Title

Baseline gut microbiota as a predictive marker for the efficacy of neoadjuvant chemotherapy in patients with early breast cancer: a multicenter prospective cohort study in the Setouchi Breast Project-14

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Abstract (238/250 words)

Purpose:

Various studies have demonstrated the causal relationship between gut microbiota and efficacy of chemotherapy, however, the impact of gut microbiota on breast cancer is not fully elucidated so far. This study aimed to evaluate the associations between the gut microbiota before neoadjuvant chemotherapy and its consequent efficacy in breast cancer.

Methods:

This prospective observational study included patients who received neoadjuvant chemotherapy for primary early breast cancer at eight institutions between October 1, 2019, and March 31, 2022. We performed 16S rRNA analysis of fecal samples and α and β diversity analyses of the gut microbiota. The primary endpoint was the association between the gut microbiota and pathological complete response (pCR) to neoadjuvant chemotherapy.

Results:

Among the 183 patients, the pCR rate after neoadjuvant chemotherapy was 36.1% in all patients and 12.9% (9/70), 69.5% (41/59), and 29.6% (16/54) in those with the luminal, human epidermal growth factor receptor 2, and triple negative types, respectively. The α

diversity of the gut microbiota did not significantly differ between patients with pCR and those without pCR. Among the gut microbiota, two species (*Victivallales*, p = 0.001 and *Anaerolineales*, p = 0.001) were associated with pCR, and one (*Gemellales*, p = 0.002) was associated with non-pCR.

Conclusions:

Three species in the gut microbiota had potential associations with neoadjuvant chemotherapy efficacy, but the diversity of the gut microbiota was not associated with response to chemotherapy. Further research is needed to validate our findings.

Keywords:

gut microbiota, predictive marker, neoadjuvant chemotherapy, early breast cancer

Introduction

International Association of Research on Cancer published global cancer burden using GLOBOCAN 2020, that reported that breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in women [1]. The incidence of breast cancer has increased since the introduction of mammography screening and continues to grow with the aging of the population [2]. The treatment of early breast cancer is complex, and it is crucial to select appropriate systemic treatments. Predictive biomarkers, such as estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), and Ki67, and approved genomic signatures has been established to help clinicians and patients determine the treatment [2]. However, more biomarkers are required to improve the benefit of chemotherapy by predicting the efficacy. It has been established that pathological complete response (pCR) to neoadjuvant treatment can be used as a surrogate marker of clinical benefit such as event-free survival and overall survival [3]. In addition, tumor-infiltrating lymphocytes (TILs) has been identified as a predictor of response to neoadjuvant chemotherapy and had been associated clinical benefits of chemotherapy in breast cancer [4]. TILs have been observed as mononuclear immune cells present within and around tumor cells and have been reported as reproducible biomarkers of immunogenicity in breast cancer [5]. Additionally, tumor-

infiltrating CD8 cytotoxic lymphocytes have been associated with patient-specific survival in breast cancer, and the total CD8 T-cell count was identified as an independent prognostic factor in multivariate analysis [6]. CD8 cytotoxic T lymphocytes were found to be an independent predictive factor for pCR in breast cancer [7]. Therefore, other immune system markers might be useful in predicting the efficacy of chemotherapy.

The human intestinal microbiome is highly complex and is predicted to contain more than 1,000 different prokaryotic species, and the collective genomes within the microbiome contains more than 3 million unique genes [8]. With the development of sequencing technology and bioinformatics, the role and relationship of the microbiome, including the gut microbiota, with human health and disease has been reported [8]. Recently, the gut microbiota had been demonstrated to affect the antitumor immunity of patients with various cancer types, such as melanoma, lung, and colon [9-16]. The gut microbiota has been identified as one of the major environmental factors that can control the development and maintenance of the immune system [10]. Various studies using mouse models have clearly demonstrated the causal relationship of commensal flora to the efficacy of chemotherapy and immunotherapy through regulation of host immunity, and these data suggested that manipulating the microbiota might improve the therapeutic effects of cancer chemotherapy and immunotherapy [11-13]. In addition, several clinical

studies revealed significant differences in the gut microbiome diversity between responders and non-responders to anticancer therapy, including chemotherapy and/or immunotherapy, in patients with various cancer types, such as melanoma, lung, and colon [9, 14-16].

However, the utility of the gut microbiota as a predictive marker of chemotherapy efficacy in breast cancer is unclear. This study investigated the associations of the gut microbiota with response to chemotherapy and patient characteristics and lifestyle before breast cancer diagnosis.

Material and methods

Trial Design

The trial design of this study (Setouchi Breast Project-14) is a multicenter, prospective, observational study conducted under the Setouchi Breast Project Comprehensive Support Organization (http://setouchi-bp.com/index.html). The primary endpoint was the association between gut microbiota diversity and pCR, and the secondary endpoints were the associations of the gut microbiota with the patient characteristics or lifestyle before initiation of any treatment for breast cancer.

