds of occurring in PFI < 6 months individuals (p-value = 0.024). B) Antibiotics within one month was negatively associated with *Lactobacillus*-dominance, but not significantly (p-value = 0.051). There were no differences between medical/health/lifestyle variables in *Prevotella* (C) and highly diverse (D) vaginal microbiomes. *Gardnerella*-dominated communities were not included in this analysis due to small sample size (n = 3).



* Postmenopausal women not on hormone replacement therapy were classified as having *likely low estrogen levels*

Figure 3

Figure 3: *Lactobacillus iners* dominates in small gross residual disease.

In individuals with Lactobacillus dominated vaginal microbiomes (n = 11), *L. iners* was at significantly higher relative abundance (p-value = 0.0359) in patients with no gross residual disease or residual disease under 1 cm.

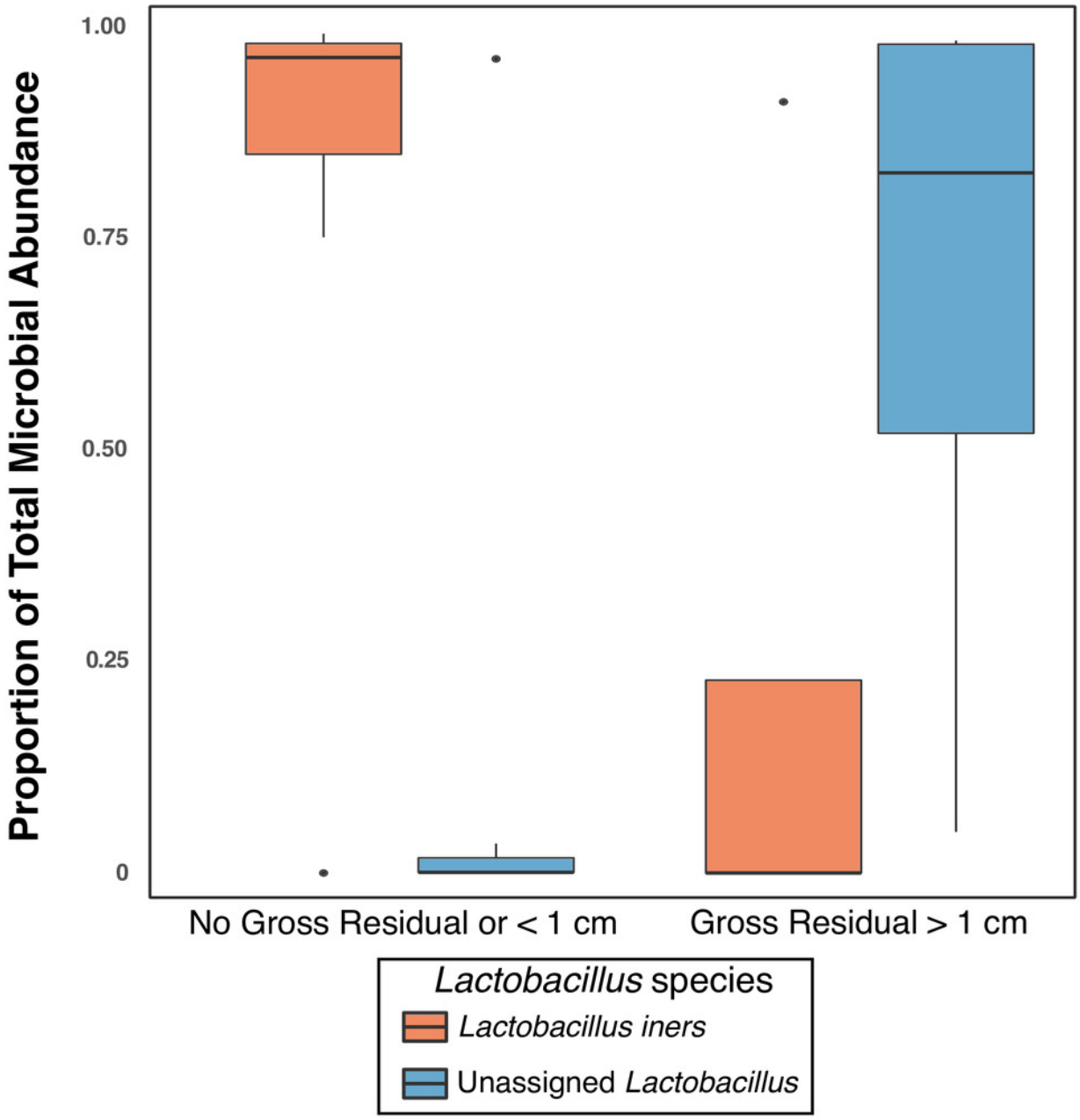

Proportion of Total Microbial Abundance
1.00
0.75
0.50
0.25
0
No Gross Residual or < 1 cm
Gross Residual > 1 cm
Lactobacillus species
Lactobacillus iners
Unassigned Lactobacillus

Figure 4

Figure 4: Unweighted UniFrac distances (PC1 and PC2) of fecal microbiomes from women with ovarian cancer.

Each shape represents a single sample and shapes clustering together have similar gut microbiome taxonomic composition. There was no significant difference in overall microbiome community structure between sample groups (PERMANOVA p-value > 0.05); however, there are nine samples (6 PFI < 6 months, 3 PFI > 24 months) that form an outlier group along PC1. These individuals are labelled.

