Effect of chemotherapy and weight change on the gut microbiome of breast cancer patients during the first year of treatment

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Research Article

Keywords: neoadjuvant, adjuvant chemotherapy, gut microbiome, alpha diversity, breast cancer

Posted Date: October 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-970564/v1

Abstract

Objective: The effects of chemotherapy and weight changes on the gut microbiome of breast cancer patients are not well understood. Methods: We conducted a 1-year follow-up study of 33 breast cancer patients and investigated gut microbiome before initiation of chemotherapy and after completion of treatment. We compared alpha diversity and mean taxa abundance at baseline and absolute changes (Δ; final-baseline) in taxa abundance by treatment (16 neoadjuvant- neoADJ, 13 adjuvant- ADJ, 4 no chemotherapy-noC) using Wilcoxon rank sum and negative binomal tests and evalauted whether these changes were affected by weight changes during follow-up. Results: Alpha diversity measures increased in the neoADJ (+16.4% in OTU p =0.03; +51.6% in Chao1 p =0.03; +7.0% in Shannon index P=0.02; +11.0% in PD whole tree p =0.09) but not in the non-neoADJ group (ADJ+noC). The difference in change in Chao1 index between groups was statistically significant (p NEOADJ vs ADJ+noC =0.04). Wilcoxon p values of 0.03 to 0.003 were observed for five taxa: Bacteroidetes (g _Alistipes) , Firmicutes (g_Clostridium, g_Eubacterium, g_Bilophila) and Preteobacteria g_Haemophilus). In the negative binomial analysis, changes in abundance differed at Bonferroni-adjusted p values ≤0.0007 for four taxa: two Bacteroidetes taxa (g_Alistipes, f _S247) and two Firmicutes taxa (g_Catenibacterium, g_Eubacterium). The negative binomial results remained largely the same when we adjusted for weight changes. Conclusions and Relevance: This pilot longitudinal study showed changes in alpha diversity measures and abundance of select taxa that appeared to differ by chemotherapy type. Further investigations are needed to confirm these findings and to assess the impact of these microbiome changes on patient outcome.

Background

A large proportion of women with breast cancer receive adjuvant (ADJ) or neoadjuvant (neoADJ) chemotherapy as part of standard care [1]. Weight gain in association with both ADJ and neoADJ is well-documented and high BMI is associated with worse outcome [2, 3]. There is a need to better understand the biology behind weight gain since it is modifiable and is known to impact breast cancer outcome [4, 5].

Advances in sequencing technologies coupled with new bioinformatics developments have produced enormous new information on the role of the gut microbiota in human health, suggesting intriguing links between the microbiota and risk of obesity, metabolic diseases and inflammatory responses [6–8]. Several studies have investigated gut microbiome of cancer patients at varying times after chemotherapy[9–13]. Because microbiome bacteria can interfere and/or enhance chemotherapy and radiotherapy treatment of cancer patients [14–16], there is a need to unravel the bidirectional relationships between the gut microbiome and cancer treatment. Higher alpha diversity and relative abundance of *Ruminococaccaceae* family were reported in anti-PD-1 responding melanoma patients[17]. Patients with lung and other cancers treated with anti-PD-1 antibodies have displayed high abundance of several *Firmicutes* including *Akkermansia* and *Alstipes*, associated with treatment response [18].

We conducted a 1 year follow-up study of 38 breast cancer patients with the objective of investigating whether the gut microbiome of breast cancer patients was altered in association with type of chemotherapy treatment and weight changes. Baseline characteristics of these participants have been reported previously [19]. We investigated whether changes in alpha diversity measures and abundance of specific taxa differed by chemotherapy. We also explored whether these changes were affected by weight changes during the follow-up period.

Materials And Methods

Data and specimen collection This 1 year longitudinal study was conducted at the University of Southern California (USC) Norris Comprehensive Cancer Center (NCCC) and at the Los Angeles County + USC Medical Center. Women of all race/ethnicities, newly diagnosed with incident invasive breast cancer were considered potentially eligible. Exclusionary criteria included recurrent breast cancer, a history of other cancers (other than non-melanoma skin cancer), celiac or inflammatory bowel disease, bariatric surgery, recent pregnancy or nursing (within past 12 months), past treatment with chemotherapy, recent antibiotic use (defined as one week or more during the month prior to baseline fecal sample collection), or use of probiotic supplements or prednisone.

After signing informed consent, eligible and willing patients donated up to four fecal specimens and completed up to four clinical visits during an average of 1 year of follow-up. Figure 1 shows the timing of baseline and final fecal sample collections with respect to diagnosis and treatment for the treatment groups. For the neoADJ group, baseline fecal samples were collected 2.5 ± 3.9 days before chemotherapy started and the final samples were collected 178.4 ± 85.0 days after chemotherapy ended. For the ADJ group, baseline fecal samples were collected 3.5 ±3.1 days before chemotherapy started and the final samples were collected 138.5 ± 55.4 days after completion of chemotherapy. For the noC group, baseline samples were collected 52.5 ± 15.7 days after surgery and the final samples were collected 127.3 ± 46.9 days after completion of radiation. The interval between date of diagnosis and the last fecal sample collection was 370.4 ± 64.0 days for the neoADJ group, 362.1 ± 90.4 days for the ADJ group, and 289.8 ± 21.3 days for the noC group. As described previously [19], participants used pre-labeled collection devices and tubes containing the nucleic acid preservative RNAlater which we provided. They collected and stored fecal samples in their home freezers until they were brought to USC at their clinic visit or the samples were picked up by the study staff. These fecal samples were stored in the -80^{o}C freezers at USC until they were sent for microbiome analysis at the completion of the study (see below). Participants had two dual-energy x-ray absorptiometry (DXA) scans at USC facilities; a baseline scan at the first clinic visit and a second scan on the same day or around the time of the last clinic visit. Participants also completed a baseline questionnaire which assessed menstrual and reproductive history, medical history (e.g., diabetes, hypertension), family history of cancer, use of medications and vitamin supplements, and usual diet. Information on lifestyle and medical history was updated at each of the clinic visits.

