Shotgun Metagenomics Identify Unique Changes of the Intestinal Microbiome in Pediatric Survivors of Acute Lymphoblastic Leukemia

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ABSTRACT

BACKGROUND: Intestinal microbiota plays an important role in human health and metabolism. Microbial dysbiosis has been observed in various chronic conditions, many of which are late effects of leukemia treatment. We previously observed significant differences in the gut microbiome of pediatric ALL survivors compared to healthy sibling controls. Shotgun metagenomic analyses were completed to better characterize the durability and metabolic implication of these changes.

PROCEDURE: Shotgun metagenomic sequencing was completed on DNA extracted from stool samples obtained from nine survivors of childhood acute lymphoblastic leukemia (ALL) and 10 healthy sibling controls.

RESULTS: Beta diversity (dissimilarity between samples) was significant with survivors' microbiomes becoming more similar to siblings further from treatment. The functional potential of gluconate-5-dehydrogenase enzyme (Ga5DH) decreased significantly with time from treatment. Relative abundance of *Faecalibacterium prausnitzii* was identified as the major contributor to differential Ga5DH expression within subjects.

CONCLUSIONS: Time from treatment has a significant effect on functional microbial recovery in ALL. Increased time from chemotherapy corresponds to microbiomes becoming more similar to sibling controls in select dyads. More significant differences were noted in patients closer to treatment. Additional, prospective studies will focus on deeper characterization of these findings and further investigate the functional role of Ga5DH in ALL survivors.

KEYWORDS: Metagenomics; gut microbiome; pediatric; children; cancer

INTRODUCTION

The treatment of pediatric acute lymphoblastic leukemia (ALL) has progressed over the last several decades with 5-year survival rates now above 90%.[1] With improved risk stratification and targeted therapy resulting in increased overall survival, minimizing late effects of treatment and decreasing survivorship-related mortality represent vital aspects of long-term care. Fifty percent of survivors of childhood ALL report at least once chronic medical condition and are 2.8 times more likely than siblings to describe having multiple chronic medical conditions.[2] Late effects of therapy include subsequent malignancy as well as musculoskeletal, cardiovascular, and neurologic diseases.[2,3]

Durable alterations of the intestinal microbiota may contribute to some of the late effects seen in survivors of childhood ALL. The gut microbiome is an integral part of maintaining human health through its impact on metabolic homeostasis and immune function.[4] It is impacted by a variety of factors, many of which overlap with standard pediatric ALL therapy, including diet, medications, environmental exposures, and infection.[5,6] Microbial dysbiosis, or imbalance of the gut microbiota following intestinal disruption, can contribute to a variety of pathologies, including cardiovascular disease, gastrointestinal disease, neurologic disease, endocrinopathies, and malignancy.[7,8]

We previously reported on 16S rRNA gene sequencing performed on gut microbiome samples from nine survivors of ALL compared to 10 healthy sibling controls. A significant difference was seen in the composition of the gut microbiome of pediatric ALL survivors compared to healthy siblings. This observation supports the hypothesis that ALL treatment regimens may result in durable changes of the gut microbiome which can contribute to the long-term health complications often observed in this population.[9]

Shotgun metagenomic sequencing is able to assess cumulative DNA within a sample and provide additional information regarding microbial gene content compared to targeted sequencing of 16S rRNA gene hypervariable regions. Such analyses allow for improved taxonomic resolution and provide a more detailed understanding of functional profiles.[10,11] We sought to better understand the functional implications of our initial findings in survivors of pediatric ALL via shotgun metagenomic analyses.

MATERIALS AND METHODS

Gut microbiome shotgun metagenomic sequencing was completed on DNA samples obtained from a previously completed, approved institutional review board, single-center cohort study.[9] Stool samples were collected to assess the gut microbiome composition of survivors of pediatric and

adolescent pre-B-cell ALL compared to their siblings. Eligible patients were between the ages of 3 and 30, had a history of pre-B-cell ALL currently in remission, and were at least six months from the completion of therapy. All participants were required to have at least one biological sibling who lived in the same home full-time. Exclusion criteria included history of bone marrow transplant and known autoimmune conditions or inflammatory bowel disease. Individuals with use of antibiotics or a diagnosis of gastrointestinal illness (defined as vomiting and/or diarrhea) within 30 days of enrollment were excluded, as well as those taking medications known to impact the gut microbiome, such as immunosuppressive agents, nonsteroidal anti-inflammatory drugs, and proton pump inhibitors. Three stool samples were collected from each study participant, each a minimum of 24 hours apart via a standardized method. Detailed description of methods and results from these analyses were previously published.[9]

