Evaluation of the Gut Microbiome and Sex Hormones in Postmenopausal Women with Newly Diagnosed Hormone Receptor-Positive Breast Cancer Versus Healthy Women: A Prospective Case-Control Study

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Abstract

Purpose

The functional composition and diversity of the gut microbiome (estrobolome) may affect breast cancer risk by modulation of systemic sex hormones. Gut bacteria with β-glucuronidase enzymatic activity may deconjugate estrogens, leading to increased estrogen reabsorption into the circulation thereby increasing breast cancer risk. We investigated the relationship between the gut bacterial microbiome and endogenous estrogens and related sex hormones in women with hormone receptor-positive breast cancer compared to healthy control women.

Methods

In this prospective case-control study, postmenopausal women (n = 46) with newly diagnosed stage I-III estrogen and/or progesterone receptor-positive breast cancer were compared with healthy postmenopausal female controls (n = 22). Bacterial composition of the gut microbiome was analyzed by 16S rRNA gene sequencing from fecal specimens. Plasma and urine sex hormones were quantified using high-performance liquid chromatography/mass spectrometry.

Results

We found evidence that some β-glucuronidase positive bacteria were enriched in the breast cancer patients compared to healthy controls, whereas abundances of some β-glucuronidase negative bacteria were reduced. There was also a wide distribution of prevalence of β-glucuronidase positive taxa in both the breast cancer subjects and healthy controls, as well as higher probability of breast cancer subjects having higher average β-glucuronidase levels. Significant differences were found in endogenous progesterone levels between the breast cancer patients and healthy controls.

Conclusion

This pilot study showed differences in the gut microbiome and endogenous progesterone levels among postmenopausal women with hormone receptor-positive breast cancer compared with healthy controls. These interesting findings may have implications for breast cancer risk and prevention and warrant further exploration.

Background

There has been growing interest in the functional role of the gut microbiome in malignancy. The human gut microbiome exerts both local and distant effects involving hormonal intermediates, metabolites, and immunologic pathways [1, 2]. Interactions between the human host and microbes have the potential to

influence carcinogenesis through mechanisms such as chronic inflammation, metabolism, induction of genotoxic responses, and alteration of the microenvironment [3, 4]. This may be mediated by the microbial ecosystem as a whole or by specific microbes, as in the case with bacterium, *Helicobacter pylori*, which is associated with an increased risk of stomach adenocarcinoma. We postulated that certain sex hormone-conjugate hydrolyzing gut bacteria microbiota could contribute to breast cancer risk by increasing circulating sex hormone levels.

In the United States, breast cancer affects one in eight women and is the second leading cause of cancer-related deaths in females [5]. The most common type of breast cancer is estrogen and/or progesterone receptor-positive (i.e., hormone receptor-positive), which comprises approximately seventy percent of patients, with the majority occurring in postmenopausal women [6, 7]. Epidemiologic studies have demonstrated a significant association between endogenous estrogen levels and risk for hormone receptor-positive breast cancer in postmenopausal women [8, 9], and the combination of exogeneous estrogens with progesterone also increased risk in the Women's Health Initiative trials [10]. On the other hand, the role of endogenous progesterone in breast cancer carcinogenesis is less well defined [11].

The 'estrobolome' is the aggregate of intestinal bacteria capable of metabolizing estrogens [12, 13]. Estrogens are primarily produced by the ovaries in premenopausal women and by the adrenal glands and adipose tissue in postmenopausal women. They circulate in the bloodstream in free or protein-bound form and first undergo metabolism primarily in the liver, where they are conjugated (sulfation and glucuronidation), oxidized, reduced, and/or methylated [14, 15]. The water-soluble conjugated estrogens are excreted from the body through the kidneys into urine or via bile into the feces [16]. However, some of the conjugated estrogens excreted in bile can be deconjugated by resident bacterial taxa in the gut with β-glucuronidase enzymatic activity, subsequently leading to estrogen reabsorption into the circulation [17–20]. Modulation of estrogen homeostasis through the enterohepatic circulation by the gut microbiome can differ amongst individuals [21–23]. It is important to investigate the composition of the gut estrobolome in breast cancer as a potential target for therapeutic or preventative interventions such as probiotics, antibiotics, and dietary modifications.

We hypothesized that the gut microbiome is different in women with hormone receptor-positive breast cancer compared with healthy control women. The bacterial composition of the estrobolome may be affected by host factors such as age, ethnicity, and body mass index (BMI), as well as lifestyle and dietary habits [24–26]. We designed a prospective case-control study with a carefully defined patient population and assessments to investigate the relationship between the gut bacterial microbiome with β-glucuronidase activity and endogenous sex hormones. We postulated that the gut microbiome in postmenopausal women with breast cancer is enriched in bacterial taxa with β-glucuronidase activity compared to control women, and differences in endogenous estrogens and related sex hormones are associated with variability in gut microbial diversity between the women with breast cancer and those without. We found evidence that some bacteria with β-glucuronidase activity are enriched in the breast cancer subjects, although there was a wide distribution seen of β-glucuronidase positive taxa in the

microbiome of both groups. Significant differences in endogenous progesterone levels between the breast cancer subjects and healthy controls were also found.

Methods

Breast Cancer and Healthy Control Subjects

Postmenopausal women with newly diagnosed histologically-confirmed breast cancer that was estrogen receptor (ER) positive and/or progesterone receptor (PR) positive and human epidermal growth factor receptor 2 (HER2) negative with resected stage I to III disease were eligible, prior to adjuvant endocrine therapy. For healthy controls, postmenopausal women without a history of malignancy were eligible. Women were defined as postmenopausal if they were at least 60 years of age, had undergone bilateral oophorectomy, or were younger than 60 years of age with cessation of regular menses for at least twelve consecutive months and plasma levels of estradiol and follicle-stimulating hormone in the postmenopausal range. Exclusion criteria included medical illnesses with potential suppressive or activating impact on immune and bowel function as determined by the treating investigator, systemic antibiotic or probiotic use within six months, use of hormone-replacement therapy within the past twelve months, or a history of gastric or intestinal surgery.