Patients

The eligible participants were those who 1) had no distant metastases, 2) underwent neoadjuvant chemotherapy for early breast cancer, 3) included all subtypes, 4) women aged ≥20 years, 5) had an Eastern Cooperative Oncology Group Performance Status of <3, and 6) were able to provide informed consent to participate in this trial. Patients were excluded if they had distant metastases, active infections (including hepatitis B virus), bilateral invasive breast cancer, or history of hypersensitivity to chemotherapy drugs containing taxanes, polyoxyethylene castor oil, anthracyclines, or alkylating drugs. We enrolled patients who received neoadjuvant chemotherapy for primary early breast cancer between October 1, 2019, and March 31, 2022 at eight institutions, including the Fukuyama City Hospital, Hiroshima City Hiroshima Citizens Hospital, Japanese Red Cross Okayama Hospital, Kagawa Prefectural Center Hospital, Kochi Health Science Center, National Hospital Organization Shikoku Cancer Center, Matsue Red Cross Hospital, and Okayama University Hospital.

Treatment

Patients received standard therapies, including surgery, neoadjuvant chemotherapy, HER2-targeted therapy, hormone therapy, and radiotherapy, based on the biomarker

results at biopsy and Japanese Breast Cancer Society Clinical Practice Guideline [17]. Neoadjuvant chemotherapy as usual care comprised sequential anthracyclines and taxanes at each institution. Dose modifications, interruptions, and discontinuations were determined in accordance with routine clinical practice. The general dosage and administration are as follows; adriamycin 60 mg/m^2 + cyclophosphamide 600 mg/m^2 or epirubicin 90 mg/m^2 + cyclophosphamide 600 mg/m^2 every 2 or 3 weeks for 4 courses, and docetaxel 75-100 mg/m^2 every 3 weeks for 4 courses or paclitaxel 80 mg/m^2/week for 12 weeks or paclitaxel 175 mg/m^2 every 2 weeks for 4 courses. For patients with HER2-positive breast cancer, trastuzumab (+ pertuzumab) should be administered with the taxane regimen. The immune checkpoint inhibitors for patients with early breast cancer were not available in Japan during that period.

Pathological diagnosis

We defined breast cancer subtypes based on the pathologic diagnosis of core needle biopsy samples before systemic treatment or surgery. ER-positive was defined as ER positivity of ≥1%, PgR-positive as PgR positivity of ≥1%, and HER2-positive as a score of 3 in immunohistochemistry or the presence of HER2 gene amplification by fluorescence in situ hybridization, according to the American Society of Clinical

Oncology/College of American Pathologists guidelines [18,19]. Breast cancer subtypes were classified as follows: Luminal type if ER positive and/or PgR positive and HER2-negative, HER2 type if HER2 positive, and Triple-negative type if ER, PgR, and HER2 were all negative.

We defined pCR as the absence of residual invasive tumor in the completely resected breast and axilla specimens. Patients were categorized into the pCR and non-pCR groups, based on the pathological assessment of the resected specimens after neoadjuvant chemotherapy. The TILs were evaluated by two board-certificated pathologists, according to the International TILs Working Group 2014 [20]. They scored independently without an access to patients' record, and discussed on a microscope about cases with discordant results until they reach an agreement. We classified TILs as follows: low for <10%, intermediate for 10%–50%, and high for >50%.

Gut microbiota analysis

We collected fecal samples from the patients before the initiation of neoadjuvant chemotherapy for breast cancer and provided them to Takara Bio. Inc. (Shiga, Japan) for 16S rRNA analysis [21]. To determine the species proportion in the sample, the QIIME2 pipeline (https://qiime2.org/) was used to analyze the bacterial flora based on sequences

obtained from polymerase chain reaction amplification of the v3–v4 region (341F-806R) of the 16SrRNA. Noise and chimeric reads were eliminated from the sequenced read sequences. Subsequently, R1R2 reads were assembled, and reads that matched completely were merged.

Clustering was performed to construct operational taxonomic unit (OTU). Each OTU was used to build the Greengenes (http://greengenes.lbl.gov) and DDBJ databases (https://www.ddbj.nig.ac.jp/index.html), and the abundance ratio was calculated based on the amount of reads in each OTU. Phylogenetic analysis was conducted based on the evolutionary distance, which was calculated from the OTU. The α and β diversity analyses were conducted using the results of the phylogenetic analysis.

Statistical analyses

We performed analyses using the software R version 3.4.2. Differences between the groups were determined using Wilcoxon's rank sum test. Categorical variables were analyzed by the Fisher's exact test, and continuous variables were examined by Spearman correlation analysis. Differences were considered significant at $P < 0.05$. Diversity is defined in ecological terms as species richness and evenness, which reflect the number of phylotypes and their relative abundance [8]. The α diversity is defined as the diversity of

species within a single sample. The β diversity is defined as the differences in species diversity across multiple samples. We used two different indices (i.e., Chao1 and Shannon) for the α or intrasample diversity analysis. The Chao1 examines the richness of different bacteria present in each sample. The Shannon evaluates importance at the evenness and richness levels. The β or intersample diversity was measured using the UniFrac distance metric. This distance was used because it incorporates information on the relative relatedness of community members by incorporating phylogenetic distances between the observed bacteria, and principal coordinates analysis (PCoA) was performed to visually compare the microbiota of the different treatment groups, considering the bacterial phylogenetic distances. A Monte Carlo two-sample t-test was employed to compare α diversity and bacterial flora structure. The composition ratio was compared between two groups using Monte Carlo two-sample t-test and among multiple groups using analysis of variance; both were corrected for Bonferroni and false discovery rate (FDR).