Metagenomic sequencing was completed by Novogene Corporation, Inc. for quality control of the DNA samples, library construction, and sequencing. Sequencing libraries were generated using NEBNext® Ultra™DNA Library Prep Kit for Illumina (NEB, USA) and sequenced on an Illumina HiSeq platform, following manufacturer's recommendations. Sequencing data was processed using the bioBakery meta'omics workflow, using the standard pipeline with default parameters.[12] KneadData v0.12.0 was used for quality control of raw reads, including trimming, adapter removal, and removal of off-target reads from the default hg37 and human contamination database. No samples fell under a 10 million final read, quality-control cutoff. Species-level taxonomic profiles were generated using MetaPhlAn v3.0.14 and the CHOCOPhlAn v30 database.[13] Functional profiling was performed using HUMAnN v3.0.0 with UniRef gene families grouped into enzyme commission number (EC) for downstream analysis.[14]

Data was analyzed using R 4.3.0. The vegan package was used for calculation of ecological diversity measurements and whole-community statistical tests. Alpha and beta diversity were quantified as Shannon diversity and Bray-Curtis dissimilarity, respectively. Permutation-based ANOVA was performed using the adonis2 function. The MaAsLin2 v1.18.0 package was used for linear modeling, for which species were filtered at a threshold of minimum 0.1% relative abundance in over 10% of samples.[15] Total sum scaling was used, and species were log-transformed with a pseudocount of half the minimum non-zero value for variance stabilization. Resulting p-values from linear modeling were adjusted for false discovery using the Benjamini and Hochberg method via the p.adjust function in R, and q-values <0.25 were reported as significant. Sequencing data is deposited in Sequencing Read Archive as BioProject PRJNA1235679.

RESULTS

Patient Information

Nine survivors of ALL and 10 siblings were enrolled [**Table 1**].[9] All survivors had a history of pre B cell ALL and were treated according to standard chemotherapy protocols at the time of diagnosis. There was a fairly equal sex distribution (4 females, 5 males). At the time of stool collection, the median age of survivors was 11 years (4–19 years), and the median age of siblings was 12.5 years (5–19). Fifty-seven stool samples were collected. Median time from end of leukemia therapy to stool collection was 24 months (6.5–114 months).

Species Composition

A total of 247 species were initially identified by Meta PhlAn v3.0.14.[13] The 15 species with highest mean relative abundance were plotted and inspected to assess the sample microbial compositions and its consistency between replicates [**Figure 1**]. Four samples were omitted from analyses. Sample RB48 (one stool sample from patient 10) had a taxonomic composition vastly different from the other two samples from the same patient, suggesting a quality issue. The taxonomic composition of three stool samples from Patient 1 was an extreme outlier compared to all other samples, possibly due to underlying diagnosis of Down Syndrome. Thus, sample RB48 and the samples from Patient 1 and their corresponding sibling were omitted from subsequent analyses. A principal coordinate analysis (PCoA) plot was created to demonstrate the relationship between survivor and sibling samples [**Figure 2**].

Table 1. Patient Demographics

Survivor ID	Sex of Survivor	Age of Survivor at Time of Stool Collection (years)	Age at Diagnosis (years)	NCI Risk stratification	Time from End of Therapy (months)	Age of Sibling at Time of Stool Collection (years)
1	F	7	4	SR	6.5	5
2	M	11	6	VHR	24	12
3	F	10	1	SR	71	11
4	M	15	10	SR	38	17
5	F	15	11	HR	24	13
6	M	9	4	SR	38.5	14, 11
7	M	19	16	HR	23	15
8	F	4	1	SR	8.5	7
9	M	17	4	SR	114	19

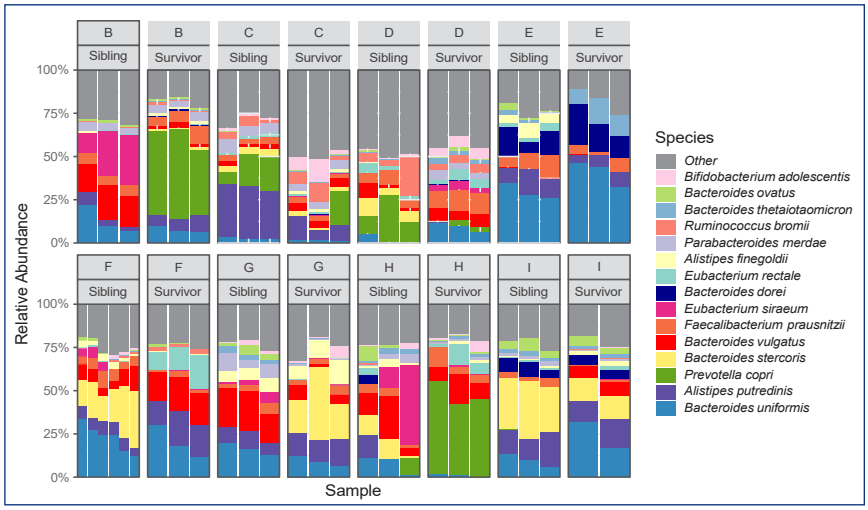
Nine survivors of childhood ALL and 10 siblings were enrolled on this study. Median time from end of therapy was 24 months. NCI= National Cancer Institute, F= female, M= male