Fecal Specimen Processing And Microbiome Analyses

Microbiome analyses were conducted in the laboratory of Dr. Jacques Ravel [19]. This included DNA extraction, 16S rRNA gene amplification of the two barcoded universal primers 319F and 806R for PCR amplification of the V3 and V4 hypervariable regions and sequenced the amplicons on the Illumina MiSeq platform. The 16S rRNA genes were amplified in 96-well microtiter plates. Negative controls without a template were processed for each primer pair. They performed taxonomic assignments and generated taxa abundance and read counts tables for each of the 144 fecal samples collected from 38 breast cancer patients. Fourteen samples failed (i.e., <100 read counts); 4 were from one patient who was excluded from all subsequent analyses. Of the other failed samples from 37 patients, two were baseline and two were last samples from four different patients (the two subjects with low baseline read counts did not contribute to analysis on alpha diversity but contributed baseline analysis on taxa abundance [19]). This current analysis on baseline and last fecal samples was based on 33 breast cancer patients (Table 1). The study protocol was approved by the USC Institutional Review Board.

Table 1
Characteristics of 33 breast cancer patients by treatment with baseline and end of study fecal samples

		Chemotherapy treatment			P value[a]	P value[b]
	All	Neoadjuvant (neoADJ)	Adjuvant (ADJ)	No (noC)		
N	33	16	13	4		
Mean age ± standard deviation (SD)	51.7±12.4	50.8±12.4	49.5±10.2	62.8±15.8	0.31	0.81
Menopausal status						
Premenopause	17	8	8	1		
Postmenopause	16	8	5	3	0.44	0.53
Race/ethnicity						
Hispanic	24	13	9	2		
Non-Hispanic	9	3	4	2	0.43	0.45
Stage at diagnosis						
I/II	18	6	8	4		
III	15	10	5	0	0.07	0.20
ER/PR status						
ER+PR+	21	8	9	4		
ER+PR-	4	2	2	0		
ER-PR-	8	6	2	0	0.34	0.41
HER2 status						
Positive	9	6	3	0		
Negative	24	10	10	4	0.29	0.40
Days of treatment (SD)[d]						
AC	48.5 (14.2)	56.0 (10.5)	37.2 (11.4)	0		0.01
Taxol or taxotere	73.3 (11.5)	77.9 (2.4)	66.5 (16.4)	0		0.20
Herceptin	271.7 (143.4)	354.9 (2.9)	240.6 (3.2)	0		0.41
TCPH	108.8 (36.8)	110.0 (43.5)	105.0 (0.0)	0		0.48
Total days of chemotherapy	107.8 (36.2)	134.0 (31.3)	102.6 (39.8)	0		0.02
Total days of radiation[d]	40.8 (10.7)	44.2 (10.0)	40.7 (7.5)	24.3 (4.2)	0.02	0.44
Days (SD) between baseline to last fecal sample	269 (83)	319 (64)	240 (74)	167 (34)	0.008	0.01
Mean (SD) Baseline BMI [e]	31.34 (7.97)	31.05 (5.56)	30.21 (8.30)	36.11 (14.52)	0.69	0.36
Change (SD) in BMI [e]	-0.22 (2.40)	-0.71 (3.03)	0.29 (1.70)	0.07 (1.22)	0.73	0.51
Mean (SD) Baseline Weight kg [e]	74.26 (16.57)	73.35 (12.37)	68.22 (8.44)	97.53 (31.52)	0.14	0.29
Change (SD) in Kg [e]	-0.39 (5.66)	-1.50 (7.00)	1.00 (3.60)	0.33 (3.43)	0.74	0.48

[a] Krushal Wallis test between the 3 chemotherapy group (neoadjuvant, adjuvant, none)

[b] Wilcoxon Signrank test: neoadjuvant vs adjuvant

[c] Wilcoxon Signrank test: HER2 positive vs HER2 negative

[d] AC (Doxorubicin + Cyclophosphamide) given to 9 neoADJ and 6 ADJ; Taxol given to 8 neoADJ and 6 ADJ, Herceptin given to 4 neoADJ and 8 ADJ, TCPH (docetaxel + Carboplatin+ Pertuzumab+ Trastuzumab) given to 6 neoADJ and 2 ADJ; radiation given to 15 neoADJ, 7 ADJ, 3 noC

[e] BMI and weight kg based on DXA

Statistical Analyses

Microbiome alpha diversity was estimated after rarefaction. We used Wilcoxon rank sum test to examine changes in alpha diversity during the 9 months of follow-up by chemotherapy treatment (16 neoADJ vs 17 non-neoADJ (13 ADJ + 4 noC); 16 neoADJ vs 13 ADJ) and by BMI change (16 lost weight vs 17 gained weight).

We conducted permutational multivariate analysis of variance (PERMANOVA) to test statistical significance of overall composition between baseline vs final samples (n=33) by treatment and by BMI change [20]. The relationship of overall gut microbiome composition by treatment and BMI change was assessed by principal coordinate analysis (PCoA) based on the unweighted and weighted phylogenetic UniFrac distance matrix[21]. PCoA plots were generated using the first two principal components by treatment and BMI change.

Turning to taxonomy, we conducted analyses to examine relationships of specific taxa to treatment and BMI change during the follow-up. We calculated change in the relative abundance of 74 specific taxa that had levels above zero, allowing comparison between baseline and last fecal microbiome by subtracting relative abundance of baseline values from final (end of study) values to represent the absolute percentage change after completion of treatment. Statistical tests by Wilcoxon rank sum were used to compare results for neoADJ vs non-neoADJ (ADJ + noC) and for neoADJ vs ADJ groups. To accommodate the sparse, non-normally distributed count data, we repeated analyses using negative binomial mixed models for longitudinal microbiome data [22], without adjustment and with adjustment for BMI change during the follow-up. To correct for multiple comparisons [23], Bonferroni-adjusted significance levels were set for 74 genera (0.05/74, $p \leq 0.0007$). Because of the modest sample sizes of this pilot study, we also considered the differences in change in genera to be suggestivie if $0.0007 < p \leq 0.007$. All data were analyzed using R (R Foundation for Statistical Computing, Vienna, Austria).

