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Brief Communication

BRIEF COMMUNICATION

Investigation of the Association Between the Fecal Microbiota and Breast Cancer in Postmenopausal Women: a Population-Based Case-Control Pilot Study

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Abstract

We investigated whether the gut microbiota differed in 48 postmenopausal breast cancer case patients, pretreatment, vs 48 control patients. Microbiota profiles in fecal DNA were determined by Illumina sequencing and taxonomy of 16S rRNA genes. Estrogens were quantified in urine. Case-control comparisons employed linear and unconditional logistic regression of microbiota α-diversity (PD_whole tree) and UniFrac analysis of β-diversity, with two-sided statistical tests. Total estrogens correlated with α-diversity in control patients (Spearman Rho = 0.37, $P = .009$) but not case patients (Spearman Rho = 0.04, $P = .77$). Compared with control patients, case patients had statistically significantly altered microbiota composition (β-diversity, $P = .006$) and lower α-diversity ($P = .004$). Adjusted for estrogens and other covariates, odds ratio of cancer was 0.50 (95% confidence interval = 0.30 to 0.85) per α-diversity tertile. Differences in specific taxa were not statistically significant when adjusted for multiple comparisons. This pilot study shows that postmenopausal women with breast cancer have altered composition and estrogen-independent low diversity of their gut microbiota. Whether these affect breast cancer risk and prognosis is unknown.

In addition to traditional factors (1–3), breast cancer risk for postmenopausal women is directly related to level of endogenous estrogens and differences in estrogen metabolism (4–11). The gut microbiota modulates estrogen homeostasis through enterohepatic circulation, with large differences among individuals (12–16). The microbiota also modulates many other metabolic and immunologic pathways (17–19). Independent of estrogen levels, cancer risk is increased with metabolic syndrome through growth factors like insulin (20,21). Inflammation probably also contributes (22), as use of nonsteroidal anti-inflammatory drugs was associated with a 20% to 30% reduced risk of postmenopausal breast cancer in some studies, albeit with inconsistency by estrogen receptor (ER) tumor expression (23–29). Noting that gut microbial differences could affect breast cancer risk through several pathways, herein we tested the hypothesis that the gut microbiota of postmenopausal women with incident breast cancer, pretreatment, differs from control women.

Following review and approval by the respective institutional review boards, with signed informed consent 48 female members of Kaiser Permanente Colorado ages 50 to 74 years who were scheduled for treatment of biopsy-proven breast cancer and 48 normal-mammography women provided data, urine (without preservative), and feces (in RNAlater), frozen at home and thereafter below -80°C until use (30–33). Microbiota profiles in fecal

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DNA were determined by amplification, Illumina sequencing, and taxonomy of 16S rRNA genes (34–38). Urinary estrogens and estrogen metabolites (EMs) were quantified by liquid chromatography/tandem mass spectrometry (39). Microbiota alpha diversity was estimated as follows. Richness: number of unique species-level taxa, unadjusted for their relative abundances. Chao1: richness, but bias-corrected for rare (singleton, doubleton) taxa. Phylogenetic diversity (PD)_whole tree: sum of the branch lengths of a phylogenetic tree constructed from all taxa in the sample. Shannon index: a conservative estimate that adjusts for relative abundance of each taxon and that is defined as (negative) the sum over taxa of the product of the relative abundance of each taxon times the natural logarithm of its relative abundance. Estrogen associations with microbiota alpha diversity were tested by Spearman rank-order correlation. Alpha diversity associations with case-control status were tested by linear and unconditional logistic regression, with adjustment for age, body mass index (BMI), and total estrogens. Composition of the microbiota (beta diversity) was compared by unweighted and weighted UniFrac analysis of the distance matrix with 10 000 permutations (40). All statistical tests were two-sided, and a *P* value of less than .05 was considered statistically significant. Detailed methods can be found in the Supplementary Materials (available online).

Case patients and control patients were 86% non-Hispanic white with mean age 62 years (SD = 6.86), mean BMI of 28 (SD 1.07), and equivalent reproductive and menstrual histories (Supplementary Table 1, available online). Two case patients (excluded from some analyses) and no control patient reported receiving an antibiotic within the previous two weeks. Two cancers were stage 3, ten at stage 2, 25 at stage 1, and 11 in situ (American Joint Committee on Cancer, Collaborative Staging Version 2.04) (41). Forty-two tumors were ER-positive, 37 were progesterone receptor–positive, and five were HER2-positive.