Study Design

This was a prospective single institution case-control pilot study. Postmenopausal women with breast cancer were recruited from breast medical oncology practices at the NYU Langone Health's Perlmutter Cancer Center (PCC) during routine office visits with their oncologists to discuss and/or initiate adjuvant endocrine therapy with an aromatase inhibitor. Postmenopausal women without breast cancer (controls) were recruited from three subject pools: (a) relatives and/or friends of patients; (b) women undergoing routine mammographic screening at PCC's facilities; and (c) female faculty or staff members. All subjects with breast cancer were planned to receive adjuvant endocrine therapy with an aromatase inhibitor (i.e., anastrozole, letrozole, or exemestane). Enrolled breast cancer subjects provided serial plasma, stool, and urine samples at baseline (prior to initiation of endocrine therapy for the breast cancer patients), and then at 1, 3, 6, and 12 months while on endocrine therapy. Samples were collected at identical time points for the control subjects, of which none received endocrine therapy while enrolled on study. Analyses are reported on the baseline samples in this publication. The study was approved by the NYU Grossman School of Medicine Institutional Review Board (IRB). Written informed consent was obtained from all subjects prior to enrollment in the study. Subjects who completed the study received a one hour-massage at the NYU Integrative Health Center in appreciation for their participation.

Sample And Data Collection

Sample collection (plasma, urine, and stool) followed standardized procedures. After providing informed consent, study subjects were provided with a specimen collection kit with instructions for collecting a fecal sample at home, and materials to transport the specimens to NYU PCC. Fecal specimens were collected in four 20-mL screw top Sarstedt tubes, including two preloaded with 5 mL RNAlater (QIAGEN) and two with 5 mL sterile PBS. After specimen collection, the fecal tubes were secured, and two frozen gel packs were added. Urine was collected in a screw-top container, without preservative in the clinic. Blood samples were obtained by venipuncture in the clinic and collected in heparin containing vacutainer tubes. All specimens were transported to the NYU biorepository, where they were processed and stored at − 80°C. The urine and plasma specimens were shipped on dry ice in batches to the laboratory (A.F.) at University of Hawaii and stored at − 80°C until analysis.

Subjects in both groups also completed a questionnaire at baseline requesting information about age, height and weight, ethnicity, medical history of immune or gastrointestinal disorders, medication use including antibiotics within past the six months, diet, alcohol use, and smoking history (**Online Resource 1**). Follow-up questionnaires were completed at each subsequent time point inquiring about any changes in the above. Subjects were also followed for development of breast cancer recurrence or new malignancies.

Fecal Microbiome Analysis

The bacterial composition of the gut microbiome was assessed using 16S ribosomal RNA gene sequencing of subject fecal samples. FASTQ files were preprocessed with QIIME2 (v. 2018.11) [27]. Following the data input, sequences underwent an error correction step with *qiime dada2 denoise-paired* (parameters: –p-trunc-len-f 0, –p-trunc-len-r 0, –p-trim-left-f 20, –p-trim-left-r 20) command. We then assigned taxonomy to the sequences by training a Naïve Bayes classifier on the V4 region of the 16S rRNA gene with *qiime feature-classifier fit-classifier-l-bayes command* based on the GreenGenes database (v 13_8). Following the taxonomy assignment, the abundance table was rarefied to the depth of 20,000 sequences and collapsed on both genus and species taxonomic levels. All downstream analysis including α-diversity, β-diversity and microbial composition was performed in R (v. 3.5.2). LefSe (Linear discriminant analysis Effect Size) [28] was used to find bacteria that explain differences between breast cancer and healthy control fecal samples with the following parameters: 0.05 alpha value and an LDA score threshold of 2.

Sex Hormone Analysis

The 11 most predominant steroidal estrogens in women, namely estrone (E1), estradiol (E2), 2-hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 2-methoxyestrone (2-MeOE1), 2-hydroxy-3-O-methylestrone (2OH-3MeO-E1), 4-hydroxyestrone (4-OHE1), 4- hydroxyestradiol (4-OHE2), 16α-hydroxyestrone (16α-OHE1), 16-ketoestradiol (16keto-E2), and estriol (E3) [29] as well as progesterone, and testosterone were measured from plasma and urine by our validated orbitrap liquid chromatography-

mass spectrometry (LCMS) (model Q-Exactive, Thermo Scientific, Waltham, MA) assay, as described in detail previously [30].

In brief, plasma or urine were mixed with ascorbic acid as preservative and deuterated or 13C labeled analytes as internal standards followed by enzymatic hydrolysis with beta-glucuronidase and sulfatase for total analyte levels (conjugated plus unconjugated analytes) or without hydrolysis for unconjugated analytes followed by extraction with methyl tertiary butyl ether. The dried extract was derivatized with 1-methylimidazole sulfonyl chloride in sodium bicarbonate followed by analysis of the tagged analytes with high-resolution accurate-mass mass spectrometry in positive mode after electrospray ionization using monoisotopic protonated analyte masses ±5ppm to account for measurement inaccuracies as detailed previously [30]. Urinary creatinine levels were determined using a clinical autoanalyzer (Roche-Cobas MiraPlus CC) and a kit from Randox Laboratories (cat. No. CR 510, Crumlin, UK) based on the Jaffé reaction with a lower limit of quantitation of < 15 μM. In this study, we found inter- and intra assay cv values of 0.8% at 187 μM. Urinary concentrations were adjusted for creatinine levels to account for differences in urine volume.

Circulating sex hormone binding globulin (SHBG) levels were measured with a well validated enzyme linked immunosorbent assay kit from R&D Systems, Inc. (Minneapolis, MN, kit# DSHBG0B, lot# P248238) following exactly the manufacturer's protocol.

Differences in measured analyte levels between cases and controls were evaluated by the unpaired t-test with common variance (Microsoft Excel version 16.54).

