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Full-length Article

Chemotherapy-induced gut microbiome disruption, inflammation, and cognitive decline in female patients with breast cancer

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ABSTRACT

Chemotherapy is notorious for causing behavioral side effects (e.g., cognitive decline). Notably, the gut microbiome has recently been reported to communicate with the brain to affect behavior, including cognition. Thus, the aim of this clinical longitudinal observational study was to determine whether chemotherapy-induced disruption of the gut microbial community structure relates to cognitive decline and circulating inflammatory signals. Fecal samples, blood, and cognitive measures were collected from 77 patients with breast cancer before, during, and after chemotherapy. Chemotherapy altered the gut microbiome community structure and increased circulating TNF-α. Both the chemotherapy-induced changes in microbial relative abundance and decreased microbial diversity were related to elevated circulating pro-inflammatory cytokines TNF-α and IL-6. Participants reported subjective cognitive decline during chemotherapy, which was not related to changes in the gut microbiome or inflammatory markers. In contrast, a decrease in overall objective cognition was related to a decrease in microbial diversity, independent of circulating cytokines. Stratification of subjects, via a reliable change index based on 4 objective cognitive tests, identified objective cognitive decline in 35% of the subjects. Based on a differential microbial abundance analysis, those characterized by cognitive decline had unique taxonomic shifts (*Faecalibacterium*, *Bacteroides*, *Fusicatenibacter*, *Erysipelotrichaceae UCG-003*, and *Subdoligranulum*) over chemotherapy treatment compared to those without cognitive decline. Taken together, gut microbiome change was associated with cognitive decline during chemotherapy, independent of chemotherapy-induced inflammation. These results suggest that microbiome-related strategies may be useful for predicting and preventing behavioral side effects of chemotherapy.

1. Introduction

Over 500,000 U.S. patients with cancer receive chemotherapy treatment every year (Maldonado et al., 2020), commonly resulting in behavioral side effects during treatment, as well as months, even years, into remission (Koppelmans et al., 2012). Breast cancer is one of the most common cancers in the world and has a high 5-year survival rate (75–90 %) (Centers for Disease Control and Prevention, 2023; Maajani et al., 2019), making treatment side effects particularly salient for this population. Up to one in three patients experience cognitive decline, including deficits in attention, memory, processing speed, and executive functioning (Janelsins et al., 2018; Whittaker et al., 2022). While this

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cognitive decline is generally more subtle than overt clinical cognitive "impairment" (e.g., dementia), it reduces quality of life and the ability to resume employment (Whittaker et al., 2022). Furthermore, the underlying mechanisms remain complex and elusive.

While the understanding of how cancer and cancer treatments alter the gut microbiome is relatively immature, preclinical (Loman et al., 2019; Sougiannis et al., 2019) and clinical (Aarnoutse et al., 2022; Montassier et al., 2015) research implicates changes in the gastrointestinal microenvironment (e.g., reduction of microbial diversity). Notably, diversity within the gut microbial community is important for the host's intestinal barrier integrity, immune function, and as a source of biomolecules (Oh et al., 2021; Yoo et al., 2020). Recent evidence that the gut microbiota can communicate to the brain via humoral and neural routes to influence behavior, including cognition (Mayer et al., 2022), suggests that the brain-regulated side effects of chemotherapy may be mechanistically linked to alterations in the gastrointestinal system (Jordan et al., 2018; Subramaniam et al., 2020).

The scant existing clinical research on the gut microbiome in the context of chemotherapy indicates that altered microbiome composition and microbial metabolites (e.g., short-chain fatty acids) relate to treatment response (Panebianco et al., 2018; Zidi et al., 2021) and fear of cancer recurrence (Okubo et al., 2020). Two studies have reported a cross-sectional association between specific gut microbiome taxa and self-reported cognitive function (Bilenduke et al., 2022; Deleemans et al., 2022); however, longitudinal studies incorporating the microbiome and cognition over chemotherapy have not been reported. An understanding of the role of the gut microbiome in chemotherapy-related cognitive decline may indicate a novel opportunity to develop inexpensive and non-invasive microbial-directed prevention and treatment options (e.g., probiotics or microbial transplantation).

One communication route between the gut microbiome and the brain is via systemic inflammatory signals. Indeed, decreased microbiome diversity and microbial community disruption are associated with increased systemic inflammation (e.g., IL-6, TNF-α) (Al Bander et al., 2020). Furthermore, inflammation can exacerbate gut microbiome disruption, resulting in a detrimental positive feedback loop (Al Bander et al., 2020; Rudzki and Maes, 2020). Inflammatory signaling is a leading hypothesized gut-brain communication route in the context of chemotherapy as circulating inflammatory markers (e.g., IL-1β, IL-6) have been previously linked to cognitive decline (Janelsins et al., 2022; Lyon et al., 2016; Zhao et al., 2020); however, this relationship is not observed across all studies (Juan et al., 2022a; Pusztai et al., 2004). Cancer and anti-cancer treatments (e.g., chemotherapy and radiation) induce inflammation, likely via multiple mechanisms, including tissue injury and endotoxin-like activity (Edwardson et al., 2019; van der Most et al., 2008). Furthermore, studies using rodent models of chemotherapy support a causal role of neuroinflammation in cognitive decline (Grant et al., 2021; Lyu et al., 2021). How chemotherapy-induced gut microbiome disruption relates to inflammation and cognitive decline remains largely understudied. Although a prior study in patients with breast cancer indicates that the use of probiotics (live microorganisms that provide a health benefit) (Hill et al., 2014) attenuate cognitive decline during chemotherapy, this effect was not related to TNF-α, IL-6, or IL-1β (Juan et al., 2022a).

Given the paucity of research, the present study addressed the effects of chemotherapy on the gut microbiome and how these changes, in conjunction with inflammation, relate to cognitive decline in patients with breast cancer. To investigate these relationships, the "Intelligut Study" enrolled patients with breast cancer and collected measures of subjective and objective cognition, as well as fecal and blood samples for analyses of gut microbiome and inflammation, respectively. Both the trajectories of these outcomes and relationships among them were investigated.

2. Materials and methods

2.1. Participants

Females were recruited from The Ohio State University Stefanie Spielman Comprehensive Breast Center in Columbus, Ohio, USA for this longitudinal observational study of chemotherapy-related cognitive decline, gut microbiome disruption, and inflammation (Intelligut Study). Recruitment occurred between November 2019 and February 2022. Female patients were eligible if they had recently been diagnosed with stage IA-IIIB breast cancer with a treatment plan including antineoplastic chemotherapy. Exclusion criteria included a history of prior malignancy (excluding basal or squamous cell skin cancer), prior chemotherapy, prior radiation, cognitive impairment, or age above 80 years. A total of 77 individuals participated in the study. Self-reported sociodemographics (age, race, education, household income) at baseline and clinical variables (stage, receptor status, BMI) were collected from the medical record (Tables 1 & 2). This study was approved by The Ohio State University Institutional Review Board and all participants provided informed consent.

2.2. Study procedure and design

Participants completed assessments including cognitive testing, symptom questionnaires, and blood and fecal sample collection at 3 timepoints in accordance with regularly-scheduled oncology clinic visits: baseline (pre-chemotherapy), at the last chemotherapy infusion (during chemotherapy; mean ± SD: 13.2 ± 4.4, range: 5.6 – 33.6 months after baseline), and after a wash-out period (post-chemotherapy; mean ± SD: 12.0 ± 8.0, range: 4 – 54.8 weeks after completion of chemotherapy treatment) (Fig. 1). Fecal samples were collected, and questionnaires and cognitive tests were completed within 3 days of the participants' visits. Participants also completed a Food Frequency Questionnaire (FFQ; Nutrition Quest, Berkley, CA, USA, Block 2000-Brief) about their diet reflective of the previous year (pre-

Table 1
Participant demographic information.

		Number (%)
Age		
	<45	28 (36 %)
	45–55	27 (35 %)
	>55	22 (29 %)
Menopausal Status		
	Pre-menopausal	41 (53 %)
	Post-menopausal	36 (47 %)
Race		
	Black or African American	9 (11 %)
	White	67 (87 %)
	Unknown/Not reported	1 (1 %)
Ethnicity		
	Latin/Hispanic	3 (3 %)
	Non-Hispanic/Latin	74 (96 %)
BMI		
	<25	18 (23 %)
	25–30	28 (36 %)
	≥30	31 (40 %)
Partner Status		
	Living with romantic partner	60 (77 %)
	Single (not living with partner, widowed, divorced)	17 (22 %)
Household Income		
	less than $50,000	10 (13 %)
	$50,000 – $150,000	38 (49 %)
	over $150,000	18 (23 %)
	Not reported	11 (14 %)
Highest Level of Education		
	High School	19 (24 %)
	Technical/Associates	14 (18 %)
	College graduate	39 (51 %)
	Not reported	5 (6 %)

Table 2
Participant clinical characteristics.