Assessment of cancer status and vital status through May 2021 was used as the outcome in proportional hazards regression models, each using one alpha diversity measure at either the baseline or the final fecal sample collection as the dependent variable. These data were analyzed using SAS 9.4 (SAS, Cary, NC).

Results

The 33 breast cancer patients with baseline and final (end of study) fecal samples were ages 51.7 ± 12.4 years, obese (mean baseline BMI of 31.4 ± 8.0 kg/m^2), mostly Hispanics (73%), and had hormone receptor (HR) positive (ER+PR+) (63.6%) and HER2 negative breast cancer (72.7%) (Table 1). About half (n=16, 48.5%) received neoADJ, 13 (39.4%) received ADJ, and 4 (12.1%) had no chemotherapy (noC). The three treatment groups were similar in distribution by HR status but all four patients requiring no chemotherapy had early (I/II) stage cancer compared with 61.5% (8 of 13) in the ADJ and 37.5% (6 of 16) in the neoADJ group (p_{2df} =0.07). Although baseline BMI did not differ by treatment and there were no overall significant changes in weight during follow-up, 17 women gained an average of 3.7± 0.5 kg while 16 women lost an average of 4.7± 1.2 kg. Weight change was not uniform by treatment; the mean weight gain was 1.0 ± 3.6 kg in the ADJ and 0.33 ± 3.4 kg in the noC groups while there was a mean weight loss of 1.5 ± 7.0 kg in the neoADJ group.

Baseline alpha diversity measures (operational taxonomic units (OTU), Chao1, Shannon index, phylogenetic diversity (PD)_whole tree) did not differ by treatment (Supplementary Table 1). However, alpha diversity measures changed nonuniformly by treatment during follow-up (Figure 2). Women in the neoADJ group showed increases: +16.4% in OTU p=0.03; +51.6% in Chao1 p=0.03; +7.0% in Shannon index p=0.02; +11.0% in PD whole tree p=0.09. Three of the four measures increased 1.6–2.4% in the ADJ group but decreased 4.4–11.3% in the noC groups; none of these changes were statistically significant. A suggestive difference in change in Chao 1 index between neoADJ and non-neoADJ (ADJ + noC) group was observed (p=0.04). Baseline alpha diversity measures did not differ between the weight loss and weight gain groups (Supplementary Table 1) but changes in alpha diversity measures appeared to differ between the two groups (Figure 2). The weight loss group (n=16, BMI decreased 2.03 ± 0.55 kg/m^2) showed increases in OTU (+17.1% p=0.02), Chao I index (+40.6% p=0.03), PD_whole tree (+15.4% p=0.04), and Shannon index (+6.7% p=0.06) while the weight gain group (n=17, BMI increased 1.49 ± 0.19 kg/m^2) showed nonsignificant decreases in alpha diversity measures. The differences in changes in alpha diversity measures between the two groups were suggestive: OTU(p=0.06), Chao 1 index (p=0.04), and PD_whole tree (p=0.08).

The neoADJ and non-neoADJ group did not differ in beta diversity at baseline using the unweighted UniFrac distance (Figure 3). However, the groups appeared to differ in their final microbiome in unweighted UniFrac distance analysis; separation differed for axis 1 (p=0.11) and axis 2 (p=0.05), each explaining 18.4% and 9.2% of the variance, respectively. The weight loss and weight gain groups did not differ in their baseline microbiome but they appeared to differ in their final microbiome using unweighted UniFrac distance analysis; separation between the groups differed for axis 1(p=0.28) and axis 2 (p=0.07), each explaining 20.0% and 9.4% of the variance respectively (Figure 4). None of the differences were statistically significant using weighted UniFrac distance (data not shown).

We compared mean taxa abundance at baseline and absolute changes (Δ) (final-baseline) in taxa abundance between neoADJ (n=16) and non-neoADJ (n=17) using Wilcoxon rank sum and negative binomal tests (Table 2). The smallest Wilcoxon P values were between 0.03 to 0.003 for five taxa: *Bacteroidetes* (g_*Alistipes), Firmicutes (g_Clostridium, g_Eubacterium, g_Bilophila) and Preteobacteria g_Haemophilus).* In the negative binomial analysis without adjustment for weight changes, changes in abundance of four taxa differed at p values ≤0.0007 and this included two *Bacteroidetes* taxa (*g_Alistipes* p =0.00003; f_*S247* p= 0.0004) and two *Firmicutes* taxa *(g_Catenibacterium* p=7.0E-06; *g_Eubacterium* p= 0.0005). Changes in eight additional taxa were also suggestive ($0.0007 < p \leq 0.007$): *Actinobacteria* (g_*Slackia), Bacteroidetes* (*f_Rikenellaceae), Euryarchaeota*

(g_*Methanobrevibacter)*, *Firmicutes (f_Germellaceae, g_Turibacter, f_Clostridiaceae; g_Acidaminococcus) and Verrucomicrobia (g_Akkermansai).* Results were similar with adjustment for BMI changes; *p* values were ≤0.0007 for four taxa: *Actinobacteria* (g_*Slackia), Bacteroidetes* (*f_Rikenellaceae;* g_*Alistipes), Firmicutes (f_Clostridiaceae)* and were 0.0007<*p*≤0.007 for seven taxa: *Bacteroidetes* (f_*S247), Euryarchaeota* (g_*Methanobrevibacter)*, Firmicutes (*f_Germellaceae, g_Turibacter, g_Acidaminococcus; g_Eubacterium) and Verrucomicrobia (g_Akkermansai).* The only noticeable change in results with BMI adjustment was that the difference in abundance of *Firmicutes (g_Catenibacterium)* was no longer observed. Results were largely similar comparing neoADJ to ADJ groups (Supplementary Table 2).