Results

Study Participants

A total of 60 breast cancer patients and 25 healthy controls enrolled in the study and provided plasma, urine, and stool samples. Fourteen breast cancer patients and 3 control subjects withdrew study consent prior to completion of serial sample collection over one year, and the analyses are therefore performed on 46 and 22 subjects, respectively. Subject demographic data and lifestyle factors are shown in Table 1 (n = 68). The breast cancer patients had a mean age of 64 (range 45–84 years), and the controls had a mean age of 58 (range 51–68). The majority of subjects were Caucasian (63–64% in both groups). African American patients comprised 15% of the breast cancer patients and 14% of the controls, and Asian patients made up 11% and 14%, respectively. In both groups, 9% of subject were Hispanic. For the breast cancer patients, the mean BMI was 26.3 kg/m^2 (range 18.0-37.1), and for the controls, the mean BMI was 25.6 kg/m^2 (range 18.9–38.7).

Table 1
Demographic and lifestyle factors for breast cancer cases and controls (n = 68)

	Breast Cancer Cases (n = 46)	**Controls (n = 22)**	**p-value**
Mean age (range)	64 (45–84)	58 (51–68)	**0.009***
Race (%)			
Caucasian	29 (63%)	14 (64%)	0.97
African American	7 (15%)	3 (14%)	0.87
Hispanic	4 (9%)	2 (9%)	0.96
Asian	5 (11%)	3 (14%)	0.76
Native Hawaiian or Other	1 (2%)	0 (0%)	0.49
Mean BMI [kg/m^2] (range)	26.3 (18.0-37.1)	25.6 (18.9–38.7)	0.59
Smoking (%)			
Yes	1 (2%)	2 (9%)	0.20
No	45 (98%)	20 (91%)	
Diet (%)			
Vegetarian	4 (9%)	4 (18%)	0.29
Probiotic Use	11 (24%)	5 (23%)	0.92
Alcohol Use (%)	20 (43%)	12 (55%)	0.53
Number drinks/week (range)	1–21	0.5-7	

The majority of subjects in both groups did not smoke (98% in the breast cancer group and 91% in the control group). About 44% of breast cancer patients and 55% of controls reported drinking alcohol, although there were large variations in the amount consumed (number of drinks per week ranged from 1–21 for the breast cancer patients and 0.5-7 for the controls). Regarding diet, 9% of the breast cancer patients were vegetarian versus 18% in the controls. A similar percentage of patients in both groups used probiotics (24% in the breast cancer patients and 23% in the controls).

Breast Cancer Patients And Tumor Characteristics

Clinical and tumor characteristics of the breast cancer patients (n = 46) are shown in Table 2. They had an ECOG performance status of 0 (52%) or 1 (48%). Invasive ductal carcinoma was the most common tumor histology at 85%, with the other 15% of tumors being invasive lobular carcinoma. The majority of tumors were grade 2 (moderately differentiated) at 63%, followed by grade 3 (poorly differentiated) at

22%, and grade 1 (well differentiated) at 15%. Seventy percent of patients had stage 1 disease, 28% had stage 2, and 2% has stage 3. All tumors (100%) were ER-positive and 89% were PR-positive. After surgery, four patients received chemotherapy and sixteen patients underwent radiation therapy to the breast prior to enrollment.

Table 2
Clinical and tumor characteristics of breast cancer cases (n = 46)

Characteristic	Number (%)
ECOG PS	
0	24 (52%)
1	22 (48%)
Tumor Histology	
Invasive Ductal Carcinoma (IDC)	39 (85%)
Invasive Lobular Carcinoma (ILC)	7 (15%)
Tumor Grade	
1 (well differentiated)	7 (15%)
2 (moderately differentiated)	29 (63%)
3 (poor differentiated)	10 (22%)
Pathologic Staging	
T1N0	31 (67%)
T2N0	9 (20%)
T1N1	1 (2%)
T2N1	4 (9%)
T3N1	1 (2%)
Overall Stage	
I	32 (70%)
II	13 (28%)
III	1 (2%)
Tumor Biomarkers	
ER positive	46 (100%)
PR positive	41 (89%)
HER2 positive	1 (2%)
Radiation Received Prior to Enrollment	16 (35%)
Chemotherapy Received Prior to Enrollment	4 (9%)

New Malignancies

Three of the breast cancer patients were later diagnosed with another malignancy after completion of longitudinal sample collection. One patient developed multiple myeloma. The second patient who was later found to have a germline mutation in the BRCA 2 gene was diagnosed with pancreatic cancer. The third patient developed both a triple-negative (hormone receptor negative and HER2-negative) breast cancer as well as rectal cancer. Of the healthy control subjects, one was diagnosed with ductal carcinoma in situ (DCIS) of the breast that was hormone receptor-positive.

Gut Microbiome Results

We first analyzed bacterial compositions in subject stool samples and calculated alpha- (within a sample) and beta- (between samples) diversity metrics. There were no significant differences in alpha diversity between breast cancer and healthy control participants (Fig. 1). In addition, we investigated whether there was a separation based on alpha diversity between tumor grade and cancer stage and found no significant differences between groups (**Online Resource 2**). We then compared sample composition by principal coordinate analysis using Bray-Curtis distance and found no clustering by our metadata variables (e.g., breast cancer vs healthy control, cancer stage, tumor grade) (Fig. 2). Of note, the two breast cancer patients that were later diagnosed with myeloma and pancreatic cancer, respectively, were found in opposite extremes on the first principal component and separated from healthy or other breast cancer subjects (Fig. 2d, **Online Resource 3**).

We then examined the relative microbial abundance on the species level of the most abundant species (abundance > 0.5%) in the two groups (Fig. 3, Fig. 4). To investigate whether these differences in taxa composition were related to differences in progesterone level (the only sex hormone found to be significantly different between cohorts, see section below), we looked at the relative taxa abundance in each sample sorted by progesterone level (Fig. 4, **Online Resource 4**). We identified a few samples dominated by one or two bacterial taxa (e.g., *Blautia and* Ruminococcaceae). We also observed altered microbial composition in samples from two breast cancer subjects with the highest progesterone levels in the urine (Fig. 4, Subjects X & Y). In order to identify microbes with statistically different abundance between breast cancer subjects and healthy controls, we used Linear discriminant analysis Effect Size analysis [31]. This revealed that *Bifidobacterium animalis* was overrepresented in breast cancer patients, while eight species were found to have higher abundance in healthy controls (Fig. 5a). *B. animalis* can produce beta glucuronidase, and therefore contribute to the deconjugation of estrogen, leading to reabsorption in the circulation.