	Number (%)	
Cancer Stage		
I	42	(54 %)
II	29	(37 %)
III	6	(7 %)
Treatment		
Adjuvant	41	(53 %)
Neoadjuvant	36	(46 %)
Chemotherapy		
Taxane	14	(18 %)
Taxane + cyclophosphamide	18	(23 %)
Taxane + AC	24	(31 %)
Taxane + AC + carboplatin	8	(10 %)
Taxane + carboplatin	13	(17 %)
Endocrine Therapy		
During chemotherapy	23	(30 %)
Post-chemotherapy	26	(34 %)
Immunotherapy		
During chemotherapy	7	(9 %)
Post-chemotherapy	4	(5 %)
Radiation		
Pre-chemotherapy (intra-operative)	1	(1 %)
Post-chemotherapy (pre-wash-out timepoint)	23	(30 %)

A participant was noted as receiving endocrine or immunotherapy if they received the treatment within 1 month of the study visit. Endocrine therapy includes trastuzumab ± pertuzumab. Immunotherapy includes pembrolizumab. AC = doxorubicin + cyclophosphamide. Adjuvant = tumor removal prior to chemotherapy initiation. Neoadjuvant = tumor removal surgery after chemotherapy.

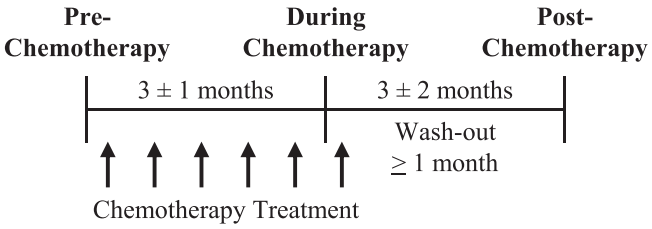

Fig. 1. Study design. Timeline of longitudinal prospective observational study with 77 female patients with breast cancer receiving chemotherapy treatment. Three study visits were completed: 1) prior to chemotherapy treatment, 2) during chemotherapy treatment, and 3) after chemotherapy treatment.

chemotherapy: n = 70) or previous week (during chemotherapy: n = 30 and post-chemotherapy: n = 40, **Supplementary Table 1**). While preliminary analyses indicated that total daily calories and dietary fiber decreased over chemotherapy treatment ($p < 0.05$ for both, **Supplementary Table 1**), the discrepancy in sample size between timepoints (due to the addition of diet data collection during and post-chemotherapy part-way through the study) did not allow for further analysis of these data over time or use as covariates. Blood samples were collected on the day of the visit in the clinic lab.

Participants in the study received a range of chemotherapy regimens with a mean and standard deviation (SD) of 10 ± 6 [range: 4–33] infusions with an infusion rate of every 7–21 days. The mean time from the most recent dose of chemotherapy to: 1) the during chemotherapy timepoint was 16.4 ± 5.1 days (range: 7 – 24) and 2) the post-chemotherapy timepoint was 2.8 ± 1.6 months (range: 1 – 8.2). If participants underwent surgery or radiation prior to or during the study, visits were completed at least 1 month after these events to minimize potential confounding effects.

2.3. Subjective cognition

Self-reported cognitive deficits were assessed using the Patient-Reported Outcomes Measurement Information System (PROMIS) Cognitive Function questionnaire (v2.0 – Abilities Subset – Short Form 8a), which has high reliability and validity in patients with cancer and breast cancer specifically (Henneghan et al., 2023; Jensen et al., 2017). This questionnaire measures facets of cognition (memory, mental acuity, and verbal fluency) and their impact on quality of life. Participants completed the questionnaire in-person electronically via REDCap or at home on paper. This questionnaire was scored using the Health Measures Scoring Service (HMSS) tool, which converts the raw questionnaire data into a validated T-score based on a calibration population. Samples sizes for subjective cognition were n = 69 pre-chemotherapy, n = 68 during chemotherapy, and n = 64 post-chemotherapy.

2.4. Objective cognitive testing

The following battery of cognitive tests was administered by a trained team member via video call, phone call, or in-person. Video and phone call cognitive testing (except for the Trail Making Test) was used for cognitive testing during the COVID-19 pandemic. Telephone administration of cognitive testing is comparable to in-person testing (Singh and Germine, 2021; Vaccaro et al., 2023). If testing was conducted the day of the participant's visit, all testing was completed prior to chemotherapy infusion. This battery of tests was chosen due to use within the context of cancer-related cognitive impairment and test validity and reliability (Wefel et al., 2011a, b). To assess overall cognition, age and education adjusted z-scores of these measures were averaged to create a cognition composite score (Castellon et al., 2004; Gullett et al., 2019). Raw scores were converted to age and education adjusted z-scores using published normative data for the Hopkins Verbal Learning Test – Revised (Benedict et al., 1998), Controlled Word Association Test (Tombaugh et al., 1999), Trail Making Test (Tombaugh, 2004), and Digit Span (Wechsler, 1997). Sample sizes for objective cognitive tests were n = 75–77 pre-chemotherapy, n = 68–70 during chemotherapy, and n = 64–70 post-chemotherapy.

2.4.1. Hopkins verbal learning test – Revised (HVLT – R)

The HVLT – R was used to assess verbal learning and memory (Benedict et al., 1998; De Jager et al., 2003). Participants were asked to immediately recall a list of 12 verbally-presented words across three learning trials. Each correctly recalled word counted as 1 point for the total learning score per trial, with a maximum of 36 points total. After a 20-min delay, participants were asked to recall as many words from the list as they could remember. The delayed recall was scored as the number of words remembered (0–12). Finally, another set of words was read to the participants, which included all the words from the original list (12), related words (6), and unrelated words (6). Participants were asked to respond with "yes" or "no" if the presented word was on the original list. The true positives (words correctly identified as on the original list), false positive-related (related words incorrectly identified as on the original list), and false positive-unrelated (unrelated words incorrectly identified as on the original list) were used to calculate a discrimination index (0–12). Alternate versions of the HTLV-R (i.e., different words) were used at each timepoint (Benedict et al., 1998).

2.4.2. Controlled oral word association test (COWAT)

The COWAT assessed verbal fluency (Benton and Hamsher, 1976; Johnson et al., 2012). Participants were asked to list as many words as they could think of that began with the letters F, A, and S in separate 1-min trials. Participants first practiced the task with the letter P to ensure understanding. The score was the total number of unique words stated across all 3 trials. Higher scores reflect better performance.

2.4.3. Trail Making test (TMT)

The TMT was used to assess psychomotor speed (TMT-A) and executive functioning (TMT-B) (Reitan and Wolfson, 1985; Tombaugh, 2004). For TMT-A, participants were asked to draw lines connecting numbers in sequence (1–25). For TMT-B, participants were asked to

connect numbers and letters in alternating sequencing (i.e., 1, A, 2, B, 3, C, etc.). Participants completed a practice trial before both aspects of the test. Both TMT-A and TMT-B were scored based on the time it took the participant to complete the task. Higher scores represent slower/worse performance.

2.4.4. Digit span (DS)

Digit Span from the Wechsler Adult Intelligence Test-III assessed simple attention and working memory (Wechsler, 1997). Starting with 2 digits, a span of numbers was read to the participant and the participant repeated the numbers in corresponding (forward) or reverse order (backward). The span length increased by one digit for every two iterations, up to 9 digits (forward) and 8 digits (backward). Once participants answered incorrectly for two trials of the same span length, that phase of the test was terminated. Each correct response received 1 point, with a maximum score of 16 (forward) and 14 (backward).