Alpha and Beta Diversity

Alpha diversity, or within sample evenness and richness, was assessed using the inverse Simpson index. There was no significant difference between survivors and siblings when tested by linear model, controlling for subject and family as random effects ($p = 0.635$). Time from treatment also did not significantly affect alpha diversity among survivors ($p = 0.309$). Beta diversity, or between-sample dissimilarity, was quantified using Bray-Curtis dissimilarity. Survivor vs. sibling status did not significantly affect beta diversity in a model controlling for family and permuting metadata blocked by subject ($R^2 = 0.034$, $p = 0.832$, permutation-based ANOVA).[16] Within survivors, time elapsed since chemotherapy was significant ($R^2 = 0.249$, $p = 0.019$, permutation-based ANOVA). Bray-Curtis dissimilarity between survivors and siblings within the same family was used as a metric of the degree of dysbiosis of survivor microbiomes. Linear modeling was used to assess the relationship between time since chemotherapy and difference between survivor and sibling microbiomes (coef = –0.0021, p = 5.36e-05). There was a downward trend, indicating that survivors' microbiomes were more similar to siblings at time points further from treatment [**Figure 3**]. While a robust measure of expected familial dissimilarity within healthy controls was not available, one pair of siblings in a single family had a mean dissimilarity of 0.36, which is comparable to the samples with the longest elapsed time since chemotherapy.

Figure 1. Taxonomic composition of samples

Bacterial separation based on taxonomic family level are represented by color variation. One hundred twenty-two species and 60 genera were present after basic filtering. The 15 species with the highest mean relative abundance were plotted for each stool sample.



Taxonomic and functional associations

The association between survivor status and the relative abundance of each microbiome feature (i.e., species and EC) was tested using the MaAsLin2 framework. Linear mixed models were constructed with patient ID and family ID as random effects to account for repeated sampling. No significant associations were observed.

When analyzing months from chemotherapy in survivors, the functional potential of gluconate-5-dehydrogenase enzyme (Ga5DH) was found to decrease significantly with time since treatment (q = 0.025) [**Figure 4**]. The species *Faecalibacterium prausnitzii* was identified as the major

Figure 2. PCoA plot of survivors of ALL and their siblings

Samples largely cluster by survivor and family with small separations between points from the same subject, which is expected from the bar plots and experimental design.

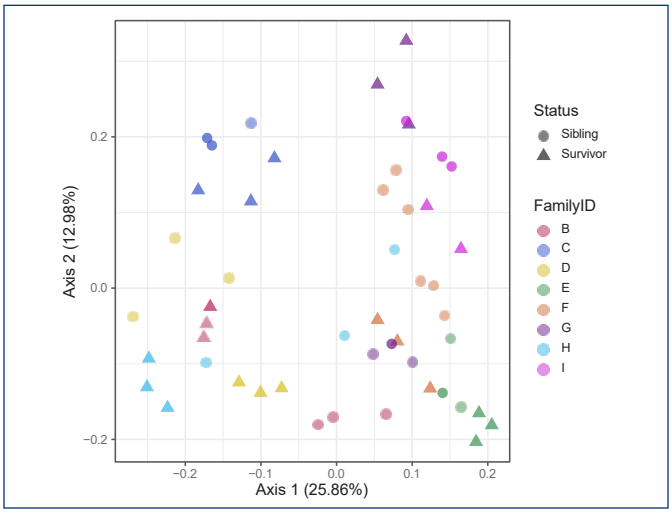


Figure 3. Beta diversity between survivors and siblings within families

The downward trend indicated microbiomes of survivors were more similar to siblings at times further from healthy siblings.