To analyze the potential estrogen-modulatory capacity of the different microbial compositions across our samples, we combined the relative abundances of all bacteria reported to possess beta glucuronidase genes [32]. The distribution of beta glucuronidase positive bacteria in samples from breast cancer subjects was slightly skewed towards larger values compared with samples from the healthy controls

(Fig. 5c, 5d, **Online Resource 5**); however, this difference was not statistically significant. Interestingly, both, breast cancer and healthy subjects showed wide distributions of the prevalence of beta-glucuronidase positivity, reaching from 0 to 33% in healthy controls and 0 to 49% in breast cancer subjects (Fig. 5c, 5d). To account for this wide variability, we conducted a Bayesian analysis to estimate the difference in the average abundances of beta glucuronidase positive bacteria indicating 74% probability that breast cancer subjects have higher average beta glucuronidase levels (**Online Resource 5**). Additionally, samples were split by progesterone tertiles to determine if any particular taxa were associated with progesterone levels. We identified four taxa (Erysipelotrichaceae, Lactobacillaceae, *Turicibacter* and *Anaerostipes*) with significantly altered abundance. Specifically, the highest progesterone tertile group had increased relative abundance of *Erysipelotrichaceae* and *Lactobacillaceae* and reduced relative abundance of *Turicibacter* and *Anaerostipes* (Fig. 6).

Sex Hormone Results

The breast cancer subjects were found to have a significantly higher progesterone level in the urine ($p = 0.036$) compared to controls **(**Table 3a**).** There was also a trend towards lower plasma progesterone in the breast cancer cases ($p = 0.054$) **(**Table 3b**).** Analyses of plasma and urine estrogens (parent estrogens and metabolites) and testosterone levels did not reveal significant differences between both groups and this applied also to circulating SHBG.

Table 3
a Urinary steroid excretion results

MEAN				
	Cases (pg/mg) n = 46	Controls (pg/mg) n = 22	Case/Control (%)	P*
E1	4.5	5.1	89%	0.57
E2	1.2	1.3	93%	0.76
2-OHE1	7.5	8.4	89%	0.71
2-OHE2	1.4	1.5	95%	0.85
4-OHE1	1.1	1.5	72%	0.30
16-OHE1	1.3	1.3	105%	0.86
16-ketoE2	0.7	3.3	21%	0.12
2-MeOE1	1.3	2.5	52%	0.17
E3	5.6	5.0	112%	0.67
Test	3.8	3.9	96%	0.82
Prog	0.2	0.1	181%	**0.036**

Table 3
b Plasma steroid and SHBG results

MEAN				
	Cases [stdev] n = 46	**Controls [stdev] n = 22**	**Case/Control**	**P***
E1				
Total (pg/mL)	199 [177]	195 [139]	102%	0.92
Total/SHBG (pg/pmol)	6 [12]	4 [4]	144%	0.50
Unconjug (pg/mL)	11[37]	10 [37]	103%	0.84
Unconjug/SHBG (pg/pmol)	0.23 [0.24]	0.21 [0.17]	106%	0.81
Free (pg/mL)	0.294 [0.193]	0.282 [0.156]	104%	0.81
E3				
Total (pg/mL)	15 [24]	17 [37]	87%	0.77
Total/SHBG (pg/pmol)	0.39 [0.99]	0.33 [0.54]	119%	0.78
Unconjug (pg/mL)	n/a	n/a		
Unconjug/SHBG (pg/pmol)	n/a	n/a		
Free (pg/mL)	n/a	n/a		
Test				
Total (pg/mL)	136 [112]	175 [175]	78%	0.27
Total/SHBG (pg/pmol)	3.2 [5.3]	3.4 [3.5]	94%	0.87
Unconjug (pg/mL)	39 [72]	44 [80]	88%	0.80
Unconjug/SHBG (pg/pmol)	1.0 [3.0]	0.9 [1.7]	114%	0.83
Free (pg/mL)	1.8 [2.1]	2.1 [2.2]	84%	0.54
Prog				
Total (pg/mL)	43 [44]	78 [108]	55%	0.054
Total/SHBG (pg/pmol)	0.9 [1.1]	1.6 [2.2]	56%	0.10
Unconjug (pg/mL)	33 [21]	51 [98]	65%	0.22
Unconjug/SHBG (pg/pmol)	0.7 [0.7]	1.1 [1.8]	63%	0.20
Free (pg/mL)	n/a	n/a		
SHBG (pmol/mL)	70 [42]	63 [37]	111%	0.50

Conclusions

A potentially important role of the gut microbiome (estrobolome) is the modulation of estrogen homeostasis through the enterohepatic circulation of estrogens, which may affect breast cancer risk. In this study, we investigated whether the pretreatment gut microbiome differed in postmenopausal patients with hormone receptor-positive breast cancer versus healthy subjects, while controlling for potential confounders and focusing upon bacterial taxa with β-glucuronidase activity. We evaluated gut microbiome diversity by analyzing how many different taxa are present, as well as how evenly distributed the taxa are amongst the two cohorts. When comparing the percentages of β-glucuronidase positive bacteria, there was no statistically significant difference, however there was evidence that some bacterial taxa with β-glucuronidase were enriched in the breast cancer patients. The breast cancer subjects had a higher abundance of *Faecalibactreium prausnitzi, Bacteroides* species, and *Bifidobacterium animalis*, which contain β-glucuronidase activity, whereas abundances of several taxa without β-glucuronidase activity were reduced, like *Blautia*, *Coprococcus*, *Roseburia faecis* and *Bifidobacterium adolescentis*. Interestingly, we also discovered a wide distribution of prevalence of β-glucuronidase positive taxa in both the breast cancer subjects and healthy controls. This suggests a wide range of potentially important effects of the gut microbiome's metabolic capacity on estrogen metabolism and host physiology, and this finding warrants further investigation. In comparison, a pilot study investigating differences in the gut microbiome among postmenopausal women found a less diverse microbiome and significantly altered microbiota composition in newly diagnosed breast cancer patients prior to treatment compared with healthy controls [33]. Levels of systemic estrogens were also higher in the breast cancer patients, although these were independent of differences in the microbiome, suggesting that the gut microbiota may affect breast cancer risk through an estrogen-independent pathway.