2.4.5. Classifying cognitive decline

To identify those with clinically-meaningful cognitive decline, and allow for the comparison of microbiome changes between those with and without cognitive decline, a reliable change index (RCI) was used as recommended by the International Cognition and Cancer Task Force (Jacobson and Truax, 1991; Wefel et al., 2011a, b; Wefel et al., 2010). An RCI is calculated to detect meaningful change in raw score outside of expected fluctuations. The calculation of the RCI was:

$$RCI = 1.64\left(SE_{diff}\right),\ where\ SE_{diff} = \left[2\left(SEM^2\right)\right]^{1/2}\ and\ SEM = SD[(1-r)^{\frac{1}{2}}]$$

where SE_{diff} is the standard error of difference, SEM is the standard error of measurement, SD is the standard deviation, and r is the test–retest reliability. The 90 % confidence interval RCI was calculated for each cognitive measure using published normative and test–retest reliability data (Benedict et al., 1998; Levine et al., 2004; Ruff, 1996). At the during and post-chemotherapy timepoints, the raw change score from baseline was calculated for each cognitive measure. Each measure was defined as reliable decline or not based on whether the change score identified a greater decline than the RCI for that measure. For all objective measures, a negative change score (i.e., a lower score than baseline) signified a decline, except for TMT–A and TMT–B, where a positive change score represented a decline because higher scores reflect worse performance on those measures. Clinically-significant cognitive decline was defined as a reliable decline in 2 or more scores across 1 or more cognitive tests.

2.5. Blood samples and assays

Approximately 8–10 mL of peripheral blood was collected via venipuncture at each timepoint. Whole blood was centrifuged for 20 min at 1,258 x g and 4 °C to isolate plasma. Inflammatory markers were quantified in plasma samples using the Human Inflammatory Panel 1 V-PLEX (interleukin [IL]-1β, IL-6, IL-8, and tumor necrosis factor [TNF]-α; Meso Scale Discovery, Gaithersburg, MD, USA, cat: K15053D-1). These markers are commonly measured in the context of chemotherapy-induced inflammation (Bower et al., 2022; Pusztai et al., 2004). Assays were run following the manufacturer's protocols. Plasma samples were thawed on the day of the assay and run in duplicate with average intra- and inter-assay CVs $\leq$ 10 %. Concentrations are presented as the natural log of pg/mL. Each participant's plasma samples across all 3 timepoints were measured on the same plate, using the same standards for all timepoints. Sample sizes for plasma samples were n = 73 pre-chemotherapy, n = 73 during chemotherapy, and n = 67 post-chemotherapy.

2.6. Fecal sample collection and processing

Participants received fecal sample collection kits containing an over-the-toilet cover, collection tube, and ice pack. Participants collected a fecal sample at home using the plastic spatula in the collection tube. Fecal samples were collected by participants within 3 days prior to each visit. Samples were kept in a sealed, insulated packet with an ice pack in their freezer (~20 °C) and then transferred on ice to the −80 °C freezer in the laboratory until analyses were completed. Sample sizes for fecal samples were n = 60 pre-chemotherapy, n = 66 during chemotherapy, and n = 65 post-chemotherapy.

2.7. Microbiota sequencing and modeling

Approximately 50 mg of each fecal sample was used for DNA extraction using QIAmp Fast DNA Mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA was quantified using the dsDNA Broad Range Assay Kit and Qubit 2.0 fluorimeter (Life Technologies, Carlsbad, CA). Library preparation and 16S high-throughput sequencing were conducted by the Genomic Services Core at the Institute for Genomic Medicine at Nationwide Children's Hospital in Columbus, OH. Quantitative Insights into Microbial Ecology (QIIME) 2.0 was used for amplicon processing, quality control, taxonomic assignment, and diversity analyses. Sequencing of samples resulted in 22,705,454 paired-end sequences and 2,736,624 high-quality sequences remained after quality control. Samples were rarefied to 4,300 sequences per sample for all analyses, including alpha and beta diversity analyses. Three beta diversity measures (Bray Curtis, unweighted UniFrac, and weighted UniFrac) were tested for significance using the ADONIS procedure, controlling for subject (repeated measures), for the entire study sample (including all 3 time points). Beta diversity distance (i.e., the distance between 2 points in 3D space) was used as a measure of the magnitude of change in microbiome composition.

Differential modeling of microbial taxa was completed using Songbird v1.0.3 and Qurro (Hill et al., 2022; Morton et al., 2019). Briefly, Songbird was used to identify microbes that were differentially abundant by treatment time point while controlling for subject. This analysis was completed at the amplicon sequence variant (ASV) level and accommodates the compositional nature of the data. This type of analysis is more robust than other commonly-used methods, such as linear discriminant effect size or direct comparison of relative abundances at a given taxonomic level, because it drastically reduces the number of comparisons made (false discovery rate) and accounts for how taxa change relative to each other (compositional nature of sequencing data) (Morton et al., 2019). To understand if changes in microbial taxa were different by cognitive decline outcomes during treatment, differentials were determined separately for: 1) all participants, 2) those without cognitive decline, and 3) those with cognitive decline. Three models were produced for comparison with the following equations: 1) Timepoint + Subject ID, 2) Timepoint + Subject ID + covariates, and 3) null model. Error (the error term in the model for the included samples) and loss (the incorrect categorical assignment of the test [left out] samples) of models were compared to the null model and each other to determine performance. All covariate-inclusive models underperformed (higher error and loss) the null models (indicating overfitting) and the models without covariates (which outperformed the null model), so the models without covariates were utilized in analyses. From these models, the log-ratio of the top 33 % of positively-associated ASVs (numerator) and the top 33 % negatively-associated ASVs (denominator), was calculated to detect significant changes in relative abundances of microbial taxa. The cutoff of 33 % was chosen as it was the minimal percentage of taxa that could be included to produce non-null values (i.e. denominator was not zero) for > 95 % of the samples. Linear mixed effects models were used to model changes in the log-ratios over time. The log-ratio was calculated by:

$$\log\left(\frac{sum\ of\ positively\ differential\ taxa\ relative\ abundances}{sum\ of\ negatively\ differential\ taxa\ relative\ abundances}\right)$$

To understand which higher-level taxa were contributing most to each model, the ASV relative abundances contributing to the log-ratios were aggregated at the genus level and shown as a relative abundance of the log-ratio numerator or denominator for each model and timepoint.

2.8. Data analysis strategy

All analyses of TNF-α, IL-6, and IL-1β used natural log-transformed values (ln). For ease of interpretation and to limit the overall number of analyses, thereby reducing the risk of Type 1 error, alpha diversity measures (Shannon entropy, observed operational taxonomic units [OTUs], and Faith's phylogenetic diversity) were combined into an alpha diversity composite score by first converting to individual z-scores, averaging the three scores for each sample, and rescaling by a constant to produce a z-score with a standard deviation of one. Linear mixed effects models were used to model changes in inflammatory markers, alpha diversity measures, and cognition measures over chemotherapy treatment (in separate models). Categorical time (pre-, during, and post-chemotherapy) was included as a fixed effect. All models included a random intercept for participant to account for within-subject correlation over time, and models for individual inflammatory markers additionally included a random effect for plate. Moderation of trajectories by age, body mass index (BMI), menopausal status (pre vs. post), and surgery status (neoadjuvant vs. adjuvant treatment) were included by adding each effect and its interaction with time in separate models. The Kenward-Roger adjustment to the degrees of freedom was used to control Type 1 error (Kenward and Roger, 1997). The ADONIS test was used to determine changes in beta diversity measures over time as well as moderation by age, BMI, menopausal status, and surgery status. With the few timepoints and lack of a cancer-free control group, the analyses did not accommodate for response shift in the subjective cognitive measure over time. To assess the extent to which the microbiome outcomes varied by timing and treatment variability, time since treatment initiation (i.e., pre-chemotherapy timepoint), time since the previous chemotherapy infusion, and the number of chemotherapy cycles were used. For these variables, the participants were split by the median values at the during and post-chemotherapy timepoints. The median values for time since treatment initiation were 98 and 170 days for the during and post-chemotherapy timepoints, respectively. For the time since previous chemotherapy infusion, the median values were 15 and 83 days for the during and post-chemotherapy timepoints, respectively. The median number of chemotherapy cycles was 8 for both the during and post-chemotherapy timepoints.

To assess relationships among gut microbiome, inflammation, and cognition measures, we used the results from the trajectory models to inform our analysis strategy. Specifically, as gut microbiome and inflammatory measures primarily changed from pre- to during chemotherapy, analyses of the relationships between these measures focused on this timeframe. As changes in cognition were more delayed, occurring from pre- to both during and post-chemotherapy, analyses were run to assess the relationship of the change in gut microbiome or inflammation measures from pre- to during chemotherapy with the change in cognition measures from both pre- to during and pre- to post-chemotherapy. Specifically, the relationship between changes in gut microbiome and changes in inflammation was assessed using linear regression models with during chemotherapy inflammatory markers as the outcome, gut microbiome measure pre- to during chemotherapy change score as the key predictor, and adjusting for baseline levels of both the outcome and predictor. In these models, adjusting for the baseline (pre-chemotherapy) level allows results to be interpreted as effects on the change from pre- to during chemotherapy. For the relation between changes in cognitive measures and gut/inflammation measures, linear mixed effects models were used, with during and post-chemotherapy cognition measures as the outcome and gut or inflammation measure pre- to during chemotherapy change score as the key predictor and adjusting for baseline levels of both the outcome and predictor. In addition, these models contained a fixed effect for time (during chemotherapy vs. post-chemotherapy) and the interaction of time with the gut or inflammation change score, allowing relationships to differ by time point. Finally, to explore the relationships between gut or inflammation measures and the presence of cognitive decline (a binary outcome), logistic regression models were used. In these models, the outcome was presence of cognitive decline, the key predictor was the gut or inflammation measure pre- to during chemotherapy change score, and the baseline predictor level was also included as a covariate. For all models using beta diversity change as a predictor, a baseline level could not be included as beta diversity is a point in 3D space rather than an individual value.