Table 2
Mean baseline (standard deviation) and absolute changes in abundance of specific taxa by treatment: neoadjuvant (neoADJ) versus non-neoADJ (adjuvant (ADJ)+ no chemotherapy (noC))

		Mean baseline (B) and absolute changes (Δ) in taxa abundance by chemotherapy				neoADJ vs non-neoADJ		
		All N=33	neoADJ N=16	ADJ N=13	No Chemo-therapy(noC) N=4	Wilconxin Test [a]	Negative Binomial [b]	
							No BMI adjustment	BMI Adjustment
Specific taxa						N=33	N=33	N=33
P__Actinobacteria f__Micrococcaceae g_Rothia	B	0.013 (.005)	0.013 (0.005)	0.017 (.011)	0 (0)	0.55		
	Δ	-0.006(.006)	-0.01 (0.003)	-0.006(.013)	0.007 (0.007)	0.11		
	P[c]	0.20	**0.008**	1.00	0.50		0.11	0.16
P__Actinobacteria f__Coriobacteriaceae g_ Slackia	B	0.292 (.086)	0.202 (0.11)	0.359 (.137)	0.313 (0.311)	0.44		
	Δ	0.138 (.121)	-0.045 (0.13)	0.359(.214)	-0.149 (0.146)	0.64		
	P[c]	0.37	0.84	0.21	0.25		**0.002**	**0.00036**
P_ Bacteroidetes f_Prevotellaceae g_Prevotella	B	7.04 (1.96)	8.19 (3.34)	7.78 (2.70)	0.071(0.065)	0.37		
	Δ	-2.11 (2.35)	-0.55 (4.34)	-4.82 (2.73)	0.516(0.504)	0.069		
	P[c]	0.23	0.93	**0.033**	0.13		0.065	0.08
P_ Bacteroidetes f_ Rikenellaceae g_	B	0.014(0.005)	0.003(0.003)	0.026(0.011)	0.014 (0.014)	**0.02**		
	Δ	0.012(0.008)	0.025(0.012)	-0.006(0.01)	0.017(0.017)	0.10		
	P[c]	0.14	**0.03**	0.73	1.00		**0.001**	**0.00037**
P_ Bacteroidetes f_ Rikenellaceae g_	B	0.791(.179)	0.793(.203)	0.683(.244)	1.135(1.08)	0.41		
	Δ	0.036(.008)	0.228(.218)	-0.114(.219)	-0.249(.213)	0.76		
	P[c]	0.92	0.50	0.91	0.25		**0.033**	0.063
P__Bacteroidetes f_Rikenellaceae g_alistipes	B	0.026 (0.009)	0.035 (0.015)	0.013(0.012)	0.033 (0.02)	0.056		
	Δ	-0.012 (0.009)	-0.032 (0.015)	0.014(0.008)	-0.014 (0.008)	**0.007**		
	P[c]	0.19	**0.016**	0.25	0.50		**0.000033**	**0.000033**
P_ Bacteroidetes f_S247 g_	B	0.26 (0.10)	0.25 (0.17)	0.35 (0.16)	0 (0)	0.37		
	Δ	-0.17(0.09)	-0.11 (0.16)	-0.31(0.13)	0.01 (0.01)	0.12		
	P[c]	0.12	0.91	**0.020**	0.50		**0.00039**	**0.008**
P_ Bacteroidetes f_Barnesiellaceae	B	0.256 (0.086)	0.337 (0.145)	0.159 (0.106)	0.247 (0.244)	0.96		
	Δ	0.05 (0.079)	0.145 (0.148)	-0.017 (0.074)	-0.111 (0.108)	0.34		
	P[c]	0.72	0.28	0.85	0.25		0.067	**0.050**
P__Euryarchaeota f_Methanobacteriaceae g_Methanobrevibacter	B	1.242 (0.87)	2.087 (1.78)	0.366 (0.258)	0.711 (0.711)	0.92		

[a] Wilcoxon rank sum (1df) comparing neoadjuvant (neoADJ) vs non-neoadjuvant (ADJ + no chemotherapy) groups at baseline and last collections, and in the change between last and baseline samples

[b] Negative binomal test model in R: glmm.nb (count at gena ~ period*weight change2 + offset(log(totreads)), random = ~ 1|SubjectID), Period (final vs baseline),

t change2 (increase weight vs decrease weight)

[c] Wilcoxon signed rank (1df) to test changes between last and baseline samples

		Mean baseline (B) and absolute changes (Δ) in taxa abundance by chemotherapy				neoADJ vs non-neoADJ		
	Δ	-0.696 (0.59)	-1.428 (1.17)	-0.202 (0.292)	0.624 (0.59)	0.80		
	P[c]	0.55	0.16	0.74	0.25		**0.003**	**0.003**
P_Firmicutes	B	0.025 (0.01)	0.025 (0.015)	0.025 (0.016)	0.002 (0.002)	0.33		
	Δ	0.01(0.014)	0.021(0.018)	-0.001(0.028)	0.002 (0.003)	**0.027**		
	P[c]	0.67	0.25	0.20	1.00		**0.001**	**0.002**
P_Firmicutes f_Gemellaceae g_	B	0.006 (0.002)	0.005 (0.002)	0.009 (0.003)	0.002 (0.002)	0.36		
	Δ	0.002 (0.004)	0.007 (0.006)	-0.003 (0.004)	0 (0.003)	0.36		
	P[c]	0.74	0.82	0.43	1.00		**0.005**	**0.006**
P_Firmicutes	B	0.137 (0.06)	0.14 (0.09)	0.16 (0.11)	0.06 (0.06)	0.62		
f_ Enteroccaceae, g_Enterococcus	Δ	0.014 (0.13)	0.13(0.27)	-0.12(0.09)	-0.02(0.03)	0.26		
	P[c]	0.18	0.92	**0.047**	0.75		**0.028**	**0.035**
P_Firmicutes f_ Lactobacillaceae, g_Lactobacillus	B	0.885 (0.60)	0.112 (0.043)	2.108 (1.502)	0.006 (0.005)	0.50		
	Δ	-0.217(0.70)	0.199 (0.176)	-0.83 (1.802)	0.11 (0.10)	0.54		
	P[c]	0.76	0.38	0.70	0.38		**0.049**	0.072
P_ Firmicutes f_ Streptococccaceae, g_Lactoccus	B	0.043 (0.016)	0.051 (0.029)	0.031 (0.017)	0.046 (0.0308)	0.63		
	Δ	0 (0.023)	0.021 (0.045)	-0.02 (0.02)	-0.025 (0.017)	0.68		
	P[c]	0.29	1.00	0.46	0.25		**0.038**	**0.043**
P_Firmicutes	B	0.042(0.022)	0.003(0.002)	0.05(0.031)	0.17 (0.15)	0.13		
f_Turibacteraceae	Δ	-0.016(0.02)	0.014(0.009)	-0.023(0.04)	-0.11(0.10)	0.26		
g_Turibacter	P[c]	0.90	0.15	0.69	0.13		**0.001**	**0.001**
P_ Firmicutes	B	0.095 (0.07)	0.013 (0.01)	0.201 (0.18)	0.08 (0.08)	0.70		
f_ Clostridiaceae	Δ	-0.043 (0.07)	0.077 (0.05)	-0.191 (0.17)	-0.04 (0.06)	0.28		
g_	P[c]	0.72	0.34	0.69	1.00		**0.003**	**3.3 E-10**
P_ Firmicutes f_ Lachnospiraceae g_ Anaerostipes	B	0.028 (0.011)	0.012 (0.006)	0.031 (0.015)	0.086 (0.078)	0.73		
	Δ	0.036 (0.019)	0.029 (0.015)	0.058 (0.04)	-0.012 (0.076)	0.64		
	P[c]	**0.038**	0.07	0.11	0.75		0.21	0.41
P_ Firmicutes	B	0.104(0.06)	0.048(0.03)	0.032(0.03)	0.567(0.46)	0.16		
f_ Lachnospiraceae	Δ	0.019 (0.06)	-0.040(0.03)	0.198(0.12)	-0.328(0.24)	**0.003**		
g_ clostridium	P[c]	0.76	0.38	**0.024**	0.13		**0.017**	**0.027**