In our study, the breast cancer subjects unexpectedly did not have significant differences in systemic estrogen levels compared to the control subjects. The reason behind this is unclear, and may be a reflection of the small sample size. Intriguingly, endogenous progesterone levels were significantly higher in the urine of breast cancer patients compared to healthy controls, and there was a nonsignificant trend toward lower plasma levels in the patients. Estrogen and progesterone are both involved in breast development during puberty, primarily through a paracrine mechanism that leads to cellular proliferation in the mammary gland [34]. Epidemiologic studies have shown a well-established increase in breast cancer risk with use of exogenous progestogens (progestins) administered together with estrogen as menopausal hormone replacement therapy [35, 36]. In contrast, the role of endogenous progesterone in breast cancer carcinogenesis is not well defined, and few studies have assessed circulating progesterone levels and breast cancer risk. One study of postmenopausal women that included over three hundred breast cancer cases and six hundred matched controls did not demonstrate an association between progesterone levels and risk for breast cancer [37]. However, this data was limited by close to 30 percent of samples having undetectable levels per the assay used for analysis. Another study in premenopausal women found a modest reduction in breast cancer risk with higher circulating progesterone levels [38]. The low levels of circulating progesterone levels in postmenopausal women and inadequate sensitivity of some assays can pose a challenge for detection, although with advances in assay technology, the assay

used in our study was adequately sensitive to detect a difference in progesterone levels. Whether progesterone levels are affected by the gut microbiome or vice versa is also unclear; we did observe an altered microbial composition in the fecal samples of two breast cancer subjects with the highest progesterone levels. Importantly, while progesterone assays capture progesterone levels in circulation at the time, they cannot assess the flux of progesterone, i.e., the rates of increase or decrease that are potentially affected by microbial metabolism in addition to homeostatic regulation. Overall, our results suggest a potential role for endogenous progesterone in postmenopausal breast cancer risk; this and a potential modulating role of the microbiome warrants further study.

Limitations of our pilot study include its small sample size, potential for selection bias, and case-control design, which preclude the ability to demonstrate causation. Four of our breast cancer subjects also received chemotherapy prior to enrollment, which may have altered the gut microbiome. Nonetheless, our findings provide an early step toward a better understanding of the role that bacteria with estrogen modulating activity may play in the risk of hormone-driven malignancies like breast cancer. We also plan to prospectively study the longitudinal role of estrogen deprivation during aromatase inhibitor therapy on the gut microbiome in our breast cancer subjects. We hope that our research will identify characteristics of the gut microbiome that could be used to develop novel and personalized approaches for breast prevention and treatment.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Maryann Kwa, Kelly Ruggles, Jonas Schluter, Adrian Franke, Karina Flores, Angelina Volkova, Adriana Heguy, and Sylvia Adams. The first draft of the manuscript was written by Maryann Kwa, Kelly Ruggles, Jonas Schluter, Adrian Franke, Angelina Volkova, and Sylvia Adams, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available due to individual privacy concerns but are available from the corresponding author on reasonable request.

Ethics Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the NYU Grossman School of Medicine Institutional Review Board.

Consent to Participate

Informed consent was obtained from all individual participants included in the study.

Consent to Publish

The authors affirm that human research participants provided informed consent for this publication.

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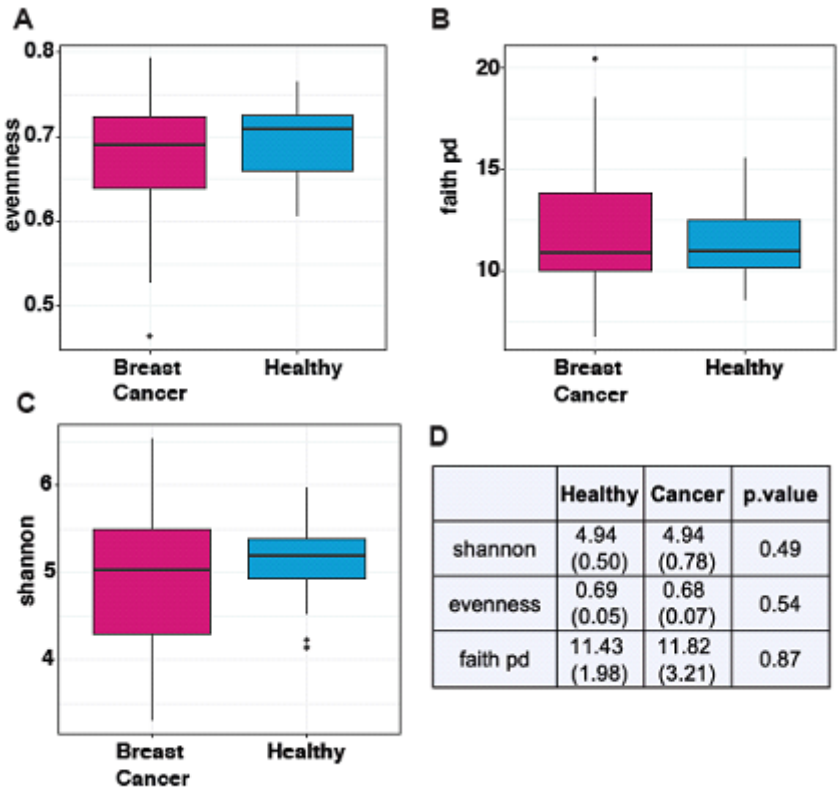
Figures



	Healthy	Cancer	p.value
shannon	4.94 (0.50)	4.94 (0.78)	0.49
evenness	0.69 (0.05)	0.68 (0.07)	0.54
faith pd	11.43 (1.98)	11.82 (3.21)	0.87

Figure 1

Alpha diversity of breast cancer (BC) and healthy control participants (HP)