3. Results

3.1. Participant characteristics

Participant demographics and clinical characteristics are shown in Tables 1 and 2. Seventy-seven female patients participated in the study, with a mean ± SD age at diagnosis of 50 ± 11 years (range: 29–74 years). Most of the participants were white (87 %) and non-Hispanic/Latin (96 %; Table 1). Fifty-four percent of participants had Stage I breast cancer and 53 % were treated with adjuvant chemotherapy (i.e., chemotherapy after tumor removal surgery). All participants received taxane therapy and 65 % also received cyclophosphamide, 42 % doxorubicin, and/or 27 % carboplatin. Thirty-four percent also received endocrine therapy (trastuzumab, pertuzumab) and 9 % also received pembrolizumab immunotherapy (Table 2). One participant received intra-operative radiation prior to the study and 23 (29 %) participants received radiation between the during and post-chemotherapy timepoints (Table 2).

3.2. Trajectories of fecal microbial diversity over chemotherapy treatment

To understand how chemotherapy treatment altered the gut microbial community, alpha and beta diversity outcomes were analyzed. Alpha diversity measures the number of different microbial taxa (richness) and/or their distribution (evenness). In general, higher alpha diversity corresponds to more types of microbial taxa. None of the three measures of alpha diversity nor their composite score changed over chemotherapy treatment (Fig. 2**A** and **Supplementary Figure 1A-D**; $p > 0.05$ in all cases). Individual participant trajectories of the alpha diversity measures are shown in **Supplementary Figure 1A-D**. Moderation analyses by age, body mass index (BMI), menopausal status, and surgery status are shown in **Supplementary Table 2**. None of these factors moderated changes over time (ps > 0.05), but on average, across all three visits, neoadjuvant status was associated with lower alpha diversity ($p < 0.05$ for all measures) and higher BMI was associated with fewer observed OTUs and lower Faith's phylogenetic diversity (ps < 0.05). The alpha diversity composite did not differ by time since treatment initiation (pre-chemotherapy timepoint), time since previous chemotherapy infusion, or number of chemotherapy cycles ($p > 0.05$ for all).

In contrast to alpha diversity, beta diversity assesses the similarity/dissimilarity of two microbial communities compared to each other (i.e., compares distances between two samples). While alpha diversity has inherent directionality, beta diversity does not. Both weighted UniFrac ($p < 0.05$, Fig. 2**B** and **Supplementary Figure 2A**) and unweighted UniFrac ($p < 0.01$, Fig. 2**C** and **Supplementary Figure 2B**) beta diversity measures changed significantly over chemotherapy treatment, whereas the Bray Curtis beta diversity measure did not ($p = 0.21$, **Supplementary Figure 2C**). The discrepancy in results of the UniFrac and Bray Curtis measures could be due to differences in the calculation, as the UniFrac measures take into account phylogenetic distances

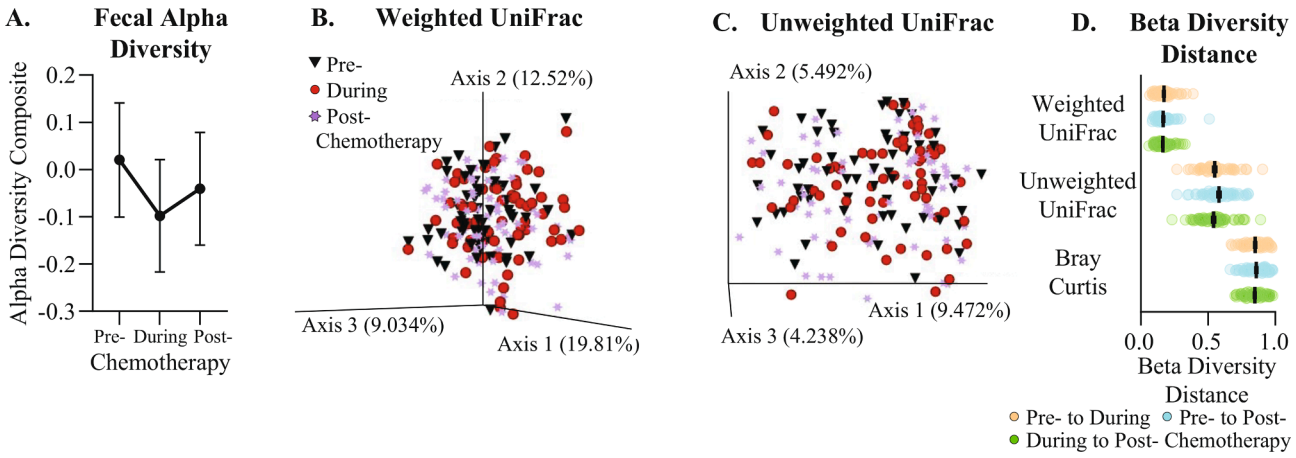

Fig. 2. Fecal microbial diversity over chemotherapy treatment. **(A)** Estimated trajectory of fecal alpha diversity composite over chemotherapy treatment. Results shown are mean ± standard error from mixed models. Beta diversity plots of **(B)** Weighted UniFrac and **(C)** Unweighted UniFrac over chemotherapy treatment. **(D)** Beta diversity distances over chemotherapy treatment.

between taxa. None of the beta diversity measures were moderated over chemotherapy (i.e., over time) by age, BMI, menopausal status, or surgery status, but all beta diversity measures were significantly associated with each of these factors independent of chemotherapy treatment (Supplementary Table 2). The distances between timepoints for the 3 beta diversity measures is shown in Fig. 2**D**. This is a measure of the magnitude of change of the microbiome over time. The beta diversity distances, either from pre- to during or pre- to post-chemotherapy, did not differ time since the previous chemotherapy treatment or number of chemotherapy cycles ($p > 0.05$ for all). While the UniFrac distances from pre- to during chemotherapy did not differ by time since treatment initiation ($ps > 0.05$), the Bray Curtis distance was greater for those with a longer time since treatment initiation ($p < 0.05$). All 3 beta diversity distance measures from pre- to post-chemotherapy were greater for those with a longer time between treatment initiation and post-chemotherapy timepoint ($p < 0.05$ for all).

3.3. *Fecal microbial relative abundance over chemotherapy treatment*

As the beta diversity analyses indicated that chemotherapy altered gut microbial communities, differential microbial abundance analysis was used to identify specific groups of microbes that shifted due to chemotherapy treatment. By convention, amplicon sequence variants (ASVs) positively associated with the metadata (in this case, changes from pre- to during or post-chemotherapy timepoint) contribute to the numerator of the log-ratio and the ASVs negatively associated with the metadata contribute to the denominator; thus, a higher log-ratio represents increases in the cumulative relative abundance of positively-associated ASVs relative to negatively-associated ASVs in a given sample. The log-ratio of the microbial taxa differential for the during chemotherapy timepoint significantly increased from pre- to during chemotherapy ($p < 0.0001$) and decreased from during to post-chemotherapy ($p < 0.001$), notably not returning to pre-chemotherapy levels ($p < 0.0001$, Fig. 3**A**). In contrast, the log-ratio of the group of microbial taxa differential for the post-chemotherapy timepoint significantly increased from pre- to during chemotherapy ($p < 0.0001$) and then further increased from during to post-chemotherapy ($p < 0.0001$, Fig. 3**B**). These microbial log ratios did not differ over time by the time since treatment initiation, time since previous treatment dose, or number of chemotherapy cycles ($p > 0.05$ for all).