Results

Patient characteristics

After applying the exclusion criteria (unwillingness to adhere to the protocol,

relocation, withdrawal of consent, progress during treatment, bilateral invasive breast cancer, and those missing 16S rRNA gene data) on the 197 patients enrolled, the full analysis set comprised 183 patients (Figure 1).

The patient characteristics at baseline are summarized in Table 1. The median age was 52 years (range, 25–77 years), and the median body mass index (BMI) was 22.6 (range, 14.8–43.8 years). The breast cancer type was luminal in 70 (38.3%), HER2 in 59 (32.2%), and triple negative in 54 (29.5%). The detailed information on the baseline lifestyle characteristics, which were available in 162 cases, is provided in Supplemental Table S1. Overall, 91.4% were non-smokers, 76.5% rarely consumed alcohol, and 56.2% exercised for less than 30 minutes per week.

The pCR rate after neoadjuvant chemotherapy was 36.1% (66/183) overall and 12.9% (9/70), 69.5% (41/59), and 29.6% (16/54) in patients with the luminal, HER2, and triple negative types, respectively.

Diversity analysis based on phylogenetic analysis

As shown in Table 2, the α diversity had no significant differences with the patient characteristics including BMI, although it showed a trend of relationship with age and TILs (age ≥50 vs <50; Odds ratio [OR]: 1.447, 95%CI, 0.980-2.179, P = 0.068 in

Shannon, OR: 1.004, 95%CI, 0.9998-1.008, P = 0.068 in Chao; TILs High vs Low; ORR: 0.640, 95%CI, 0.351-1.157, P = 0.130 in Shannon, OR: 0.992, 95%CI, 0.983-1.000, P = 0.067 in Chao). However, α diversity had a significant relationship with lifestyle characteristics, particularly exercise (≥3 h vs. <30 min, OR 1.007, 95% CI 1.001–1.013, P = 0.030 in Chao; Supplemental Table S2). The detailed results of the relationship between lifestyle characteristics and α diversity are shown in Supplemental Table S2. As shown in Figure 2, there was no significant difference in α diversity between the pCR and non-pCR groups, both overall and by breast cancer subtype. Similarly, the α diversity did not significantly differ among the breast cancer subtypes (Supplemental Figure S1). Figure 3 shows the β diversity analysis of the gut microbiota. The PCoA of the OTUs showed no obvious separation between the pCR and non-pCR groups in all samples and among the breast cancer subtypes.

Microbial order and response to neoadjuvant chemotherapy

We investigated the relationship between bacterial flora structure (order: 59 species), excluding 15 that were unclassified, and the efficacy of neoadjuvant chemotherapy, which was adjusted by breast cancer subtype. Table 3 shows the relationship between bacterial flora structure (order: 59 species) and neoadjuvant chemotherapy efficacy. The

pCR group was associated with two species, including *Victivallales* (p = 0.001, FDR 0.030, Bonferroni 0.031), which was significantly increased across all breast cancer subtypes, and *Anaerolineales* (p = 0.001, FDR 0.030, Bonferroni 0.071), which was present only in patients with the luminal type. The non-pCR group was associated with one species, particularly *Gemellales* (p = 0.002, FDR 0.030, Bonferroni 0.089), which was significantly increased across all samples.

The detailed results of the relationship between bacterial flora structure (order: 44 species), including three species with FDR <0.1, and neoadjuvant chemotherapy efficacy are provided in Supplemental Table S3. The relationships of patient characteristics/lifestyle with *Victivallales*, *Anaerolineales*, and *Gemellales* are shown in Table 3 (FDR <0.1). To further analyze the microbial orders that were significantly associated with chemotherapy response, we performed class comparisons of the response to chemotherapy. There were no significant differences between patient characteristics/lifestyle and *Victivallales* and *Anaerolineales*, but the use of supplements was significantly associated with *Gemellales* (OR 1.018, 95% CI 1.004–1.034, P = 0.016) (Supplemental Table 4); notably, abundance of *Gemellales* was associated with resistance to chemotherapy (Table 3).

Discussion

As far as we know, this is the largest clinical study that assessed the relationship between gut microbiota and various factors, including response to chemotherapy, in breast cancer. Based on our analyses, gut microbiota diversity had no significant associations with chemotherapy response and baseline characteristics among patients with breast cancer. However, frequent exercise was associated with increased gut microbiota diversity. Preclinical studies have shown important associations between the gut microbiota and response to chemotherapy, including cyclophosphamide [12,22], doxorubicin [23], and oxaliplatin [11].