[a] Wilcoxon rank sum (1df) comparing neoadjuvant (neoADJ) vs non-neoadjuvant (ADJ + no chemotherapy) groups at baseline and last collections, and in the change between last and baseline samples

[b] Negative binomal test model in R: glmm.nb (count at gena ~ period*weight change2 + offset(log(totreads)), random = ~ 1|SubjectID), Period (final vs baseline),

t change2 (increase weight vs decrease weight)

[c] Wilcoxon signed rank (1df) to test changes between last and baseline samples

		Mean baseline (B) and absolute changes (Δ) in taxa abundance by chemotherapy				neoADJ vs non-neoADJ		
P_ Firmicutes	B	1.50 (0.26)	1.55 (0.45)	1.45 (0.31)	1.41 (0.85)	0.63		
f_ Ruminococcaceae	Δ	0.06 (0.24)	0.51 (0.30)	-0.42(0.44)	-0.22(0.27)	0.13		
g_	P[c]	0.69	0.14	0.50	0.63		**0.036**	**0.048**
P_ Firmicutes	B	6.48 (0.72)	6.40 (1.02)	6.10 (1.14)	8.05 (2.69)	0.73		
f_ Ruminococcaceae	Δ	1.58 (0.89)	1.67 (0.81)	2.65(1.92)	-2.25(1.68)	0.35		
g_Faecalibacterium	P[c]	0.10	**0.044**	0.19	0.38		0.61	0.67
P _Firmicutes	B	0.44 (0.241)	0.872 (0.48)	0.033 (0.032)	0.036 (0.035)	0.40		
f_ Veillonellaceae	Δ	0.056 (0.328)	-0.41 (0.552)	0.626 (0.459)	0.072 (0.064)	0.31		
g_Acidaminoccoccus	P[c]	0.30	0.84	0.13	0.50		**0.001**	**0.001**
P__Firmicutes	B	0.038 (0.021)	0.05 (0.041)	0.034 (0.016)	0.001 (0.001)	0.62		
f_Mogibacteriaceae	Δ	-0.002 (0.21)	0.018 (0.04)	-0.029 (0.016)	0.008 (0.007)	0.26		
g_Mogibacterium	P[c]	0.80	0.69	0.16	0.50		**0.049**	0.062
P_ Erysipelotrichaceae	B	0.143 (0.062)	0.094 (0.066)	0.247 (0.134)	0.001 (0.001)	0.52		
g__Catenibacterium	Δ	-0.012 (0.08)	0.169 (0.106)	-0.241 (0134)	0.005 (0.006)	0.09		
	P[c]	0.93	0.16	0.13	1.00		**7.0 E-06**	0.24
P__Firmicutes	B	0.37 (0.11)	0.09(0.03)	0.80 (0.24)	0.10 (0.09)	**0.032**		
f_ Erysipelotrichaceae	Δ	-0.13(0.11)	0.16(0.07)	-0.57(0.23)	0.12(0.08)	**0.003**		
g__[Eubacterium]	P[c]	0.95	**0.042**	**0.020**	0.13		**0.00048**	**0.001**
P__Proteobacteria	B	0.092(0.02)	0.133(0.033)	0.042(0.012)	0.085(0.074)	0.08		
f_ Desulfoybrionaceae	Δ	0.086(0.059)	0.019(0.047)	0.214(0.132)	-0.062(0.063)	**0.006**		
g__Bilophila	P[c]	0.23	0.50	**0.002**	0.75		**0.015**	**0.019**
P__Proteobacteria	B	0.356 (0.164)	0.431 (0.29)	0.371 (0.223)	0.009 (0.008)	0.39		
g__Haemophilus	Δ	-0.187(0.173)	-0.16(0.308)	-0.318(0.229)	0.126(0.096)	**0.030**		
	P[c]	0.76	0.21	0.19	0.25		0.54	0.600
P_Verrucomicrobia	B	0.491 (0.371)	0.976 (0.758)	0.042 (0.036)	0.008 (0.006)	**0.018**		
f_ g_Akkermansai	Δ	-0.138 (0.407)	-0.738 (0.784)	0.370 (0.277)	0.605 (0.578)	0.15		
	P[c]	0.52	0.45	0.16	0.50		**0.005**	**0.008**