The relative abundance of the specific ASVs contributing to the differential microbial log-ratios are shown aggregated at the genus level (Supplementary Table 3). Due to the type of analysis used, taxa contributing to the log-ratio must be described as positively or

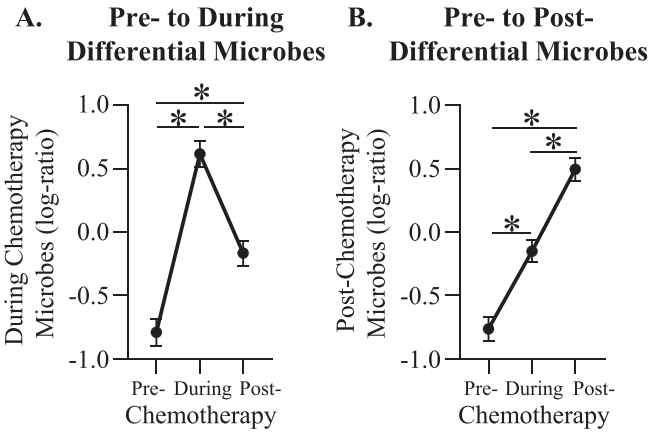

Fig. 3. Differential microbial abundance analysis over chemotherapy treatment. Log-ratios of the most differential taxa associated with **(A)** the during chemotherapy timepoint and **(B)** post-chemotherapy timepoint. Results shown are mean ± standard error from linear mixed effects models. *$p < 0.05$.

negatively associated with a certain timepoint rather than increasing or decreasing. Indeed, higher relative abundances in the numerator (and/or lower in the denominator) means a higher log-ratio, but for each individual or the overall cohort this analysis cannot determine if the relative abundances actually changed. The strength of this analysis is that it accounts for the compositional nature to find associated microbes, but the weakness is that limited assumptions about the changes in absolute abundance can be made. Most of the differential microbial taxa at the genus level were split between positively and negatively associated at both the during and post-chemotherapy timepoints, reflecting that individual ASVs within that taxon responded differently to treatment. ASVs in the genera of *Bacteroides*, *Collinsella*, *Escherichia-Shigella*, and *Eubacterium halli* accounted for the highest proportion of the positively-associated taxa and *Blautia*, *Akkermansia*, *Anaerostipes*, *Bacteroides*, *Methanobrevibacter*, and *Ruminococcus torques* the negatively-associated taxa at the during chemotherapy timepoint. At the post-chemotherapy timepoint, ASVs in the genera of *Bacteroides*, *Streptococcus*, *Ruminococcus torques*, *Eggerthalla*, and *Blautia* accounted for the highest proportion of the positively-associated taxa and *Blautia*, *Bacteroides*, *Subdoligranulum*, *Anaerostipes*, *Methanobrevibacter*, and *Streptococcus* the negatively-associated taxa.

Of note, *Blautia* and *Akkermansia* accounted for more of the relative abundance of the negatively-associated taxa at the during chemotherapy

than post-chemotherapy timepoint. *Eubacterium halli* and *Escherichia-Shigella* accounted for a greater proportion of the positively-associated taxa at the during chemotherapy timepoint than post-chemotherapy, with an opposite pattern for *Ruminococcus torques*, *Streptococcus*, and *Eggerthella*. Remarkably, *Methanobrevibacter*, the only contributing Archaea taxa (non-bacterial), was negatively associated with both the during and post-chemotherapy timepoints.

3.4. Trajectories of circulating inflammatory markers over chemotherapy treatment

Thirty-two and 29 participants received a corticosteroid pre-chemotherapy infusion medication, known to be anti-inflammatory, within 1 day prior to the pre- and during chemotherapy blood draws, respectively, but no participants received corticosteroids at the post-chemotherapy timepoint. As expected, corticosteroid treatment reduced circulating IL-1β, IL-6, and TNF-α, ($p < 0.05$ for all; Fig. 4**A-C**). For participants who did not receive corticosteroids, on average TNF-α significantly increased from pre- to during chemotherapy and remained elevated post-chemotherapy ($p < 0.05$ for both; Fig. 4**A**). In contrast, chemotherapy did not significantly alter the trajectories of IL-1β or IL-6 ($p > 0.05$ for both; Fig. 4**B-C**). Moderation analyses by age, BMI, menopausal status, and surgery status are shown in **Supplementary Table 4**. No interactions of these moderators over time were observed, but multiple main effects were present. TNF-α and IL-6 were associated with surgery status, such that neoadjuvant status was associated with higher inflammation independent of chemotherapy ($p < 0.05$ for both). IL-6 was also associated with BMI such that higher BMI was related to higher IL-6 levels ($p < 0.01$).

3.5. Associations between inflammation and gut microbiome disruption

Given that in this study, chemotherapy altered both the gut microbiome and inflammatory markers, and also gut microbiome disruption is associated with circulating inflammation in other disease contexts, we next assessed the relationships between gut microbiome and inflammatory outcomes. Even though alpha diversity and IL-6 did not significantly change over chemotherapy treatment, they were included in these analyses based on previously reported chemotherapy effects (Aarnoutse et al., 2022; Bower et al., 2022). A decrease in the fecal microbial alpha diversity composite score related to an increase of circulating TNF-α (estimate: −0.27, SE: 0.13, $p < 0.05$) and IL-6 (estimate: −0.43, SE: 0.21, $p < 0.05$) from pre- to during chemotherapy. In contrast, the change in beta diversity (unweighted or weighted UniFrac) from pre- to during chemotherapy did not relate to the change of TNF-α or IL-6 ($p > 0.05$ for all) over the same time period. Furthermore, the change in the log-ratio of microbes differential for the during chemotherapy timepoint from pre- to during-chemotherapy timepoints positively related to the change of TNF-α over the same time (estimate: 0.16, SE: 0.07, $p < 0.05$), meaning that a greater shift of the chemotherapy-associated microbial taxa was related to a greater increase of circulating TNF-α. This relationship was strongest for TNF-α, as it was not significant for IL-6 (estimate: −0.01, SE: 0.12, $p > 0.05$).

3.6. Trajectories of subjective and objective cognition over chemotherapy treatment

To understand the extent to which chemotherapy altered cognition in this cohort of patients with breast cancer, subjective cognition was measured via the PROMIS cognitive function questionnaire and objective cognition was measured via a battery of cognitive tests (Hopkins Verbal Learning Test − HVLT-R, Digit Span − DS, Trail Making Test − TMT, and Controlled Word Association Test – COWAT; **Supplementary Table 5**). Two cognitive measures changed over chemotherapy treatment. Subjective cognitive function robustly decreased over time ($p < 0.001$), from pre- to during chemotherapy and remained reduced post-chemotherapy ($p < 0.01$ for both, Fig. 5**A**). Subjective cognitive function was associated with BMI, independent of chemotherapy ($p < 0.05$, **Supplementary Table 6**), with higher BMI associated with lower subjective cognition. The discrimination index of the HVLT, a measure of memory, likewise decreased from during to post-chemotherapy ($p < 0.01$, Fig. 5**B**) and was not associated with age, BMI, menopause, nor surgery status (**Supplementary Table 6**). Furthermore, to assess overall cognitive function, age- and education-normalized objective cognitive measures were combined into a cognition composite score, which did not change over chemotherapy treatment, likely due to high variability ($p > 0.05$, Fig. 5**C**). The cognition composite score was, however, related to age and menopause status with younger age and premenopausal status associated with a lower cognition composite score ($p < 0.02$ for both, **Supplementary Table 6**). While other cognitive measures did not significantly change on average over chemotherapy, they were significantly associated with various factors (**Supplementary Table 6**): DS (surgery status, $p < 0.05$), TMT–A (age and menopause status, $p < 0.05$ for both), and TMT–B (age, age × time, menopause status, and menopause status × time, $p < 0.05$ for all).