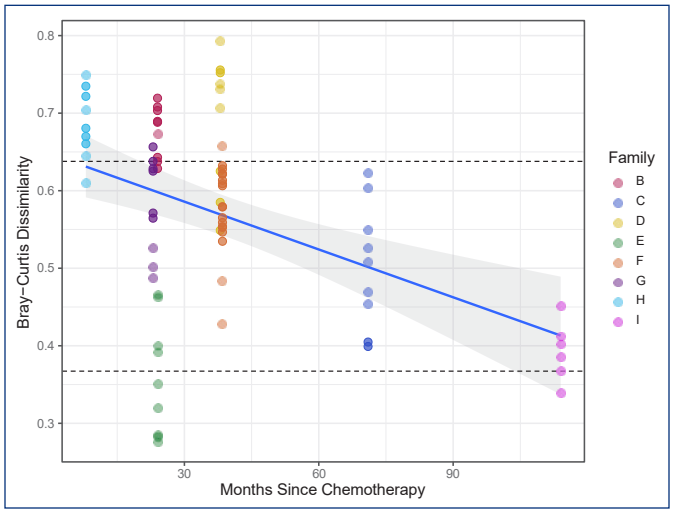


Figure 4. Abundance of Gluconate-5-dehydrogenase enzyme in survivors, as a function of time since chemotherapy

The functional potential of gluconate-5-dehydrogenase in survivors decreases the further away from chemotherapy.

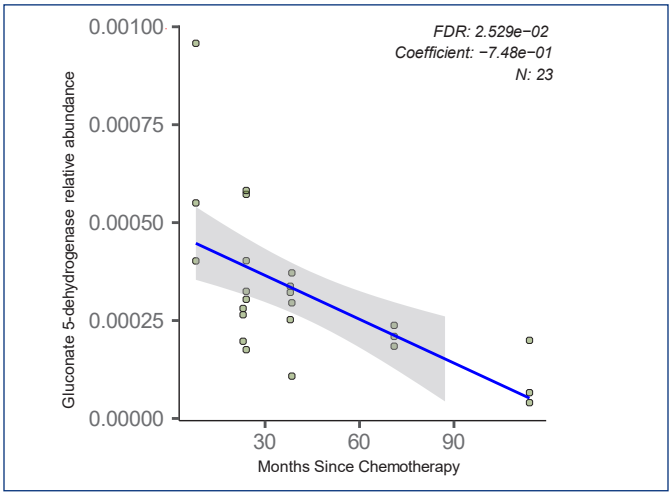
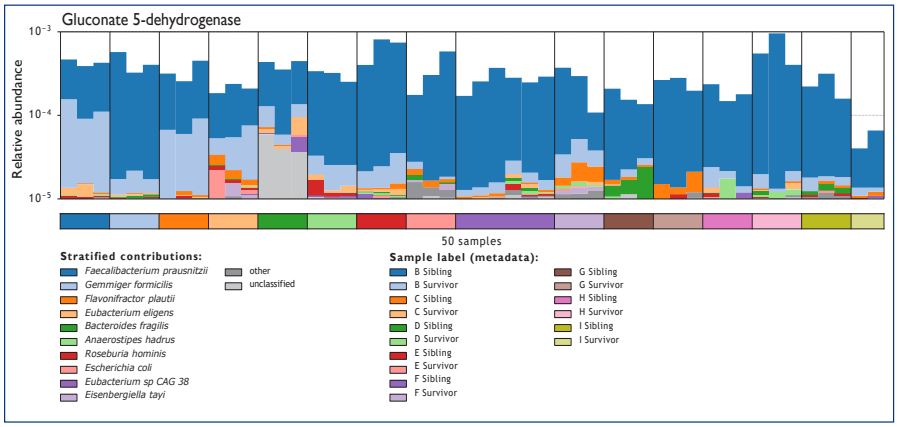


Figure 5. Contributions of microbial species to measured gluconate-5-dehydrogenase, stratified by subjects

The species Faecalibacterium prausnitzii was identified as the major contributor in most samples to the relative abundance of Ga5DH in subjects.



contributor in most samples to the relative abundance of Ga5DH in subjects [**Figure 5**]. However, the overall abundance of *Faecalibacterium prausnitzii* was not associated with time from treatment (p = 0.08).