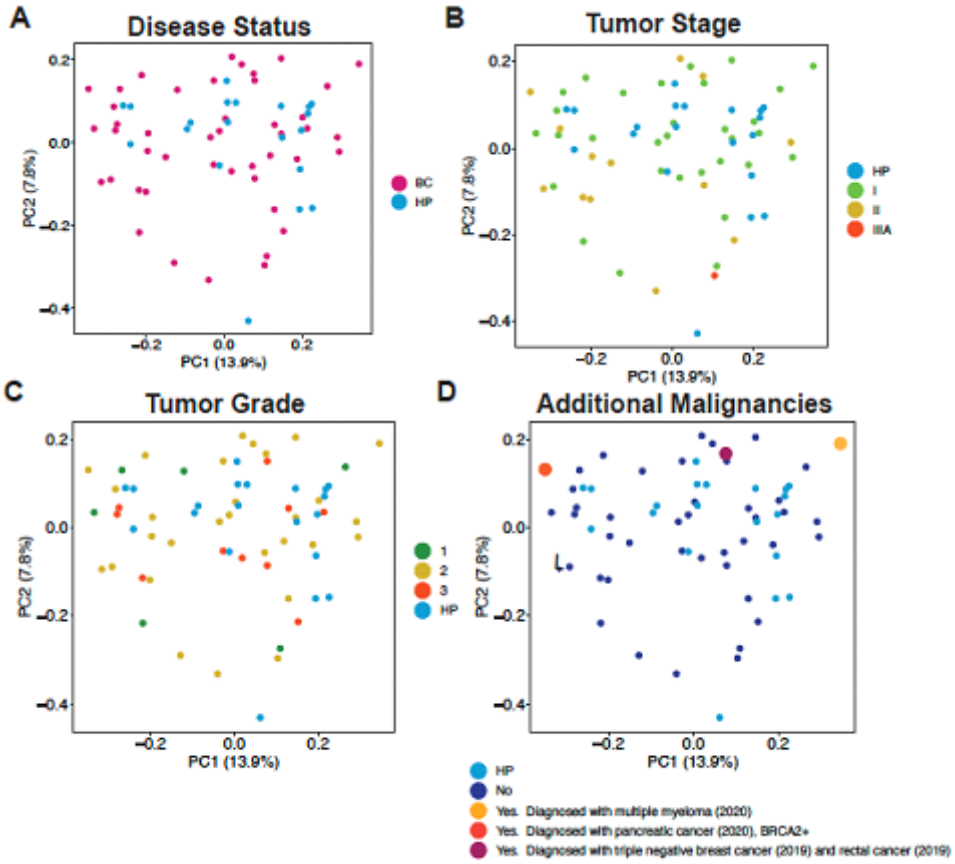


Figure 2

Beta diversity of breast cancer (BC) and healthy control participants (HP) by disease status (a), tumor stage (b), tumor grade (c), and additional malignancies (d)

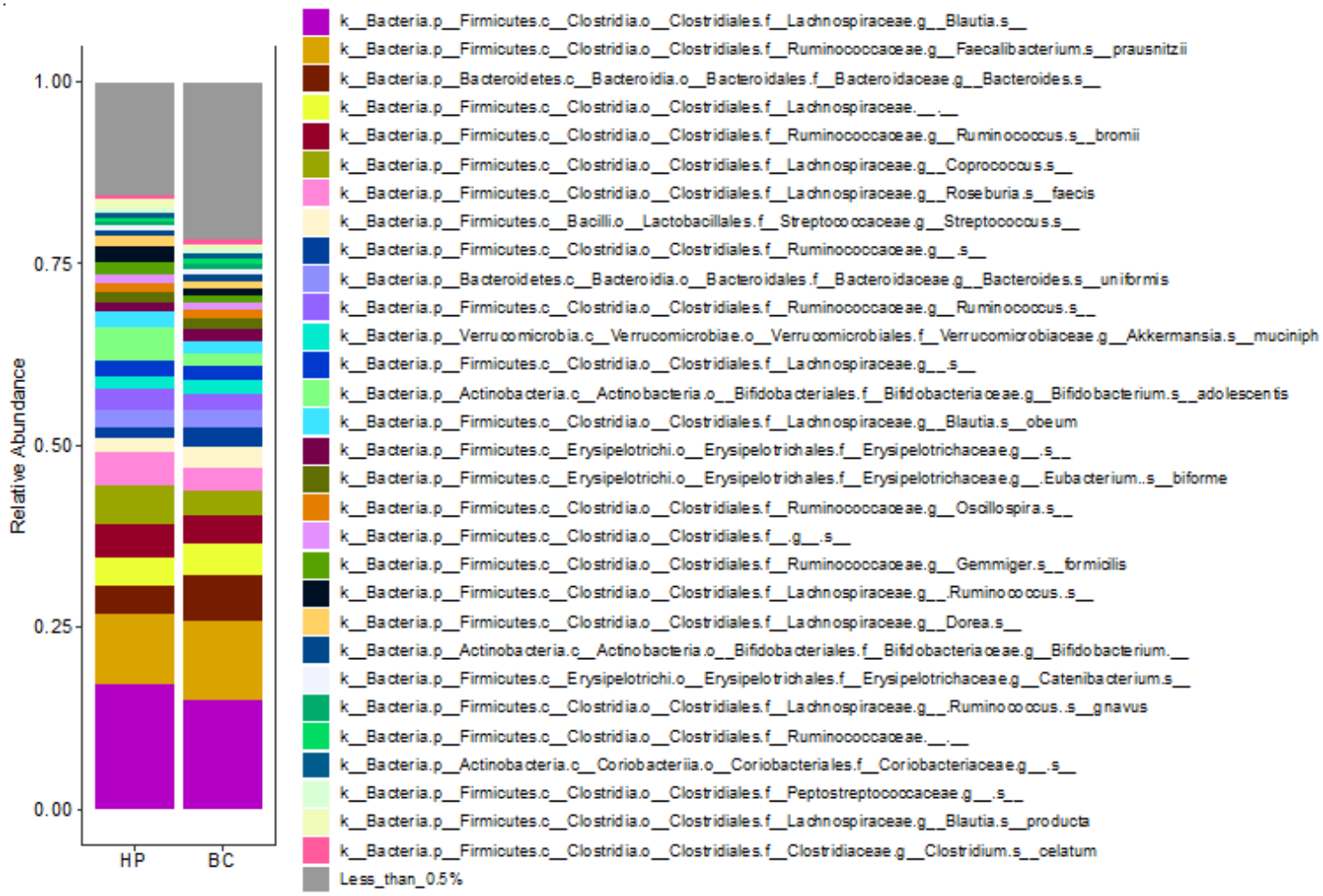


Figure 3

Taxa composition of breast cancer (BC) and healthy control participants (HP)