The reliable change index (RCI) was used to identify those with clinically-meaningful cognitive decline. This categorical stratification was necessary to assess how chemotherapy-induced gut microbiome changes were different between those with and without cognitive decline. Twenty-seven participants (35 %) met the criteria for cognitive

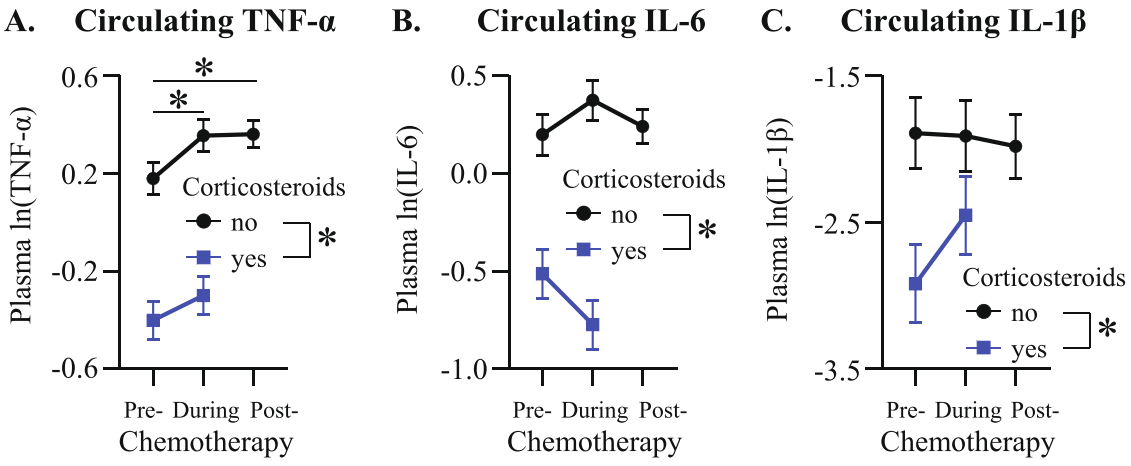

Fig. 4. Circulating inflammatory markers over chemotherapy treatment. Estimated trajectories of circulating **(A)** TNF-α, **(B)** IL-6, and **(C)** IL-1β. Results shown are mean ± standard error from linear mixed effects models. *$p < 0.05$.

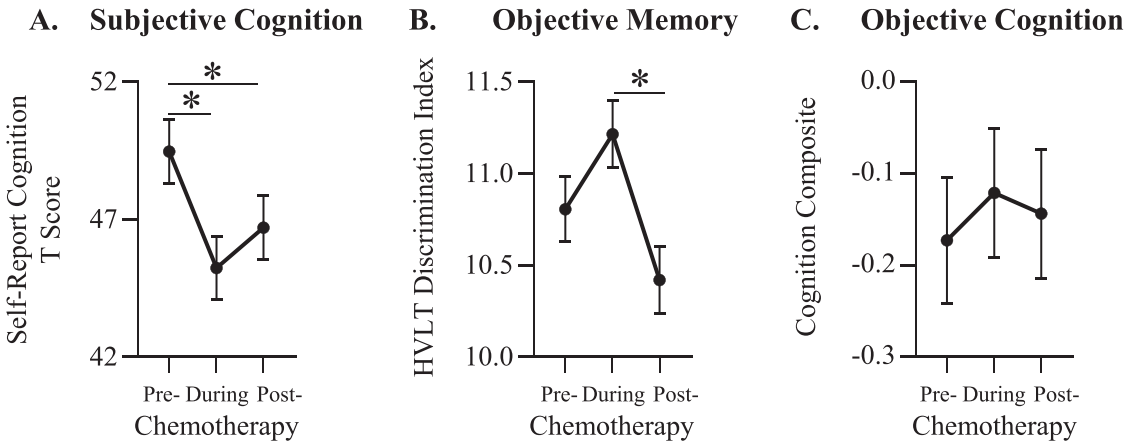

Fig. 5. Subjective and objective cognition over chemotherapy treatment. Estimated trajectories of **(A)** subjective cognition and **(B)** Hopkins Verbal Learning Test Discrimination Index, **(C)** overall cognition composite. Results shown are mean ± standard error from linear mixed effects models. *p < 0.05.

decline. The most commonly-declined measures were those in the HVLT, with 48 % of cognitively declined participants presenting a decline in at least one score of the HVLT during chemotherapy and 70 % post-chemotherapy. The next most commonly-declined measures were those in the TMT, followed by DS and COWAT during chemotherapy and COWAT, followed by DS and TMT post-chemotherapy. Those who met criteria for cognitive decline did not differ by any assessed demographics including age, BMI, menopausal status, corticosteroid use, nor surgery status compared to those who did not meet cognitive decline criteria ($p > 0.05$ for all, data not shown).

3.7. Associations among gut microbiome disruption, inflammation, and cognition measures

Next, how chemotherapy-induced gut microbiome disruption and inflammation related to cognition was investigated. Measures that changed over chemotherapy, in addition to IL-6 and the alpha diversity composite score, were used for these analyses. IL-6 and the alpha diversity composite score did not change over time but were used based on previous reports (Aarnoutse et al., 2022; Bower et al., 2022). First, the decrease of subjective cognition observed over chemotherapy treatment was neither related to alterations in gut microbiome measures (alpha diversity composite, unweighted or weighted UniFrac, or during or post-chemotherapy differential microbes) nor increases in TNF-α or IL-6 from pre- to during chemotherapy ($p > 0.05$ for all). In contrast, the objective cognitive measure that decreased over chemotherapy, the HVLT discrimination index, was inversely related to the shift in the microbial community differential relative abundance from pre- to during chemotherapy (estimate: −0.34, SE: 0.16, $p < 0.05$). This relationship was driven by the decrease in the HVLT discrimination index at the post-chemotherapy timepoint (estimate: −0.46, SE: 0.20, $p < 0.05$). Thus, a greater shift in the microbial relative abundance toward the chemotherapy-associated differential taxa was associated with decreased memory for this test. Likewise, a reduction in the overall cognition composite score over chemotherapy was associated with a decrease in the alpha diversity composite score (estimate: 0.24, SE: 0.11, $p < 0.05$). The same change in alpha diversity was not significantly related to the presence of cognitive decline as determined by the RCI (estimate: −0.87, SE: 0.60, $p = 0.15$), although the relationship followed the same direction. In contrast, neither the changes in beta diversity, the microbial log-ratio, nor TNF-α or IL-6 related to the overall cognition composite or the presence of cognitive decline ($p > 0.05$ for all).

3.8. Chemotherapy impacts the microbiome community differently in those with and without objective cognitive decline

Next, microbial differential abundance analysis was completed separately for those with and without cognitive decline to understand the extent to which chemotherapy-induced changes in the microbiome composition differed between these groups (Table 3 **and Supplementary Table 7**). For those subsets with and without cognitive decline, many of the differential microbial taxa followed similar patterns as observed in the full cohort, including alterations in the relative abundances within microbial taxonomic genera of *Eubacterium halli*, *Collinsella*, *Anaerostipes*, *Blautia*, and *Methanobrevibacter* during chemotherapy (Table 3) and *Eggerthella*, *Streptococcus*, *Ruminococcus torques*, *Collinsella*, *Subdoligranulum*, *Bifidobacterium*, and *Methanobrevibacter* post-chemotherapy (**Supplementary Table 7**). Further, select taxa changed with chemotherapy only within the subset without cognitive decline. These taxa often followed a similar pattern as the overall population, including *Escherichia-Shigella*, *Erysipelatoclostrium*, and *Akkermansia* during chemotherapy (Table 3) and *Erysipelatoclostridium* after chemotherapy (**Supplementary Table 7**). However, the ASVs in some genera had different patterns for those with cognitive decline as compared to those without. For those with cognitive decline *Faecalibaterium*, *Fusicatenibacter*, and *Erysipelotrichaceae UCG-003* were primarily positively associated with the during chemotherapy timepoint and *Bifidobacterium*, *Dorea*, *Subdoligranulum*, *Ruminococcus gnavus*, *Ruminococcus torques*, and *Ruminococcaceae CAG-352* were primarily negatively associated (Table 3). *Blautia* and *Agathobacter* were primarily positively associated with the post-chemotherapy timepoint for those with cognitive decline and *Faecalibacterium* and *Bacteroides* were primarily negatively associated. Those without cognitive decline had either the opposite pattern or these genera were evenly split between positively and negatively associated (**Supplementary Table 7**).

4. Discussion

Effective prevention and treatment options for chemotherapy-induced cognitive decline remain elusive, due in part to a limited understanding of the underlying mechanisms. The goal of this study was to investigate chemotherapy-induced gut microbiome disruption and inflammation and assess their potential relationships with cognitive decline in patients with breast cancer. Here, chemotherapy significantly altered the gut microbial community over the course of treatment, specifically measures of beta diversity. This finding is generally consistent with the few other studies of chemotherapy-induced gut microbiome disruption in patients with hematologic cancers (Galloway-Peña et al., 2020; Montassier et al., 2015), whereas others in patients with

Table 3
Relative abundance of differential ASVs during chemotherapy by cognitive decline.