Two clinical studies have previously reported the association between chemotherapy efficacy in breast cancer and gut microbiota diversity [10,24], but their sample size was smaller. In the study by Li Y et al on 23 patients who received a heterogeneous regimen for all breast cancer subtypes, the gut microbiota diversity was lower in the noneffective group (n = 5) than in the effective group (n = 18) [24]. In another clinical study on patients treated with neoadjuvant trastuzumab for HER2-positive breast cancer (n = 25), the gut microbiota α diversity before treatment initiation was significantly higher in responders (n = 16) than in non-responders (n = 7) [10]. On the contrary, our results did not show significant association between gut microbiota α

diversity and neoadjuvant chemotherapy efficacy. This difference may be accounted for by the smaller sample sizes of the preceding studies, racial differences, and different neoadjuvant regimens, such as the fact that pertuzumab was not used as antiHER2 therapy in the previous study. Conversely, some clinical studies showed similar results on the absence of association between neoadjuvant chemotherapy efficacy and the gut microbiota. One clinical study reported that the pathological response was not associated with the baseline gut microbiota richness, diversity, and composition in patients who underwent neoadjuvant chemotherapy for ER-positive breast cancer (n = 18) [25]. We speculate that ethnic and geographical influences affected the results of these studies, including ours, even under different situations [26,27]. Therefore, the association between anticancer therapy and gut microbiota diversity remains controversial, and further validation is required.

Our results showed a trend of relationship between low α diversity and high TILs. Some reports showed associations between gut microbiota and the host immune system. One study demonstrated that fecal microbiota transplantation modulated immune responses, such as peripheral and intratumoral CD8+ T cell activation [28]. Li Y et al attributed the relatively high levels of peripheral blood CD4+ T cells and absolute numbers of CD4+ and CD8+ TILs in tumor tissues to the relatively abundant

Coprococcus, *Dorea*, and uncultured *Ruminococcus* sp. in responders [24]. These results suggested that the gut microbiota modulate the efficacy of neoadjuvant chemotherapy through interactions with immune cells.

Moreover, we found a significant association between gut microbiota diversity and a lifestyle of frequent exercise before diagnosis. Lifestyle factors, including exercise, age, smoking, and antibiotic use, had been reported to be associated with the gut microbiota. Previous studies found that exercise might have positive effects on gut microbiota diversity and health [29,30]. One study reported that the gut microbiota was significantly less diverse among centenarians than among elderly or young adults and implied that the aging process significantly influenced the human gut microbiota structure and homeostasis with the host immune system [31]. Alternatively, another study demonstrated no significant difference in the α diversity between premenopausal and postmenopausal patients with breast cancer [32]. Our results showed a trend of relationship between α diversity and age. These results implied the possibility of age-related changes in the gut microbiota diversity among patients with breast cancer. In the future, it is necessary to investigate and validate whether interventions for lifestyle factors including exercise, age, weight control and dietary habits, have an impact on the response to therapy and prognosis of breast cancer.

We found three species that were associated with pCR. The order Victivallales includes Gram-negative bacteria that belong to the phylum Lentisphaerae and class Lentisphaeria. Recent research has shown that Lentisphaerae was relatively abundant in patients with inflammatory bowel disease, suggesting a close association between these microbial communities and immune regulation [33]. The order Anaerolineales includes Gram-negative bacteria that belong to the phylum Chloroflexi and class Anaerolineae. One study showed that Chloroflexi were the dominant microbes in hepatocellular carcinoma tissues [34]. In addition, another study demonstrated that the *Lentisphaerae* and *Victivallaceae* gut microbiota were associated with clinical response to immunotherapy in various patients with cancer in China [35]. The order Gemellales includes Gram-positive bacteria that belong to the phylum Firmicutes (Bacillota) and class Bacilli. One study reported that certain gut microbiota, such as the *Faecalibacterium* genus and Firmicutes phylum, were relatively abundant among the responders to immunotherapy [9]. Similar to *Gemellales*, *Enterococcus faecium*, which was reported to be relatively abundant in immunotherapy responders [15], belongs to the phylum Firmicutes (Bacillota) and class Bacilli. In contrast, our study found that *Gemellales* was significantly increased in the non-pCR group across all samples. The differences might be accounted for by the exact classification of the bacterial species,

given that there are five subclasses within the phylum Firmicutes. The more we investigated the associations between three species with significances and patient characteristics and lifestyles, and demonstrated only supplement use was associated with *Gemellales*. Although supplements come in various forms, including probiotics and multivitamins. Further research is needed to determine the effect of specific bacterial taxa, such as *Victivallales*, *Anaerolineales*, and *Gemellales*, on treatment response in breast cancer.