[a] Wilcoxon rank sum (1df) comparing neoadjuvant (neoADJ) vs non-neoadjuvant (ADJ + no chemotherapy) groups at baseline and last collections, and in the change between last and baseline samples

[b] Negative binomal test model in R: glmm.nb (count at gena ~ period*weight change2 + offset(log(totreads)), random = ~ 1|SubjectID), Period (final vs baseline),

t change2 (increase weight vs decrease weight)

[c] Wilcoxon signed rank (1df) to test changes between last and baseline samples

We then compared baseline mean taxa abundance and absolute changes (Δ) (final-baseline) in taxa abundance between the weight loss (n=16) and weight gain (n=17) groups using Wilcoxon and negative binomal analysis. None of the taxa showed changes in abundance that met the $p<0.0007$ threshold, but one taxa, *Firmicutes (g_Lachnobacterium)* emerged in all three tests (Wilcoxon $p=0.007$; negative binomial $p= 0.002$ without adjustment for chemotherapy; and $p=0.006$ with adjustment for chemotherapy).

As of May 2021, 8 (6 neoADJ, 2 ADJ) of the 33 breast cancer patients developed a recurrence or metastases of whom five were deceased or in hospice care at this date. There were no significant associations between risk of recurrence/metastases and baseline alpha diversity measures or changes in alpha diversity (p's were ≥ 0.18).

Discussion

To our knowledge, this is the first study to follow a group of breast cancer patients with preplanned analyses to examine changes in gut microbiome during their first year of treatment in relation to type of chemotherapy, taking into account changes in weight during the study period. In all three groups, the final fecal sample was collected more than 100 days after the end of chemotherapy (for neoADJ and ADJ groups) or radiation (for noC group), representing a recovery period. We found striking differences in gut alpha diversity changes by treatment, increases in all four alpha diversity measures in the neoADJ group that was not observed in the ADJ or noC groups. Increases in alpha diversity were also observed in the weight loss but not in the weight gain groups. However, there were no significant changes in alpha diversity in relation to risk of recurrence/metastases. There were notable changes in taxa abundance that differed by treatment; one taxa, *p Bacteroidetes (g_Alistipes*) showed changes in abundance that reached the Bonferroni threshold of $p<0.0007$, while changes in abundance of 12 taxa showed p values that ranged from <0.001 to 0.0007 without or with adjustment of weight changes. In contrast, there were far few changes in taxa differences between the weight loss and weight gain groups, but one taxa, *p_Firmicutes (g_Lachnobacterium*) showed changes that were consistent in all three statistical analyses.

Although average weight changes were modest during this first year of treatment, our finding of a significant increase in the alpha diversity measures in the neoADJ group may be related in part, to weight loss in the neoADJ group (-1.48 kg) but weight gain in the ADJ (+1.01 kg) and noC (+0.33 kg) groups. Alpha diversity measures have been used to assess health habits including body composition, and low gut alpha diversity has been associated with obesity in some studies [24, 25]. In a study of 26 cancer patients (7 with breast cancer) who were treated with cytotoxic, targeted chemotherapy, or a combination of chemotherapy with immununotherapy, gut microbiome Shannon index was higher in responders than in nonresponders, who also displayed higher abundance of *Alistipes*, a genus member of the *Rikenellaceae* family within the *Bacteroidales* order [13]. In a study of non-small cell lung cancer patients treated with immune checkpoint inhibitors, responders showed higher diversity of gut microbiome as well as an enrichment of *Alistipes* [26]. In our analysis, *Alistipes* emerged to be important in both Wilcoxon rank sum and negative binomal analyses but its abundance decreased in the neoADJ group but not in the ADJ group after completion of treatment. The significance of our finding on *Alistipes* is unclear but this genus has been found to be correlated with both healthy phenotypes as well as having pathogenic roles [27] in colorectal cancer [28] and liver diseases [29]. It has been suggested that decrease in *Alistipes* contributes to the decrease in short chain fatty acids which have anti-inflammatory properties.

We also found suggestive differences in changes in taxa of select *Erysipelotrichaceae* genera (*Catenibacterium, Eubacterium and Clostridium*), abundance increased in the neoADJ but decreased in the non-neoADJ groups. In a small study of patients with breast or gynecological cancers, *Erysipelotrichaceae* abundance also increased but this was mainly among women who gained weight following treatment [12]. The immunogenic properties of some members of the *Erysipelotrichaceae* family may lead to gut inflammation and weight gain [30]. Our findings on changes in taxa abundance of *Verrucomicrobia (g_Akkermansai)*, in particular, a reduction in abundance in the neoADJ group but an increase in the non-neoADJ group adds to the literature of the importance of this butyrate-producing bacteria [31, 32]. In a study of breast cancer patients treated by neoadjuvant chemotherapy, breast tumor tissue microbiome profile was impacted by treatment but a comparable group of patients treated with adjuvant chemotherapy was not included in this study [33]. Nevertheless, results from this [33] and our study suggest that type of chemotherapy may impact breast and gut microbiome changes.

Our finding of a difference in the abundance of *Lachnobacterium* between the weight gain and weight loss groups needs confirmation. There is scant information on this genus. A recent Swedish cross-sectional study found that high intake of sugar and sweet bevarages was significantly inversely associated with abundance of *Lachnobacterium* [34]. However, another cross-sectional study found abundance levels of *Lachnobacterium* was higher in obese subjects than normal weight subjects and higher among individuals with low physical activity than those with high physical activity [35].