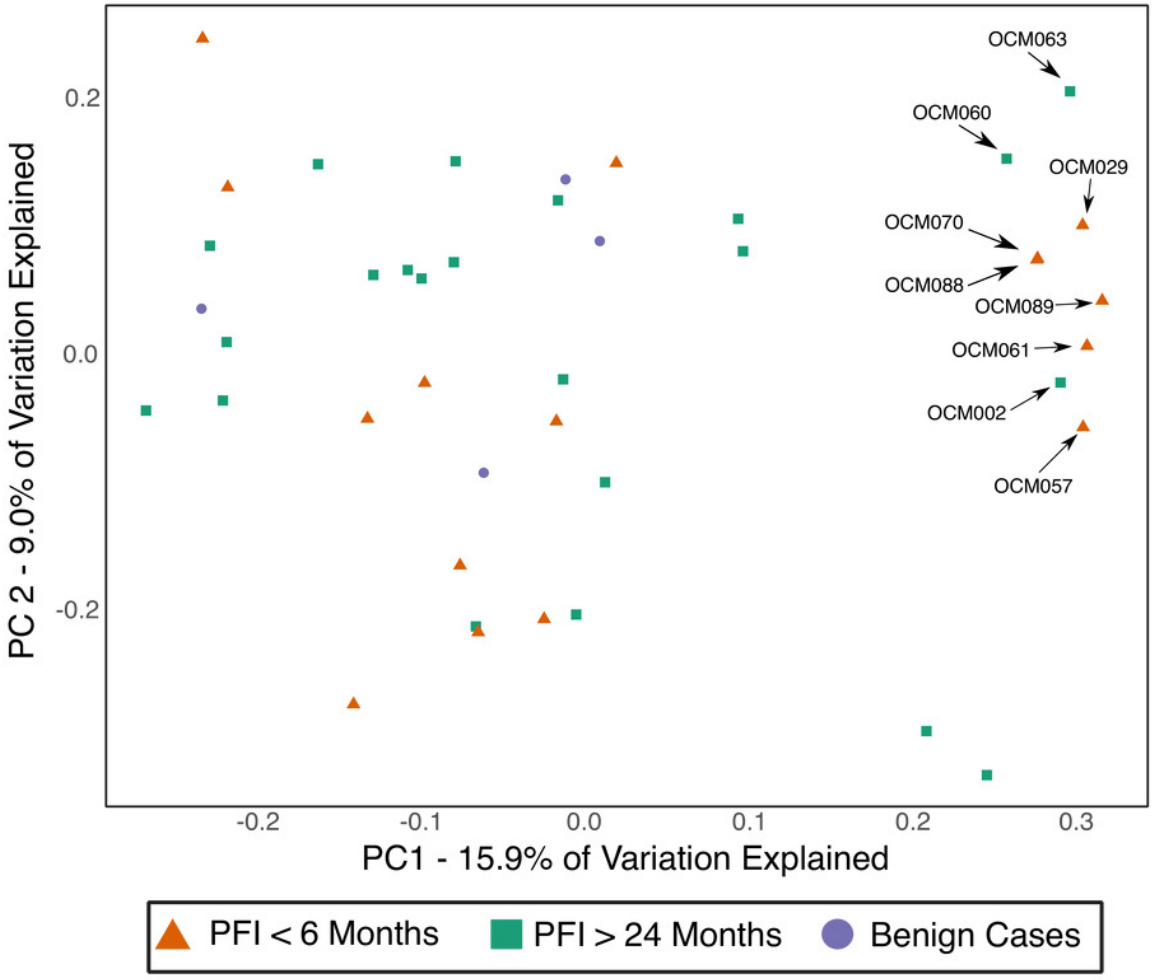
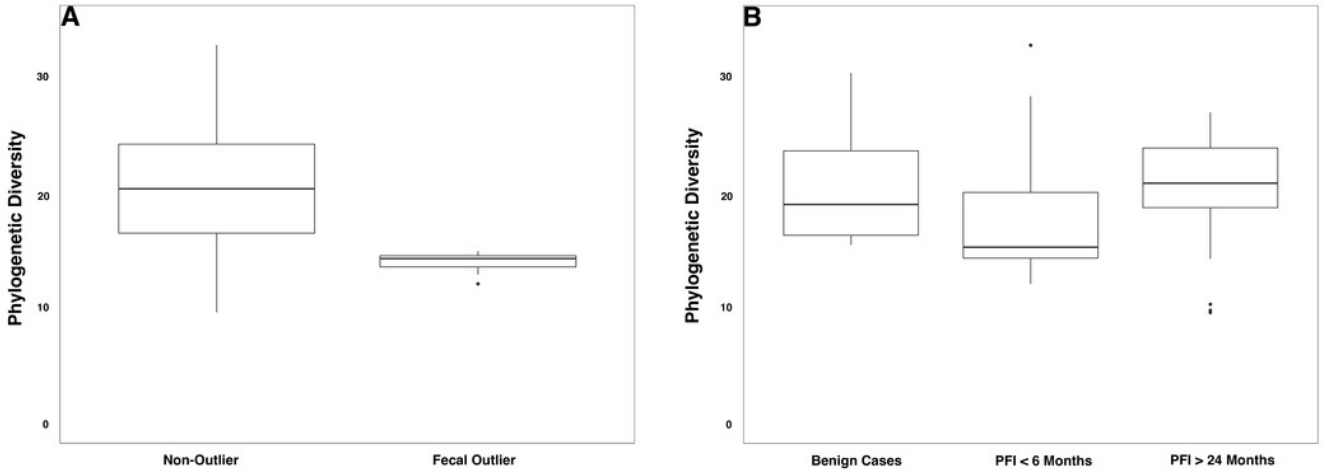


Figure 5

Figure 5A-B: Phylogenetic diversity in fecal microbiomes.

A) Samples that formed the outlier group in Figure 4 (n = 9) had lower phylogenetic diversity compared to the remainder of the gut microbiome samples (p-value = 0.0001). B) Patients with PFI < 6 months had lower phylogenetic diversity than benign and platinum-sensitive patients but this was not a significant result (p-value = 0.18).