There were some limitations in this study. First, the number of cases was not statistically examined, because this was an exploratory study. Consequently, the number of pCR events might be low, especially in the luminal type, and this potentially affected the results. Nevertheless, the overall number of cases in our study surpassed that in previous reports, and the distribution of breast cancer subtype was consistent with prior studies [10]. Notably, our study's strength lies in its exploration of the luminal type. Second, although evaluating the intestinal microbiota before and after treatment might have been beneficial in identifying the microbial influences on treatment efficacy, we limited our examination to baseline because of the potential impact of other factors, such as steroids and antibiotics, on neoadjuvant chemotherapy. Third, our study included only Japanese patients with breast cancer, and cautious interpretation is

advised. Further prospective studies are required to investigate the relationship between the gut microbiota diversity and chemotherapy response, incorporating intestinal regulators and lifestyle interventions, such as diet and exercise.

Conclusions

This study revealed potential associations between three species in the gut microbiota and efficacy of neoadjuvant chemotherapy. However, no clear correlations were found between the gut microbiota diversity and chemotherapy efficacy or lifestyle. The vast differences in the gut microbiota composition among races and regions, along with potential environmental factors, may contribute to the divergence from overseas data and warrant further research.

DECLARATIONS

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Author contributions

NT, YK, MI, HD, and TI contributed to the study conception and design. SN, NT, YK, YM, TK, MY, DT, SK, HH, YO, HD, and TI prepared the material preparation and collected the data. AH and KT performed pathological studies of the specimens in this study. SN and TI performed the formal analysis. SN, NT, MI, HD, TS, TI, and ST contributed to the interpretation and discussion of the results. SN wrote the first draft of the manuscript, and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

SN: Lecture fee: Daiichi Sankyo, Eisai and Chugai, AH: Honoraria: Daiichi Sankyo, Pfizer, Chugai; Advisory Board: Daiichi-Sankyo, Exact Science; Research grant: Visiopharm, MI: Research grant: Lilly, Honoraria: Daiichi Sankyo, Lilly, Pfizer, Ezai, Kyowa Kirin, Astra Zeneca, Taiho, Celltrion Healthcare Japan, MSD, Tsumura, Nihon Mediphysics, Exact Science, Chugai, Nihon Kayaku; Advisory Board: Daiichi-Sankyo; Manuscript fee: Pfizer, Chugai, Medic Media, TI: Research grant: Pfizer. The other authors declare no conflicts of interest associated with this manuscript.

Ethics approval

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. This study received an approval from the Ethics Committee of Okayama University Hospital (1909-032, 4, October 2019) and the respective institution, and each participant provided written informed consent.

Consent to participate and publish

Informed consent was obtained from all individual participants included in the study. All authors consent to the publication of this manuscript. The patient informed consent

also included a section on consenting to the publication of the patients' anonymized data.

Data availability statemen

The data that support the findings of this study are available from the Setouchi Breast Project Comprehensive Support Organization but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of the Setouchi Breast Project Comprehensive Support Organization.

Figure legends

Figure 1. Participants' flowchart

Figure 2. α-diversity of the gut microbiota in patients with pCR (n = 66) and non-pCR (n = 117) to neoadjuvant chemotherapy by (a) Shannon and (b) Chao, and α-diversity of the gut microbiota by breast cancer subtype in patients with pCR and non-pCR to neoadjuvant chemotherapy by (c) Shannon and (d) Chao.

Abbreviations: HER2, human epidermal growth factor receptor type2; pCR, pathological complete response; TN, triple negative.

Figure 3. Principal Coordinate Analysis (PCoA) of gut bacterium data in response to neoadjuvant chemotherapy; (a) PCoA analysis plots of all groups, (b) luminal group, (c) HER2-positive group, and (d) triple-negative group.

The blue ball represents patients with pathological complete response, and the red ball represents patients with residual disease.

Supplemental Figure 1. α-diversity of the gut microbiome by breast cancer subtypes with (a) Shannon and (b) Chao.

Abbreviations: HER2, human epidermal growth factor receptor type2; pCR, pathological complete response; TN, triple negative.

Tables

Table1. Patient characteristics

Abbreviations: A/T, anthracycline and/or taxane-based regimen; BMI, body mass index; HER2, human epidermal growth factor receptor type2; TILs, tumor-infiltrating lymphocytes; TC, docetaxel plus cyclophosphamide.

*: Measurable cases only

**: HER2-positive cases only

Variables	Total
	N = 183 (%)
Median age, years (range)	52 (25–77)
BMI (kg/m^2) (range)	22.6 (14.8–43.8)
Menopausal status	
Premenopausal	88 (48.1)
Postmenopausal	95 (51.9)
Tumor size	
T1	23 (12.6)
T2	115 (62.8)
T3	29 (15.8)
T4	15 (8.2)
TX	1 (0.5)
Lymph node status	
N0	49 (26.8)
N1	83 (45.4)
N2	24 (13.1)
N3	27 (14.8)
Clinical Stage	
I	8 (4.4)
II	104 (56.8)