All mean urinary estrogens were two-fold higher in the case patients, although these were not statistically significant ($P \geq .10$) (Table 1). In control patients, fecal microbiota alpha diversity (phylogenetic diversity [PD]_whole tree) correlated directly with total estrogens (Spearman Rho = 0.37, $P = .009$). In contrast, PD_whole tree was not correlated with total estrogens in case patients (Spearman Rho = 0.04, $P = .77$). PD_whole tree was weakly correlated with EM:parent estrogen ratio in control patients (Spearman Rho = 0.26, $P = .08$). There was no such correlation between PD_whole tree and EM:parent estrogen ratio in case patients (Rho = -0.11, $P = .45$).

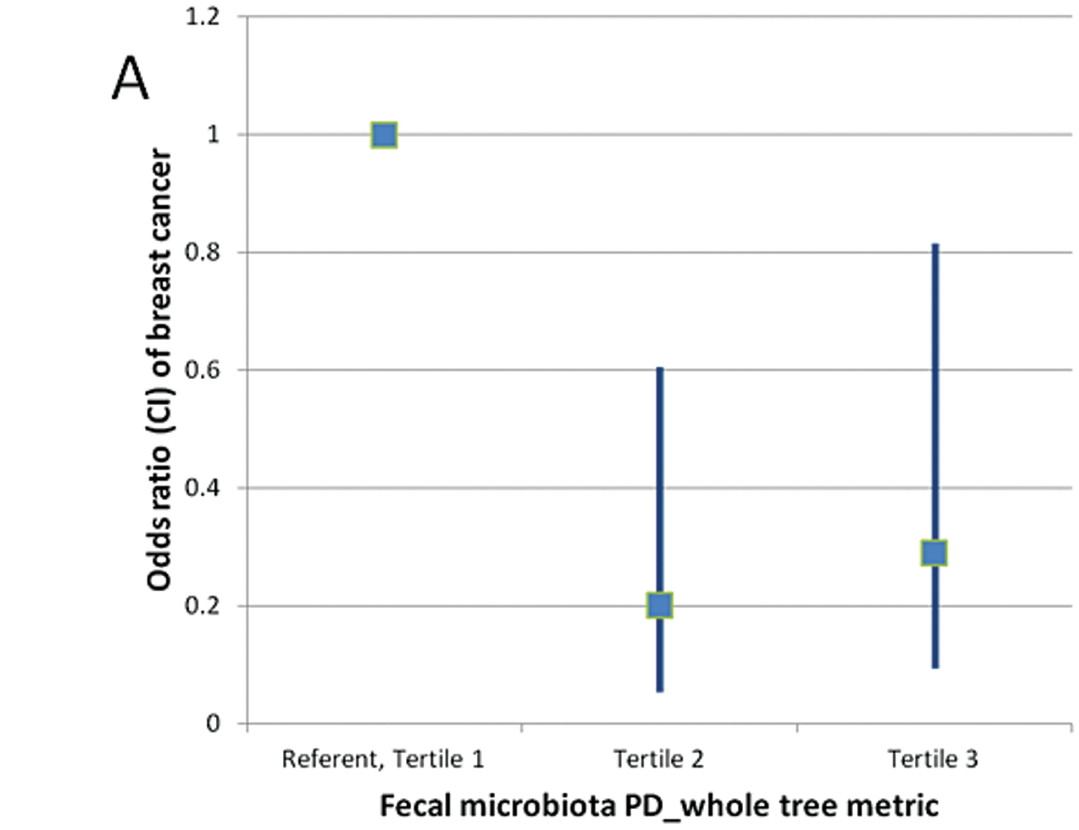
The fecal microbiota of case patients, compared with control patients, had statistically significantly lower alpha diversity ($P \leq .004$), except Shannon index ($P = .09$) (Table 1). Adjusted for age, BMI, and total urine estrogens, the breast cancer odds ratio (OR_{adj}) per tertile category increase in PD_whole tree was 0.50 (95% confidence interval [CI] = 0.30 to 0.85), and nearly identical for richness and Chao1 (Table 1). The association was not linear. Rather, compared with lowest-tertile levels, breast cancer OR_{adj} was reduced 70% to 80% with both middle-tertile and highest-tertile levels of these measures (Table 1 and Figure 1A).