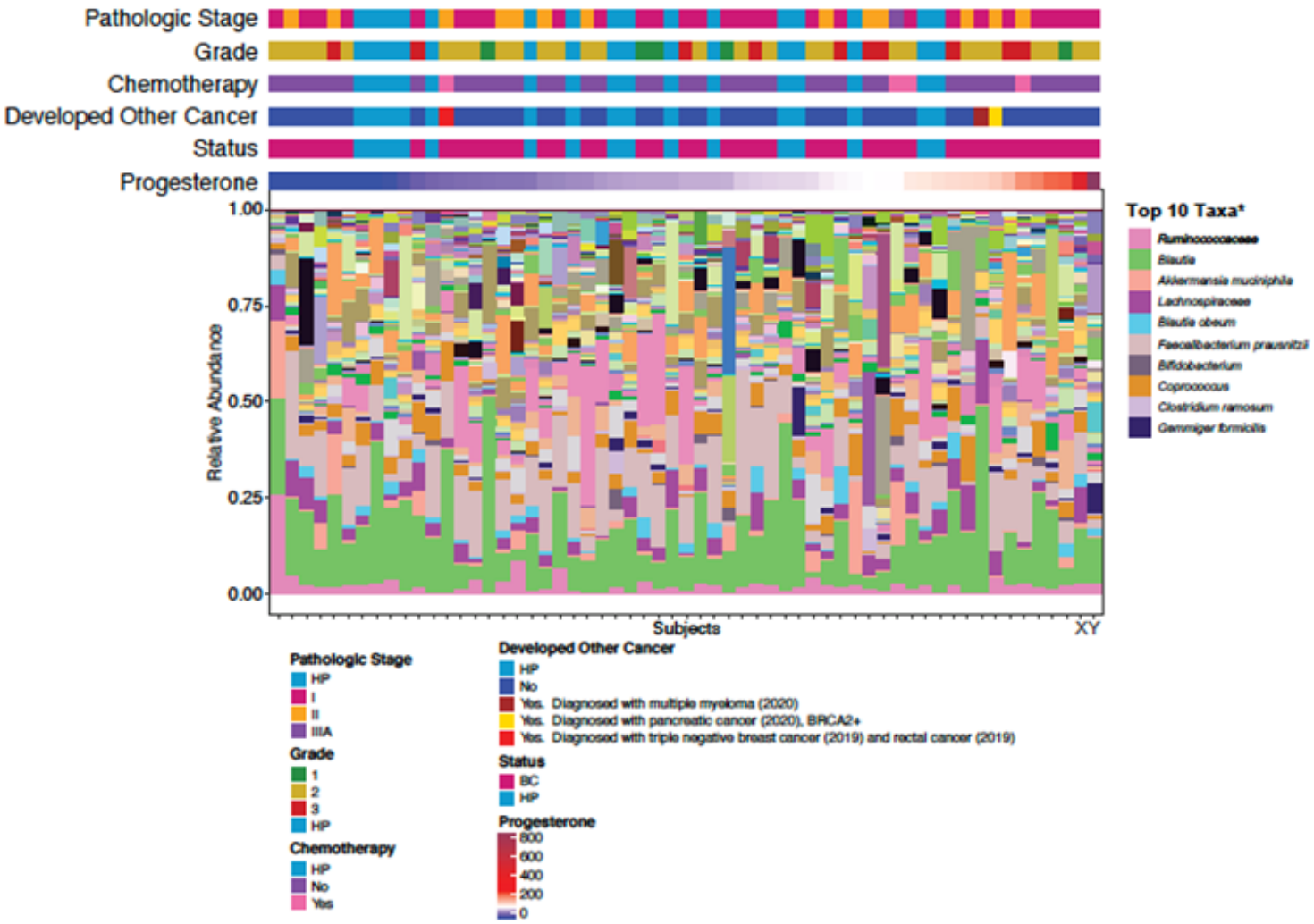
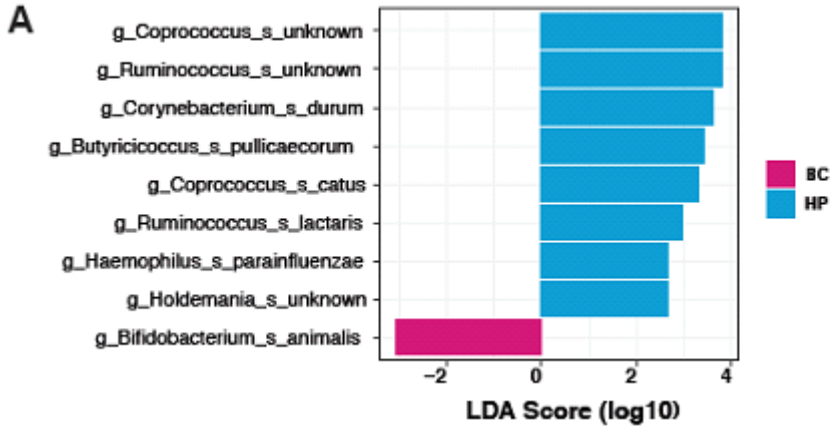


Figure 4

Relative abundance of taxa across breast cancer samples

*Additional taxa key available in Online Resource 4



B

Taxa	ß-Glucuronidase	ß-Galactosidase
Coprococcus sp	+	-
Ruminococcus sp	-	+
Butyricicoccus pullicaecorum	-	+
Coprococcus catus	-	+
Ruminococcus lactaris	-	+
Bifidobacterium sp	+	+