III	71 (38.8)
Pathological grade	
I	9 (4.9)
II	42 (23.0)
III	30 (16.4)
Unknown	102 (55.7)
Subtype	
Luminal type	70 (38.3)
HER2 type	59 (32.2)
Triple negative type	54 (29.5)
Median Ki67 (%) (range)	42.8 (3.4–97.4)
Median TILs* (%) (range)	20 (1.0–90)
Low	33 (24.4)
Intermediate	82 (60.7)
High	20 (14.8)
Neoadjuvant chemotherapies	
A/T	179 (97.8)
TC	4 (2.2)
Anti-HER2 therapies**	
Trasutuzumab	3 (5.1)
Trasutuzumab + Persutuzumab	56 (94.9)
Dose-dense treatment	67 (36.6)
Dose reductions/interruptions	
Treatment completed	150 (82.0)
Reductions	12 (6.6)
Interruptions	21 (11.5)
Pathologic complete response	66 (36.1)

Table2. Patient characteristics and α-diversity of gut microbiota

Abbreviations: BMI, body mass index; CI, confidence interval; HER2, human epidermal growth factor receptor type2; TILs, tumor-infiltrating lymphocytes; TN, triple negative.

*: Measurable cases only

	Shannon				Chao			
	Odds ratio	95% CI		*P*	Odds ratio	95% CI		*P*
Age								
≥50 vs. <50	1.447	0.980	2.179	0.068	1.004	0.9998	1.008	0.068
BMI								
≥25 vs. <25	1.066	0.710	1.638	0.762	0.999	0.995	1.003	0.746
Pathological grade *								
II vs. I	0.561	0.163	1.392	0.290	0.995	0.986	1.003	0.216
III vs. I	0.567	0.140	1.751	0.369	0.993	0.982	1.004	0.236
III vs. II	1.177	0.677	2.166	0.572	1.001	0.995	1.007	0.770
Clinical Stage								
II vs. I	0.468	0.130	1.312	0.196	0.995	0.987	1.004	0.283
III vs. I	0.572	0.147	1.704	0.383	0.997	0.988	1.008	0.599
III vs. II	1.298	0.871	1.989	0.212	1.003	0.999	1.007	0.167
Breast cancer subtypes								
HER2 vs. Luminal	0.817	0.505	1.307	0.401	1.000	0.996	1.005	0.915
TN vs. Luminal	1.149	0.712	1.906	0.574	1.001	0.996	1.006	0.613
TN vs. HER2	1.345	0.844	2.234	0.227	1.001	0.996	1.006	0.710
TILs*								
Intermediate vs. Low	0.890	0.559	1.397	0.613	0.998	0.993	1.002	0.342
High vs. Low	0.640	0.351	1.157	0.130	0.992	0.983	1.000	0.067
High vs. Intermediate	0.496	0.207	1.128	0.099	0.993	0.983	1.002	0.163

Table3. Microbial order and response to neoadjuvant chemotherapy (FDR<0.1)

Abbreviations: FDR, false discovery rate; HER2, human epidermal growth factor receptor type2; pCR, pathological complete response; TN, triple negative.

Bacterial species	Average percentage						P	FDR	Bonferroni
	Luminal		HER2+		TN				
	non-pCR	pCR	non-pCR	pCR	non-pCR	pCR			
Victivallales	1.796E-05	2.009E-04	1.511E-05	2.27792E-05	1.224E-05	2.18548E-05	0.001	0.030	**0.031**
Anaerolineales	0	3.818E-06	0	0	0	0	0.001	0.030	**0.071**
Gemellales	4.13317E-05	3.735E-05	1.253E-04	9.34998E-05	2.366E-04	2.81999E-05	0.002	0.030	**0.089**

Supplemental Table1. Patient characteristics of lifestyle

		N = 162 （%）
Smoking (within 1 month)	Smoking	14 (8.6)
	Non-smoking	148 (91.4)
Alcohol	Almost no alcohol	124 (76.5)
	several times a week	21 (13.0)
	almost every day	17 (10.5)
Antibiotics (last 1.5 months)	Yes	4 (2.5)
	No	158 (97.5)
Intestinal regulator	Yes	17 (10.5)
	No	145 (89.5)
Fermented foods	almost every day	75 (46.3)
	several times a week	75 (46.3)
	Less than a few times a month	12 (7.4)
Supplements	Yes	33 (20.4)
	No	129 (79.6)
Bedtime (sleep for 2 hours or more once a week)	Yes	60 (37.0)
	No	102 (63.0)
Exercise (per week)	Less than 30 minutes	91 (56.2)
	30 minutes to 3 hours	51 (31.5)
	3 hours or more	20 (12.3)

Supplemental Table2. Patient characteristics of lifestyle and α-diversity of gut microbiota