Strengths of this pilot study include the longitudinal collection of gut microbiome data on 33 breast cancer patients at multiple (baseline, during, and at the completion) time points during the first year of treatment with either neoADJ, ADJ, or noC treatment. In addition to the detailed information on breast cancer treatment, tumor characteristics, and lifestyle information that was updated at each clinic visit, body composition was assessed using DXA at baseline and at the completion of study. Our results on gut microbiome changes were analyzed using complementary statististical methods, Wilcoxon rank sum test and negative binomial mixed models for longitudinal microbiome data with adjustment for select covariates including changes in weight. We also considered multiple comparisons and used a Bonferroni-adjusted type I error rate to evaluate p-values. Participants included whites and nonwhites, reflecting the catchment area of USC. However, we are limited by a modest sample size and the no chemotherapy group was based on only 4 patients. Although we conducted results separately for the three treatment groups, our main analysis was based on comparing neoADJ to non-neoADJ groups (i.e, ADJ + noC). Because of the inherent differences in timing of treatment between neoADJ, ADJ, and no chemotherapy groups, we were not able to collect fecal samples at a standardized interval and the period of enrollment and length of follow-up were not identical in the three groups (Figure 1). Nevertheless, the baseline fecal samples were collected before initiation of chemotherapy for the neoADJ and ADJ groups or radiation for the no chemotherapy group and the final fecal samples were collected when there was a recovery period of at least 100 days after completion of chemotherapy or radiation. Because this was funded as a pilot study, we only monitored patients during the first year of treatment and did not collect information on additional treatment (e.g., hormone therapy).

In conclusion, this pilot longitudinal study found significant increases in gut microbiome alpha diversity measures in the neoADJ group but not in the non-neoADJ group and also intriguing changes in select *Bacteroidetes* and *Firmicutes* taxa. The dynamic nature of the gut microbiome in association with chemotherapy and weight changes highlight the need to better understand the significance of these findings and how to harness this information to identify a gut microbiome profile that would have lasting beneficial effects among women with breast cancer. Given the very modest sample size of this pilot study, we view these taxa changes as potentially informative and worthy of investigation in future studies with larger sample sizes of breast cancer patients and with longer duration of follow-up.

Abbreviations

adjuvant (ADJ)
body mass index (BMI)
dual-energy x-ray absorptiometry (DXA)
estrogen/ progesterone receptor (ER/PR)
hormone receptor (HR)
human epidermal growth factor receptor 2 (HER2)
negative binomial regression (NBR)
neoadjuvant (neoADJ)
no chemotherapy (noC)

operational taxonomic units (OTU)

permutational multivariate analysis of variance (PERMANOVA)

phylogenetic distance (PD)

principal coordinate analysis (PCoA)

ribosomal RNA (rRNA)

University of Southern California (USC)

Declarations

Acknowledgements: We gratefully acknowledge all the women who participated in this study, Wendy Cheng for coordinating this study; Diana Chingos for serving as patient advocate; helpful advice from Dr. Wendy Cozen and Dr. Jacques Ravel during the early phase of the study; and invaluable support from Dr. Jacques Ravel and Dr. Mike Humphrys at the University of Maryland for performing the 16S rRNA gene sequencing work.

Funding: This study was funded by the California Breast Research Program (20IB-0105) and supported by the USC Norris Comprehensive Cancer Center Core Support grant (P30 CA14089) (Wu).

Availability of data and materials: Request for additional details of the data used in this manuscript can be directed to the corresponding author.

Ethics approval and consent to participate: Informed consent was obtained from all individual participants included in this study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by the USC Institutional Review Board.

Consent for publication: Not applicable

Conflict of interests: All authors have approved the final version of the manuscript. The authors declare that they have no conflict of interests.

Authors' contribution statement: AH Wu conceived the study and obtained funding with advice from C Vigen, D Spicer, and A Garcia. D Spicer and A Garcia supervised the recruitment of patients. C Tseng and C Vigen performed the statistical analyses. AH Wu, C Vigen and CTseng interpreted the data. AH Wu and C Vigen were the primary contributors to the manuscript. All authors reviewed and approved the final manuscript.

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Figures

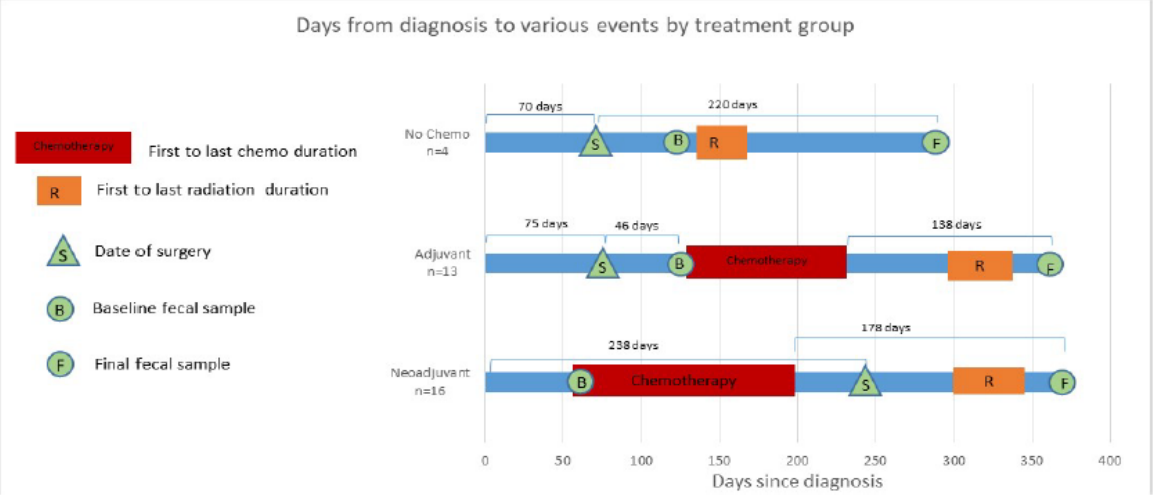

Figure 1

Timing of baseline (B) and final (F) fecal sample collection in relation to date of diagnosis and standard treatment