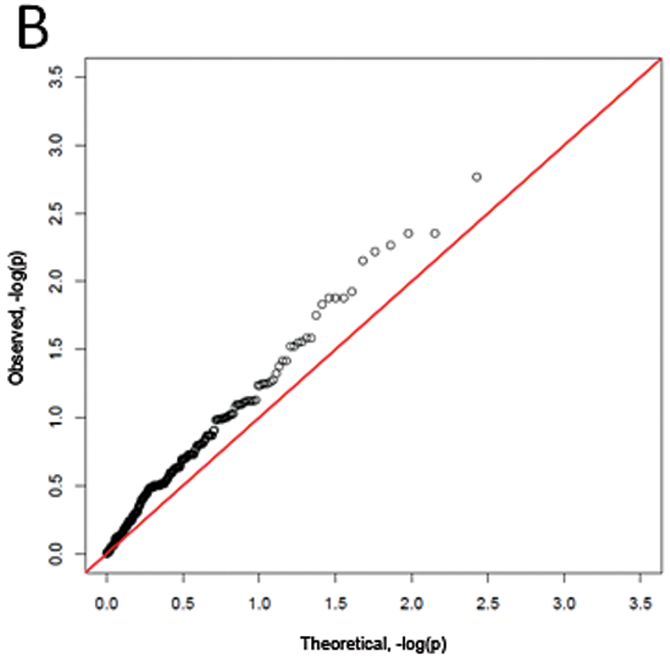
Fecal microbiota composition (beta diversity) also differed between case patients and control patients overall (unweighted UniFrac $P = .009$), across all genus-level taxa (Figure 1B), and on the first principal coordinate (PC1) of the

Table 1. Urinary estrogens and estrogen metabolites (EM) and fecal microbiome alpha diversity in postmenopausal breast cancer cases and controls

Variable/outcome	Case patients, N=48	Control patients, N=48			P*
Estrogen, EM levels, mean (SD)†					
Total estrogens and EM	45.40 (106.94)	22.36 (17.79)			.12
Parent estrogens	16.89 (44.38)	7.30 (5.93)			.12
Estrone	12.97 (31.76)	5.83 (4.81)			.11
Estradiol	3.92 (12.86)	1.47 (1.31)			.17
Total EM	28.51 (63.33)	15.07 (12.42)			.15
2-Hydroxylation pathway	12.94 (28.83)	6.51 (5.59)			.10
16-Hydroxylation pathway	14.43 (32.12)	7.99 (6.81)			.21
4-Hydroxylation pathway	1.13 (2.41)	.56 (.47)			.11
Estrogen, EM ratios					
EM/parent	2.15 (.87)	2.35 (2.01)			.56
2-pathway/parent	.97 (.40)	.96 (.44)			.90
16-pathway/parent	1.10 (.45)	1.30 (1.59)			.41
4-pathway/parent	.09 (.04)	.09 (.04)			.95
2-pathway/16-pathway	.89 (.16)	.85 (.15)			.14
Fecal microbiome richness, alpha diversity, mean (SE)					
No. observed species	78.6 (23.1)	91.2 (16.6)			.004
Chao1	909.5 (24.4)	1053.8 (174.9)			.001
PD_whole tree	33.1 (7.9)	37.5 (6.1)			.004
Shannon index	6.0 (.7)	6.2 (.6)			.09
Breast cancer risk, by tertile, odds ratio (95% confidence intervals)‡		Tertile 1	Tertile 2	Tertile 3	
No. observed species	.50 (.30 to .84)	Referent	.24	.29	
Chao1	.53 (.31 to .89)	Referent	.28	.30	
PD_whole tree	.50 (.30 to .85)	Referent	.20	.29	
Shannon index	.83 (.50 to 1.37)	Referent	.89	.68	

* Linear regression with (log-transformed estrogen and EM values) and adjustment for age.
† Picomoles/mg creatinine.
‡ Logistic regression, tertiles among controls, adjusted for age, body mass index, and total urine estrogen level.