Abbreviations: CI, confidence interval

	Shanon				Chao			
	Odds ratio	95%CI		*P*	Odds ratio	95%CI		*P*
Smoking (within 1 month)								
Smoking vs Non-smoking	1.339	0.675	2.446	0.363	1.005	0.997	1.014	0.239
Alcohol								
several times a week vs Almost no alcohol	1.066	0.613	2.019	0.831	1.001	0.993	1.010	0.729
almost every day vs Almost no alcohol	1.067	0.563	2.255	0.854	1.001	0.994	1.007	0.872
almost every day vs several times a week	0.992	0.421	2.386	0.986	0.999	0.993	1.005	0.761
Antibiotics (last 1.5 months)								
Yes vs No	0.584	0.095	2.128	0.503	0.998	0.986	1.012	0.763
Intestinal regulator								
Yes vs No	1.337	0.703	2.379	0.340	1.005	0.998	1.013	0.227
Fermented foods								
several times a week vs less than a few times a month	1.407	0.693	2.692	0.306	0.999	0.991	1.007	0.720
almost every day vs less than a few times a month	1.667	0.778	3.561	0.179	1.667	0.993	1.008	0.954
almost every day vs several times a week	1.113	0.726	1.724	0.624	1.002	0.276	1.761	0.423
Supplements								
Yes vs No	1.132	0.695	1.949	0.636	0.999	0.993	1.004	0.605
Bedtime (sleep for 2 hours or more once a week)								
Yes vs No	1.139	0.754	1.716	0.531	1.002	0.997	1.006	0.442
Exercise (per week)								
30 minutes to 3 hours vs less than 30 minutes	0.933	0.594	1.481	0.764	0.9997	0.995	1.004	0.888
3 hours or more vs less than 30 minutes	1.968	0.913	4.751	0.106	**1.007**	**1.001**	**1.013**	**0.030**
3 hours or more vs 30 minutes to 3 hours	1.557	0.843	3.315	0.199	**1.006**	**0.9999**	**1.013**	**0.059**

Supplemental Table3. Microbial order and response to neoadjuvant chemotherapy (n=44) *.

Abbreviations: FDR, false discovery rate; HER2, human epidermal growth factor receptor type2; pCR, pathological complete response; TN, triple negative.

*: We excluded 15 "unclassified" bacterial flora structure

Bacterial species	Rate of bacterial flora structure						P	FDR	Bonferroni
	Luminal		HER2+		TN				
	non-pCR	pCR	non-pCR	pCR	non-pCR	pCR			
Victivallales	1.796E-05	2.009E-04	1.511E-05	2.278E-05	1.224E-05	2.185E-05	0.001	0.030	**0.031**
Anaerolineales	0	3.818E-06	0	0	0	0	0.001	0.030	**0.071**
Gemellales	4.133E-05	3.735E-05	1.253E-04	9.350E-05	2.366E-04	2.820E-05	0.002	0.030	**0.089**
Actinomycetales	3.387E-04	2.295E-04	0.001	3.647E-04	0.001	0.001	0.024	0.349	1
Turicibacterales	0.002	0.009	0.002	0.001	0.004	0.008	0.047	0.508	1
Rickettsiales	0	0	0	0	0	4.348E-06	0.061	0.508	1
Cardiobacteriales	0	0	0	0	0	2.983E-06	0.061	0.508	1
Sphingomonadales	1.211E-06	0	0	0	0	2.783E-05	0.076	0.509	1
Campylobacterales	5.823E-06	0	6.957E-05	4.523E-05	2.268E-05	1.859E-05	0.088	0.509	1
Bifidobacteriales	0.106	0.082	0.034	0.071	0.067	0.067	0.125	0.606	1
Bacillales	0.001	0.001	0.000	0.002	0.000	0.001	0.200	0.846	1
Rhodobacterales	0	0	2.363E-06	0	5.8678E-07	0	0.204	0.846	1
Pasteurellales	0.001	0.002	0.001	0.000	0.003	0.001	0.222	0.858	1
Lactobacillales	0.017	0.026	0.028	0.018	0.022	0.063	0.257	0.932	1
Verrucomicrobiales	0.010	0.001	0.022	0.003	0.005	0.004	0.430	0.935	1