DISCUSSION

This study compared the intestinal microbiota of pediatric ALL survivors and healthy siblings via next generation sequencing with shotgun metagenomic analysis. Prior studies have been limited and relied on 16S rRNA sequencing to demonstrate that certain gut microbiome changes can be seen in ALL survivors.[17-19] Thomas et al found that members of *Ruminococcaceae, Lachnospiraceae,* and *Faecalibacterium* were significantly depleted in survivors compared to siblings.[17] Chua et al also reported a decrease in *Faecalibacterium* and alterations in immune regulation in survivors of ALL.[19] Our previous work added to these studies and demonstrated a significant difference in alpha and beta diversity in ALL survivors compared to healthy sibling controls.[9]

Shotgun metagenomic sequencing allowed for better understanding of the differences between our two cohorts with higher species resolution, assessment of functionality, and improved accuracy due to less amplification during library preparation. While survivor status alone was not a significant predictor of overall microbiome community signatures, there was evidence that time from treatment had a significant effect on recovery. For within-family comparisons, the Bray-Curtis dissimilarities for each survivor sample against each sibling sample were used as an approximation of deviation from a baseline state. Previous work has established that shared environment and kinship exert a stronger influence than genetics on microbial similarity amongst family members.[20] Therefore, large differences between survivors and siblings may indicate treatment-induced dysbiosis.[21] Upper and lower reference points were established by comparing unrelated study participants and a pair of untreated siblings, respectively. Our study demonstrated that longer duration from chemotherapy exposure is associated with increasing similarity of subjects' microbiome composition compared to their siblings. Only two families had sample pairs as similar as untreated siblings, one of which was the survivor sampled furthest from treatment. For select survivors closer to treatment, the difference between sibling microbiomes was approximately as large as the difference between unrelated study participants. This supports the notion that microbial dysbiosis may persist for years following completion of chemotherapy.[22]

Microbiome feature associations with time within the survivor cohort should provide reasonable estimates of longitudinal trends, particularly if a feature is relatively conserved across healthy subjects. This is more likely to be observed in functional profiles rather than species.[23] Using sibling comparators as a survivor baseline in interaction models was precluded by limited sample size. The relative abundance of Ga5DH enzyme was found to have a significant negative association with time from end of therapy. Ga5DH catalyzes a reversible reduction of 5-ketogluconate to D-gluconate.[24] D-gluconate is an important source of carbon for many microorganisms and may play a role in bacterial survival and virulence.[25] Additional studies are needed to

verify a within-patient decrease in Ga5DH and investigate its role in the gut microbiome in survivors of ALL. Further work will be needed to better understand if there are clinical implications of Ga5DH disruption in survivors of ALL.

Our study had several strengths and limitations. Our design pairing survivors with healthy siblings allowed for the control of various factors known to impact the gut microbiome such as genetics, environment, and diet. By usings siblings as the comparator group, a per-patient microbiome baseline could be created where one would expect the microbiome of survivors to regress to over time after treatment. Collecting three different samples from each participant also allowed for better assessment of outliers and technical artifacts. Shotgun metagenomic sequencing allowed for more precise taxonomic assignments than previous 16S rRNA studies, as well as functional profiling. Our primary limitation was sample size precluding further powered analyses to expand on some of the perturbations seen. Samples from one patient (patient 1) in our survivorship cohort were removed due to a significant difference in the taxonomic composition compared to other participants, likely due to underlying Down Syndrome. Their corresponding sibling was also removed from analyses. One sample from patient 10 (labeled RB48) was also omitted from analyses as its taxonomic composition varied significantly compared to the other samples from that same patient, raising concern for a technical or experimental error as such variations would not be expected in samples collected within one month of one another. By allowing samples to be taken at different times after the completion of chemotherapy, we were less powered to see consistent associations between subjects and treatment history at specific time points. Conversely, this allowed for better assessment of treatment recovery, albeit not as well as true longitudinal sampling. General aspects of microbiome recovery from chemotherapy are not currently well described because of the difficulty of sample acquisition. Longitudinal designs require a long sampling time so many studies are either ongoing or utilize older microbiome sequencing and analysis methodologies. Increasing sample size and length of time from end of therapy to stool collection will be critical to better understand the changes demonstrated in our study.

While the cure rate of pediatric ALL is high in children, the multimodal treatment regimen needed to maximize survival is intense and associated with long-term effects. Microbial dysbiosis from various treatment exposures may represent a pathologic mechanism for the development of long-term side effects of such therapy. The microbiome is known to have important clinical implications, especially with regards to immune development and function. Early life perturbations in the microbiome have been demonstrated to have long lasting effects, so disruptions are particularly concerning if recovery of the microbiome is slow compared to conditions such as illness and antibiotic usage.[26] Furthermore, survivors' microbiomes might never return to their pre-treatment state, instead developing a new equilibrium that is either more or less healthy.[27] Additional studies are required to understand how microbial dysbiosis is associated with specific multi-modal ALL treatment and whether interventions can be utilized to support microbiome recovery during and following treatment. The current results are a strong step in determining this relationship and will serve as the basis for prospective research in survivors of childhood ALL.

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