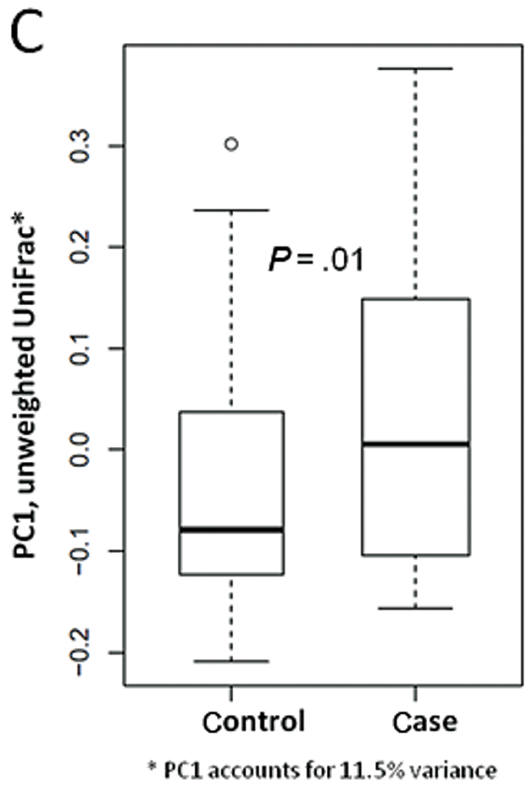


Figure 1. Fecal microbiota differences between postmenopausal breast cancer case patients and control patients. **A)** Odds ratio (**square**) and 95% confidence interval (**bar**) of breast cancer by tertile of alpha diversity (phylogenetic diversity [PD]_whole tree). Odds ratios by tertile are presented. **B)** Beta diversity, quantile-quantile plot of two-sided Wilcoxon rank-sum *P* values for all genus-level taxa. The distribution (x-axis expected, y-axis observed) diverges from the null (**diagonal line**) for many taxa. **C)** Beta diversity, distribution of the first principal coordinate values (PC1, 11.5% of the variance*) of the unweighted UniFrac distance matrix. **Boxes** are the interquartile range; median values are bands within the boxes; **whiskers** are 1.5-times the IQR; **open circle** is an outlier value. CI = confidence interval; PD = phylogenetic diversity.

beta diversity distance matrix (P = .01) (Figure 1C). The difference was larger with exclusion of the two antibiotic-exposed case patients (unweighted UniFrac P = .006). Without adjustment for multiple comparisons, relative abundance of several taxa differed between case patients and control patients by 1% or more (Supplementary Table 2, available online). Particularly in the order *Clostridiales*, case patients had higher levels of *Clostridiaceae*, *Faecalibacterium*, and *Ruminococcaceae*; and they had lower levels of *Dorea* and *Lachnospiraceae* (Supplementary Figure 1, available online).

This population-based case-control study found that the fecal microbiota was less diverse and compositionally different in postmenopausal women who were awaiting treatment for biopsy-proven breast cancer compared with similar women without breast cancer. As expected (5–11), the cancer case patients also had higher levels of systemic estrogens, although these were not statistically significant in this small study. Importantly, the difference in estrogens, and statistical adjustment for this difference, did not alter the cancer-microbiota association.

Our findings are consistent with the 40-year literature on the gut microbiota's effects on systemic estrogens (12–16) and with our previously observed correlations of alpha diversity with systemic estrogen level and EM:parent estrogen ratio (16,33). The novel implication of our current study is that breast cancer was statistically significantly associated with other functions of the gut microbiota, unrelated to systemic estrogen levels. Low gut microbial diversity occurs with adiposity, insulin resistance, dyslipidemia, leukocytosis, and elevated C-reactive protein (17), some of which are associated with breast cancer (20,42–47).

The infant's gut microbial composition may influence breast cancer risk in adulthood. In both mice and people, microbiota composition is acquired directly from the mother during birth (48–53). The distinct microbiota of adults who were born by cesarean vs vaginal delivery (54), as well as the similarity of microbial composition within adult dizygotic twin pairs (55–57), implies that composition is stable for decades. Breast cancer risk is affected by obscure early-life effects that also are transmitted through the maternal line (3). Such maternal effects could reflect differences in systemic estrogens, but genetic determinants of estrogen levels have been inconsistent (58–60). We postulate an effect for maternal transmission of the microbiota.

The strengths of our study include representative control patients, careful clinical staging and histopathology, optimal specimens collected prior to treatment among case patients, state-of-the-art assays, and rigorous statistical analysis methods. Weaknesses include the small sample size, which precluded assessment of minor taxa and of interactions between microbiota metrics and known risk factors, particularly estrogens. Estrogen parameters were correlated with microbiota alpha diversity, although only in control patients (16,33). Our case-control design is another important weakness, precluding exclusion of reverse causality—that cancer caused the microbiota distinction. To minimize this possibility, our case patients received only an outpatient breast biopsy, no surgical or systemic therapy prior to specimen collection. Excluding case patients who reported antibiotic exposure, perhaps biopsy-related, had no major impact on the microbiota associations.

In summary, postmenopausal women with newly diagnosed breast cancer had a fecal microbiota that was less diverse and compositionally different compared with similar women without breast cancer. The cancer case patients also had higher levels of urinary estrogens, but these were independent of microbiota differences. The findings imply that the gut microbiota may affect breast cancer risk and may do so through estrogen-independent pathways.

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