Streptophyta	0	0	0	2.085E-05	3.7467E-06	0	0.444	0.935	1
Clostridiales	0.58070962	0.51163121	0.63015699	0.594232928	0.59138859	0.559449754	0.449	0.935	1
Brachyspirales	3.975E-06	1.338E-05	0	0	0	0	0.464	0.935	1
Fusobacteriales	0.003	0.019	0.007	0.006	0.009	0.005	0.514	0.935	1
Enterobacteriales	0.014	0.048	0.015	0.017	0.015	0.012	0.549	0.935	1
Saprospirales	0	0	0	0	4.592E-06	0	0.581	0.935	1
Chlamydiales	0	0	0	0	1.610E-05	0	0.581	0.935	1
Gammaproteobacteria	0	0	0	0	9.769E-07	0	0.581	0.935	1
Synergistales	0.000	3.8128E-05	1.549E-04	0.002	9.268E-05	0.001	0.611	0.935	1
Thermoanaerobacterales	1.83104E-06	0	0	1.305E-04	4.055E-06	0	0.631	0.935	1
Rhodocyclales	0	0	0	7.512E-07	0	0	0.634	0.935	1
Neisseriales	1.327E-06	0	0	1.938E-06	1.488E-05	0	0.639	0.935	1
Methanobacteriales	8.824E-06	4.293E-05	0	2.114E-05	4.549E-05	5.020E-05	0.668	0.935	1
Pseudomonadales	7.755E-05	7.531E-06	0.000	0.000	0.001	4.853E-05	0.694	0.935	1
Bacteroidales	0.211	0.253	0.203	0.235	0.225	0.217	0.768	0.935	1
Coriobacteriales	0.024	0.015	0.026	0.021	0.024	0.032	0.793	0.935	1
Rhizobiales	3.408E-06	0	0	1.038E-06	0	2.439E-06	0.810	0.935	1
Aquificales	7.806E-07	0	0	0	0	0	0.853	0.935	1
Flavobacteriales	4.840E-07	0	0	0	0	0	0.853	0.935	1
Caulobacterales	3.856E-06	0	0	0	0	0	0.853	0.935	1
Methylophilales	1.291E-06	0	0	0	0	0	0.853	0.935	1
Alteromonadales	9.782E-06	0	0	0	0	0	0.853	0.935	1
Oceanospirillales	1.012E-05	0	0	0	0	0	0.853	0.935	1
Xanthomonadales	1.544E-05	0	0	0	0	0	0.853	0.935	1
Deinococcales	6.688E-07	0	0	0	0	0	0.853	0.935	1
Aeromonadales	5.168E-05	0	0	8.031E-05	3.133E-05	0	0.870	0.935	1
Burkholderiales	0.011	0.009	0.012	0.009	0.010	0.013	0.879	0.935	1
Erysipelotrichales	0.014	0.018	0.016	0.016	0.017	0.013	0.907	0.939	1

Desulfovibrionales	0.003	0.002	0.002	0.002	0.002	0.002	0.981	0.981	1

Supplemental Table4. Patient characteristics/lifestyle and bacterial species associated with response to chemotherapy

Abbreviations: CI, confidence interval; NA, not available.

	Victivallales				Anaerolineales				Gemellales			
	Odds ratio	95%CI		*P*	Odds ratio	95%CI		*P*	Odds ratio	95%CI		*P*
Age												
≥50 vs <50	0.995	0.969	- 1.023	0.713	61.276	0.000	- NA	0.987	0.998	0.986	- 1.011	0.776
Body Mass Index												
≥25 vs <25	1.005	0.976	- 1.032	0.715	0.018	NA	- 1.81E+27	0.988	1.003	0.989	- 1.015	0.691
Smoking (within 1 month)												
Smoking vs Non-smoking	1.011	0.970	- 1.142	0.751	46.781	0.000	- NA	0.993	1.006	0.985	- 1.050	0.704
Alcohol												
several times a week vs Almost no alcohol	1.002	0.945	- 1.036	0.899	0.018	NA	- 1.156E+46	0.992	1.005	0.985	- 1.020	0.569
almost every day vs Almost no alcohol	1.009	0.962	- 1.041	0.593	0.019	NA	- 1.223E+46	0.993	0.988	0.938	- 1.013	0.509
almost every day vs several times a week	1.008	0.955	- 1.072	0.745	NA	NA	- NA	NA	0.985	0.938	- 1.014	0.401
Antibiotics (last 1.5 months)												
Yes vs No	842.926	0.000	- NA	0.996	42.718	0.000	- NA	0.996	1.018	0.976	- 1.197	0.682
Intestinal regulator												
Yes vs No	0.980	0.951	- 1.014	0.179	49.798	0.000	- NA	0.993	0.998	0.982	- 1.024	0.835
Fermented foods												
several times a week vs less than a few times a month	0.991	0.944	- 1.069	0.750	54.617	0.000	- NA	0.992	1.006	0.986	- 1.046	0.647

almost every day vs less than a few times a month	0.995	0.960	-	1.060	0.781	NA	NA	-	NA	NA	0.988	0.947	-	1.043	0.601
almost every day vs several times a week	0.999	0.968	-	1.029	0.918	0.014	NA	-	1.436E+27	0.987	0.988	0.970	-	1.002	0.141
Supplements															
Yes vs No	1.016	0.987	-	1.048	0.247	139.129	0.166	-	NA	0.990	**1.018**	**1.004**	**-**	**1.034**	**0.016**
Bedtime (sleep for 2 hours or more once a week)															
Yes vs No	1.010	0.982	-	1.053	0.559	59.572	0.000	-	NA	0.987	0.999	0.986	-	1.013	0.854
Exercise (per week)															
30 minutes to 3 hours vs less than 30 minutes	1.062	1.011	-	1.149	0.066	82.516	0.000	-	NA	0.986	1.004	0.989	-	1.018	0.593
3 hours or more vs less than 30 minutes	1.072	0.990	-	1.188	0.112	NA	NA	-	NA	NA	1.004	0.980	-	1.023	0.669
3 hours or more vs 30 minutes to 3 hours	0.990	0.936	-	1.021	0.592	0.014	NA	-	8.971E+45	0.992	1.000	0.975	-	1.020	0.998

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