	No Cognitive Decline		Cognitive Decline	
Taxa	**"+" associated**	**"-" associated**	**"+" associated**	**"-" associated**
Blautia	5.3 (3)	11 (2.3)	9.6 (7.9)	16.4 (6.8)
Anaerostipes	3.3 (2.8)	5.4 (2.3)	8 (11.8)	10.8 (10.2)
Bacteroides	9.8 (2.7)	4.3 (1.5)	3.2 (5.6)	7.3 (5.3)
Subdoligranulum	8.8 (3.8)	.	2.9 (3.6)	12.4 (7.7)
Streptococcus	4.5 (2.6)	1.4 (1.3)	10.8 (6.6)	6.4 (6.5)
Methanobrevibacter	.	4.6 (2.6)	.	14.8 (9.6)
Eggerthella	0.5 (1.6)	7 (2.1)	5.2 (5.3)	6.7 (16.6)
Fusicatenibacter	3.6 (3.2)	4.4 (3.4)	7.7 (8.7)	2.1 (3.6)
Agathobacter	.	5 (3.6)	5.2 (5.3)	4.6 (5.9)
Faecalibacterium	1 (1.2)	6.1 (1.8)	5.3 (3.2)	1.7 (2.7)
Dorea	5.2 (5.4)	2.8 (2.4)	2 (3.4)	3.4 (1.9)
Ruminococcus torques	5.7 (3.4)	6.7 (2.6)	.	0.7 (3)
Collinsella	5.5 (3.3)	0.5 (1)	6.2 (6.4)	.
Eubacterium hallii	4.2 (3)	2.1 (1.1)	4.3 (4.4)	1.1 (3.4)
Akkermansia	.	10.9 (3.3)	.	.
Erysipelotrichaceae UCG-003	.	2 (1.7)	8.5 (6.2)	.
Bifidobacterium	2.1 (4)	0.9 (2.2)	2.9 (6.1)	4.3 (8.1)
Phascolarctobacterium	2.6 (2.1)	1.9 (2.9)	2.9 (4.7)	1.4 (2.4)
Peptostreptococcaceae Unknown	3.6 (3.6)	.	5.2 (5.5)	.
Incertae Sedis	2.4 (4.3)	0.6 (0.8)	4.3 (5.8)	.
Escherichia-Shigella	7.2 (2.8)	.	.	.
Ruminococcus gnavus	3.4 (3.2)	1.7 (2.7)	.	1.3 (4.1)
Ruminococcaceae CAG-352	.	.	2 (4.1)	3.9 (7.3)
Dialister	1.9 (2.3)	.	2.8 (3.9)	.
Erysipelatoclostridium	3.2 (2.6)	1.2 (0.7)	.	.
Adlercreutzia	1 (2.8)	2.4 (2)	0.9 (3.4)	.
Lachnospiraceae ND3007	0.5 (2.6)	2.8 (3.1)	.	.
Ruminococcaceae Unknown	.	3.1 (3.3)	.	.
Faecalitalea	.	3.1 (1.2)	.	.
Monoglobus	1.1 (1.7)	1.6 (0.9)	.	.
Butyricicoccus	1.8 (3.7)	0.5 (1.1)	.	.
Parabacteroides	2.2 (2.2)	.	.	.
Clostridium sensu stricto 1	2.1 (3.9)	.	.	.
Coprococcus	1.3 (4.3)	.	.	0.7 (1.6)
Eubacterium coprostanoligenes	1.3 (3.5)	0.5 (0.9)	.	.
Ruminococcus	1.3 (1.8)	0.2 (0.6)	.	.
Intestinimonas	0.5 (1.8)	0.8 (4)	.	.
Roseburia	1.3 (4.7)	.	.	.
Clostridium innocuum	0.9 (2.3)	0.2 (1)	.	.
Ruminococcus gauvreauii	.	1 (1.5)	.	.
Ruminococcaceae UBA1819	.	0.8 (2.3)	.	.
Actinomyces	.	0.7 (1.2)	.	.
Alistipes	.	0.7 (1.5)	.	.
Oscillospiraceae UCG-002	.	0.6 (1.2)	.	.
Lactococcus	0.5 (1.7)	.	.	.
Intestinibacter	.	0.5 (0.7)	.	.
Candidatus Stoquefichus	0.3 (3.8)	.	.	.
Lachnoclostridium	.	0 (0.3)	.	.

Relative abundance of differential ASVs attributed to genus-level taxa contributing to the differential log-ratio at the during chemotherapy timepoint as compared to the pre-chemotherapy timepoint. Results are presented as % (standard deviation).

breast cancer report no change in beta diversity over chemotherapy (Aarnoutse et al., 2022; Juan et al., 2022a). Chemotherapy did not significantly decrease alpha diversity here, which corroborates some previous reports (Bilenduke et al., 2022; Kong et al., 2019; Shrode et al., 2023), but conflicts with others (Aarnoutse et al., 2022; Deleemans et al., 2022; Galloway-Peña et al., 2020; Montassier et al., 2015). These slightly inconsistent diversity results may reflect inter-individual variability, differences in the timing of sample collection, the patient populations, type of malignancy, and/or chemotherapeutic agents among these studies. Given the large individual variability in the human gut microbiome, the present study included a pre-chemotherapy sample, allowing for the within-subjects analysis of how the gut microbiome changes over chemotherapy treatment, whereas most of the previous studies lack this timepoint (Bilenduke et al., 2022; Deleemans et al., 2022).

Interestingly, the groups of microbes that were most different during chemotherapy did not return to baseline post-chemotherapy, indicating that the effects of chemotherapy on the gut microbiome persist for at least several months after chemotherapy cessation. Furthermore, the microbes that were most different post-chemotherapy had already started to shift during chemotherapy, suggesting that the momentum for microbial change during chemotherapy continued even after chemotherapy cessation. Our analyses indicate that these microbial changes are driven by chemotherapy regardless of duration or intensity of treatment. Longer-term studies are necessary to determine the persistence of altered microbial communities after chemotherapy, as well as the physiological changes that support them. The differentially abundant taxa were similar to other previous reports of chemotherapy-induced changes in fecal microbial communities. Specifically, taxa in the genera of *Bacteroides*, *Colinsella*, *Escherichia-Shigella*, *Eggerthella*, *Faecalitalea*, and *Parabacteroides* were primarily positively associated (Deleemans et al., 2022; Montassier et al., 2015; Shrode et al., 2023; Walker et al., 2023) and those in the genera of *Blautia*, *Anaerostipes*, and *Ruminococcus* were primarily negatively associated with the during and post-chemotherapy timepoints (Galloway-Peña et al., 2020; Montassier et al., 2015). Of note, these studies include various types of cancer (e.g., breast, colorectal, hematogenous), chemotherapy regimens (e.g., anthracycline-based, taxane-based), and lengths of time from chemotherapy treatment (1 week to 5 years post-chemotherapy). This consistency in results despite significant variation of relevant factors indicates that a variety of anti-neoplastic chemotherapeutics used for different cancer types may have similar and potentially lasting effects on the gut microbiome. Overall, these gut microbiome analyses indicate that chemotherapy induces a temporally-dynamic shift in the gut microbiome that may be due more to changes in the actual types and abundances of microbes than to a difference in the number (richness) or dispersion (evenness) of different types of microbes. Furthermore, our analyses suggest that chemotherapy, regardless of timing and intensity, drives alterations in the microbiome.

As the existing literature demonstrates that the gut microbiome is intimately related to the immune system, circulating inflammatory markers were measured. Similar to other studies of chemotherapy-induced inflammation (Bower et al., 2022), chemotherapy increased circulating TNF-α. Corticosteroid drugs significantly suppress circulating proinflammatory cytokines and over one-third of the participants in this study were taking transient corticosteroids at the pre- and during chemotherapy timepoints. Because of this, the sample size of subjects without confounding corticosteroids was reduced, thereby potentially masking a predicted chemotherapy-induced increase in IL-6 (Bower et al., 2022; Liu et al., 2012). Interestingly, standard-of-care corticosteroid use during chemotherapy is not often reported in the existing literature. Additionally, chemotherapy-induced inflammation is not universally observed in clinical studies and can be transient and dose- and regimen-dependent (Juan et al., 2022a; Pusztai et al., 2004). As the during and post-chemotherapy timepoints were 7 – 21 days and at least 1 month from the most recent chemotherapy infusion, respectively, transient cytokine increases (e.g., IL-6 and IL-1β) may have been missed in the present study. Furthermore, other treatments including endocrine and immunotherapies may have immune-related effects, but the current study was not powered to assess these.

Consistent with the reported bidirectional relationship between gut

microbiome disruption and circulating inflammation, decreased alpha diversity and more extreme shifts in the groups of microbes identified as differential with chemotherapy were each associated with increases in circulating inflammatory markers. Similarly, our team previously reported an association between chemotherapy-induced inflammation and gut microbiome disruption using a mouse model of paclitaxel chemotherapy (Grant et al., 2021); however, this association remains understudied in clinical cohorts. For example, previous studies have included measures of inflammatory markers and changes in the gut microbiome over chemotherapy treatment but did not relate the two (Juan et al., 2022b; Walker et al., 2023).