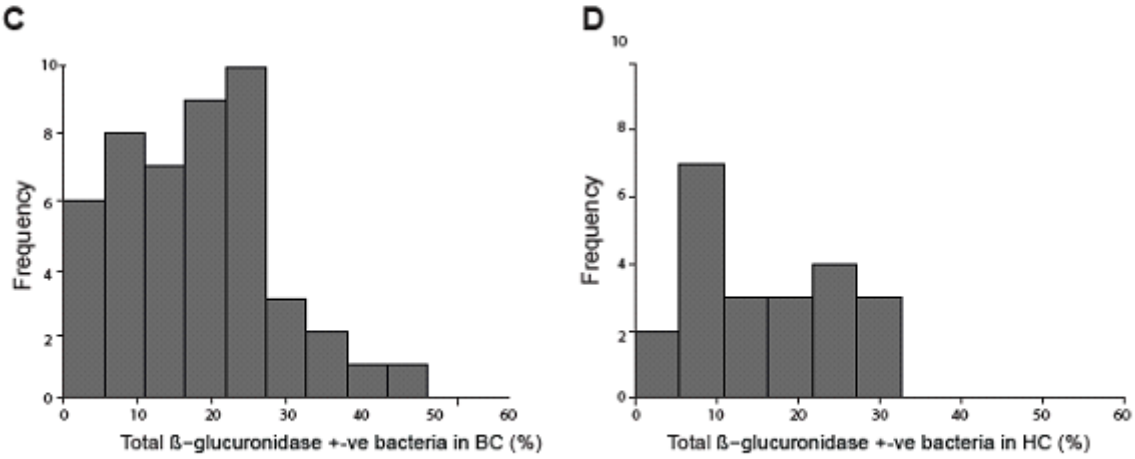


Figure 5

Differential taxa abundance (a) and distribution of selected beta glucuronidase positive (b) bacteria in cancer and control cohorts. Histograms show summed relative abundances of beta glucuronidase bacteria in a sample for breast cancer patients (c) and healthy controls (d).

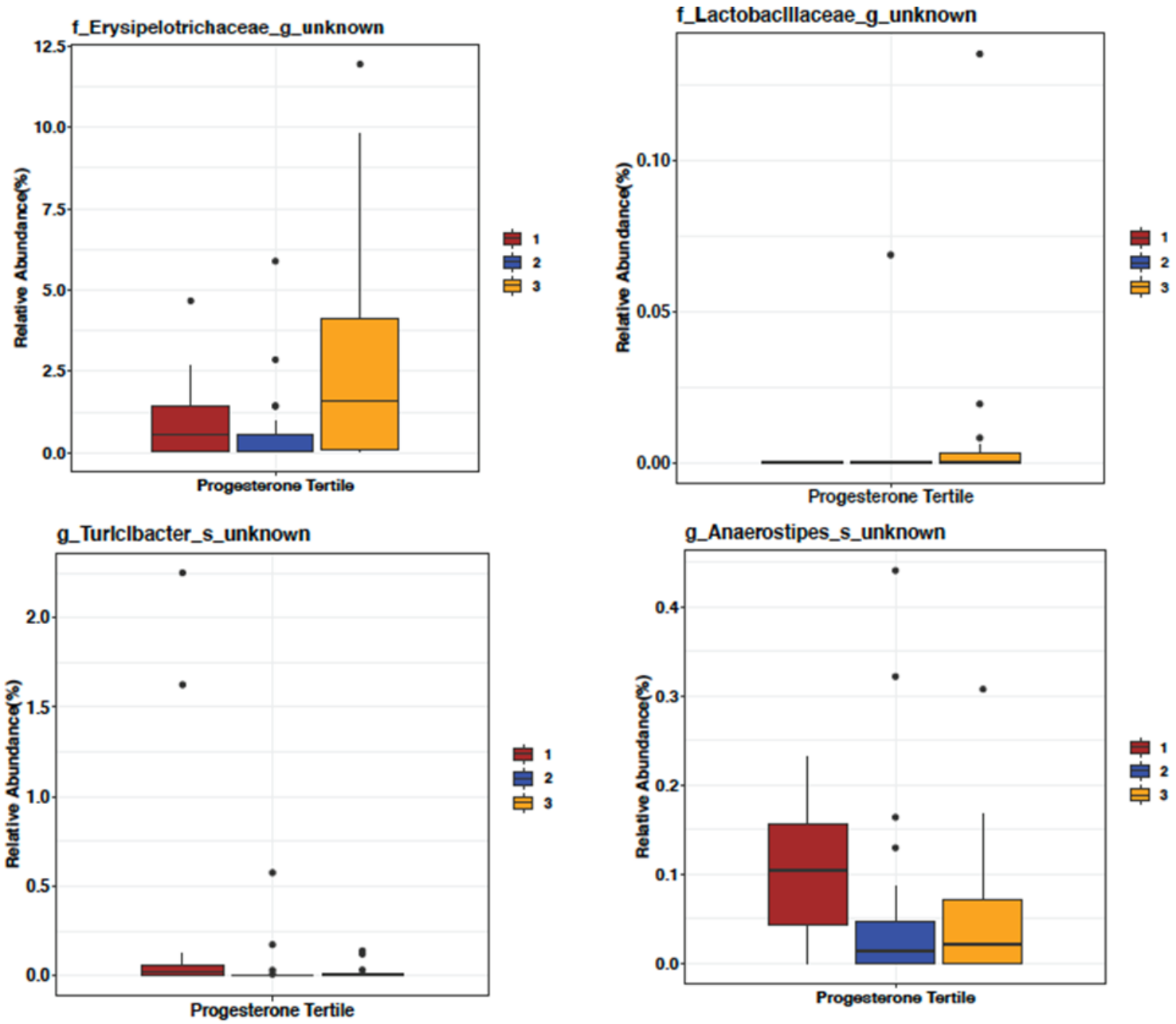


Figure 6

Altered taxa abundance by progesterone tertiles. Cancer samples were divided by progesterone group into tertiles (1-Low, 2-Medium, 3-High). Pairwise Kruskal-Wallis test was performed to compare the progesterone tertiles with each of the bacteria identified previously. Bacteria with a p value < 0.05 were selected for the post-hoc Mann-Whitney test between the progesterone tertiles with Benjamini-Hochberg p value adjustment. The reported bacteria had at least one comparison with p value < 0.05.