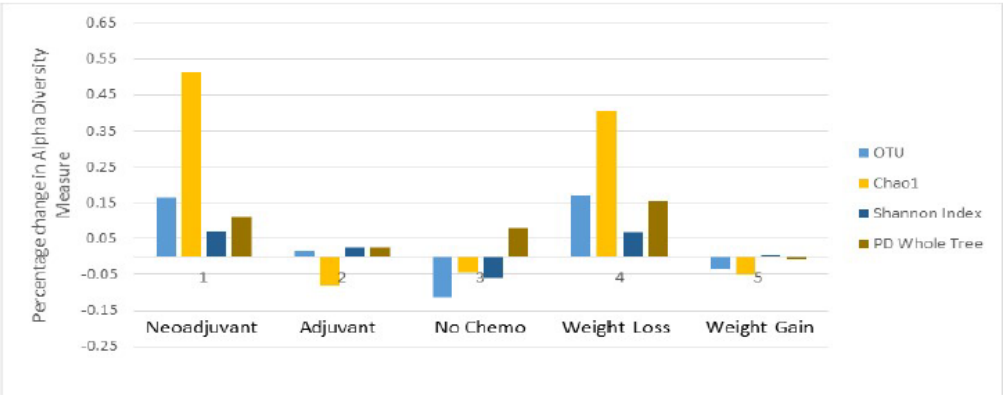

Figure 2

Percentage change in alpha diversity measures (OTU, Chao 1 index, Shannon index, and PD Whole tree) by chemotherapy treatment (neoADJ n=16, ADJ n=13, no chemotherapy n=4), and by weight changes (weight loss n=16; weight gain n=17).

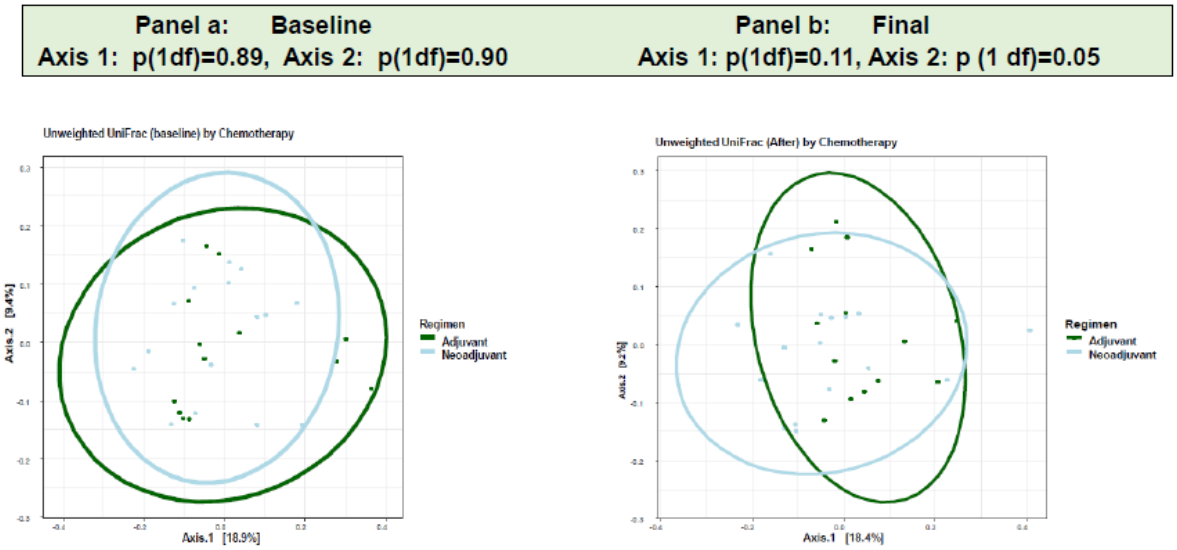

Figure 3

Beta-diversity results at baseline and final collection are shown based on unweighted UniFrac-based principal component analysis plot of the first two principal coordinates categorized by neoadjuvant (neoADJ n=16) and non-neoADJ (ACT n=13 + no chemotherapy n=4). At baseline collection, axis 1 explained 18.9% while axis 2 explained 9.4% of the variance. (B) At final collection, axis 1 explained 18.4% and axis 2 explained 9.2% of the variance.

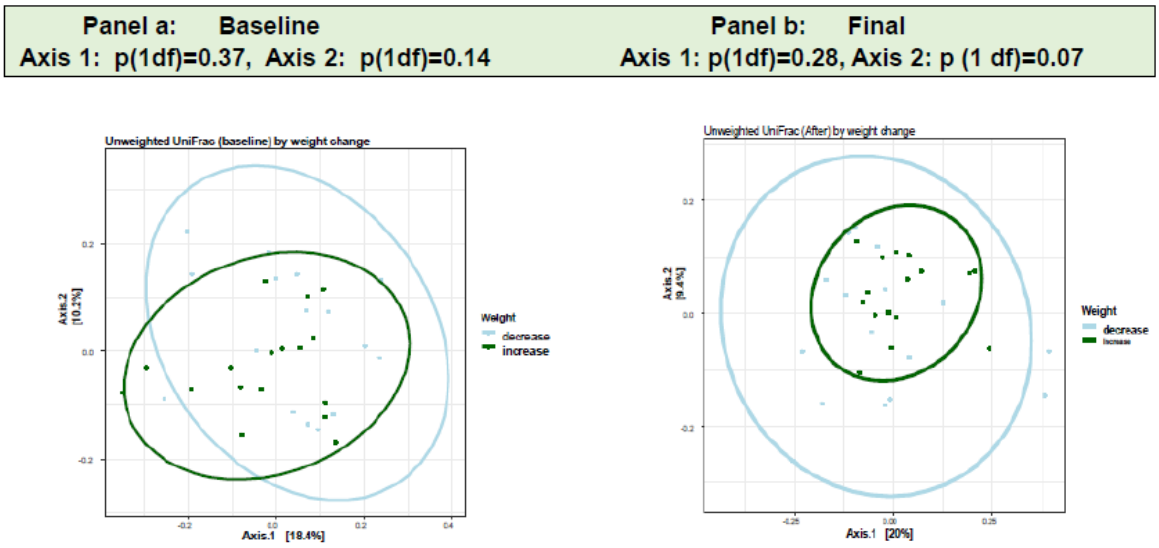

Figure 4

Beta-diversity results at baseline and final collection are shown based on unweighted UniFrac-based principal component analysis plot of the first two principal coordinates categorized by weight loss (n=16) and weight gain (n=17). At baseline collection, axis 1 explained 18.4% while axis 2 explained 10.2% of the variance. (B) At final collection, axis 1 explained 20.0% and axis 2 explained 9.4% of the variance.