We also assessed changes in subjective and objective cognitive function over the course of chemotherapy. In general, subjective cognitive assessments are more vulnerable to chemotherapy than objective tools (Kim et al., 2022) and often do not correlate with objective measures (Dhillon et al., 2018), but rather with mood (Veal et al., 2023). Furthermore, the decline in subjective cognition observed in the present study was neither related to increased inflammation nor gut microbiome outcomes, whereas two previous studies of chemotherapy-associated subjective cognitive decline reported significant relationships with relatively lower alpha and beta diversity (Bilenduke et al., 2022; Deleemans et al., 2022). However, these studies compared chemotherapy-treated patients with cancer to healthy controls rather than following the same patients over time as in the present study. Thus, numerous cancer-related factors (stress, tumor, surgery) beyond chemotherapy may be contributing to the observed relationships between self-reported cognitive decline and microbiome diversity.

The modest effects of chemotherapy on objective cognitive testing here manifested as a post-chemotherapy decline in memory of one test. Minor cognitive reductions observed with the other objective cognitive measures were leveraged using an RCI, resulting in the identification of a subset of participants with clinically-meaningful cognitive decline. Twenty-seven participants (35 %) met the criteria for measurable cognitive decline, primarily in the domains of verbal learning and memory, which is similar to reported rates in the literature (Cerulla et al., 2017; Whittaker et al., 2022). Notably, our chemotherapy and post-chemotherapy timepoints were relatively close together (average ~ 3 months) and correspond to the period when the greatest cognitive decline is anticipated (Kim et al., 2022).

Remarkably, the chemotherapy-induced decline in the HVLT discrimination index was associated with shifts in the gut microbial community. Furthermore, a decrease in the overall cognition score over chemotherapy was related to a decrease in microbiome alpha diversity, but not increases in inflammatory markers. These findings suggest that greater shifts in the gut microbiome due to chemotherapy may contribute to cognitive deficits, potentially via a mechanism other than circulating inflammatory signaling (e.g., short-chain fatty acids, gastrointestinal damage or functional change, tryptophan metabolites). Further studies are needed to elucidate these mechanisms. Given that the present 16S rRNA gene amplicon sequencing does not provide adequate information for functional microbiome analyses (Matchado et al., 2024), future studies would benefit from shotgun metagenomic sequencing or RNAseq analyses to understand the functional changes in the gut microbiome induced by chemotherapy. While few clinical studies have assessed associations of chemotherapy-induced gut microbiome disruption and cognition, one previous study observed that chemotherapy patients who reported subjective cognitive decline had increased relative abundance of the genera *Eggerthella* and family *Erysipelotrichaceae* (Bilenduke et al., 2022). Notably, *Erysipelotrichaceae UCG-003* (family *Erysipelotrichaceae*) and *Eggerthella* were uniquely positively associated with the during and post-chemotherapy timepoints, respectively, in the present group with cognitive decline as compared to the group without cognitive decline. Both the genus *Eggerthella* and family *Erysipelotrichaceae* are heightened in older adults with mild cognitive impairment and Alzheimer's disease (Hatayama et al., 2023; Zhu et al., 2022). Finally, *Erysipelotrichaceae* is also associated with gastrointestinal inflammation and colorectal cancer (Chen et al., 2012; Palm et al., 2014). In another study, subjective cognitive decline was related to decreased *Bacteroides* and *Faecalibacterium* in young adult cancer survivors within 5 years of chemotherapy treatment (Deleemans et al., 2022). Similarly, for those with cognitive decline in our study, *Bacteroides* and *Faecalibacterium* were uniquely negatively associated with the during and post-chemotherapy timepoints as compared to those without cognitive decline. In the present study, some genera had different relationships with cognitive function than in these previous studies. For example, an increase of the genus *Subdoligranulum* was related to a decrease in subjective cognitive function (Deleemans et al., 2022), but was uniquely negatively associated with the post-chemotherapy timepoint in the present group with cognitive decline. Furthermore, previous studies have seen relationships of *Intestinibacter* and *Odoribacter* with subjective cognitive decline over chemotherapy (Bilenduke et al., 2022; Deleemans et al., 2022), but these were not differential over chemotherapy treatment for those with cognitive decline in the present study. Overall, mounting evidence indicates that chemotherapy-induced alterations of specific groups of microbes may be related to both subjective and objective cognition, but additional corroborating studies that similarly include pre-chemotherapy baseline samples are necessary. A better understanding of the role of the gut microbiome in cognitive decline is important because microbial-directed therapies may provide inexpensive, non-invasive, and potentially effective treatment options (e.g., microbial transplant, probiotics) that may minimize potential interactions with concurrent cancer-related medications. To our knowledge, only a single trial using probiotics over chemotherapy treatment and assessing cognition has been reported to date (Juan et al., 2022a), and thus additional research is imperative. Our results indicate that approaches aimed at bolstering overall alpha diversity and certain genera, such as *Faecalibacterium* and *Bifidobacterium*, while blunting increases of others, such as *Eggerthella* and *Erysipelotrichaceae* may be promising next steps.

Notable strengths of this study include the medium-sized sample, with 77 participants. In addition, the longitudinal, pre/post design with 3 within-subjects assessments was a strength that allowed for analyses of the change in outcomes over chemotherapy treatment. Indeed, the initial studies of chemotherapy-induced gut microbiome disruption with inflammation or cognitive decline are either lacking a baseline sample and/or have a smaller sample size (15 – 40 participants) (Aarnoutse et al., 2022; Bilenduke et al., 2022; Deleemans et al., 2022; Walker et al., 2023). Finally, in addition to gut microbial diversity measures, differential abundance analysis was also used to assess the relative abundance of different microbial taxa over chemotherapy treatment and differences between those who developed cognitive decline and those who did not. A limitation of the present study was the lack of a cancer-free control group, which limited the ability to assess the roles of tumor biology and cancer diagnosis on the outcomes. This study was also not powered to differentiate among the various chemotherapy treatment regimens nor the endocrine/immunotherapies that occurred infrequently during the final visit. Other notable limitations include the variability in the timepoints (e.g., variable length of chemotherapy treatment and time between chemotherapy infusions and outcomes measurements) and lack of gut symptoms data.

Cognitive decline and other chemotherapy-induced behavioral side effects reduce patients' quality of life and contribute to treatment interruptions and dose reductions, increasing morbidity and mortality (Dranitsaris et al., 2005; Hanna et al., 2020). The current study provides evidence and direction for future studies to assess the impact and clinical benefit of microbial-directed treatments (e.g., microbial transplant, probiotics) on chemotherapy-induced cognitive decline.

5. Declaration of competing Interest

M.T.B. is a scientific cofounder and stock owner of Scioto Biosciences.

CRediT authorship contribution statement

L.D. Otto-Dobos: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation. **C.V. Grant:** Writing – review & editing, Project administration, Investigation, Data curation. **A.A. Lahoud:** Writing – review & editing, Project administration, Methodology, Investigation, Data curation, Conceptualization. **O.R. Wilcox:** Writing – review & editing, Project administration, Investigation, Data curation. **L.D. Strehle:** Writing – review & editing, Investigation, Formal analysis. **B.R. Loman:** Writing – review & editing, Visualization, Investigation, Formal analysis. **S. Adarkwah Yiadom:** Writing – review & editing, Visualization, Formal analysis. **M.M. Seng:** Writing – review & editing, Validation, Data curation. **N.R. Halloy:** Writing – review & editing, Investigation, Data curation. **K.L.G. Russart:** Writing – review & editing, Investigation. **K.M. Carpenter:** Writing – review & editing, Methodology. **E. Dawson:** Writing – review & editing, Formal analysis. **S.D. Sardesai:** Writing – review & editing, Resources. **N.O. Williams:** Writing – review & editing, Resources. **M.E. Gatti-Mays:** Writing – review & editing, Resources. **D.G. Stover:** Writing – review & editing, Resources. **P.K. Sudheendra:** Writing – review & editing, Resources. **R. Wesolowski:** Writing – review & editing, Resources, Methodology. **J.K. Kiecolt-Glaser:** Writing – review & editing, Resources, Methodology. **M.T. Bailey:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **R.R. Andridge:** Writing – review & editing, Visualization, Methodology, Formal analysis. **L.M. Pyter:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2024.05.039.

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