STATISTICAL ANALYSIS PLAN

Anaerobic Antibiotics and the Risk of Graft-Versus-Host Disease after Allogeneic Hematopoietic Stem Cell Transplantation: Pediatric Gut Microbiome Subgroup Analysis

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Version History

Version	Date	Reason for Update	Time Stamp
1.0	OCT-31-2019	Original version of SAP	OCT-31-2019
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BACKGROUND:

The gut microbiota interacts extensively with the host immune system and thus may modify the risk of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT).[1] During the post-transplant period, the majority of allogeneic HSCT recipients receive empirical broad-spectrum antibiotics for febrile neutropenia. Previous studies have shown an association with lower relative abundances of commensal anaerobes, specifically from the order of Clostridiales, and acute GVHD.[2,3] Additionally, an adult study with supplemental murine GVHD model showed that anaerobic antibiotics decrease the relative abundance of these commensal anaerobes and were associated with an increase in GVHD-related mortality.[4] It has been proposed that butyrate, a product of the commensal aerobes, plays a role in the development of acute intestinal GVHD.[3,4] We hypothesize that receipt of an antibiotic regimen with activity against anaerobes would be associated with increased risk and severity of acute GVHD through depletion of *Clostridiales*, which promote immune tolerance through production of butyrate and other short chain fatty acids (SCFA), as compared to regimens that do not include anti-anaerobic agents.

STUDY OBJECTIVES:

Aim 1: To evaluate for changes in the diversity and composition of the gut microbiota among pediatric HSCT recipients receiving anaerobic or aerobic-only antibiotics for fever and neutropenia.

Aim 2: To evaluate for associations between antibiotic regimens for fever and neutropenia and the relative abundances of genes for production of butyrate and secondary bile acids in pediatric HSCT recipients.

Aim 3: To evaluate for temporal changes in the diversity and composition of the gut microbiota and the relative abundances of genes for production of butyrate and secondary bile acids in HSCT recipients who did and did not develop acute GVHD of the gut and/or liver.

STUDY DESIGN AND METHODS:

Study Design

Retrospective cohort study

Study Population

We included patients <18 years of age who underwent allogeneic HSCT at Duke University starting in October 2015. Fecal samples were collected from patients from the pre-transplant evaluation until day +100 after transplant. While participants were admitted to the inpatient HSCT unit, nurses collected stool samples on as many days as possible. If the patient was discharged home, participants and their parent/guardian were asked to collect a weekly stool sample through day +100 using the EasySampler® Stool Collection Kit. Stool samples were stored in 4ºC refrigerators. The study team transported fecal samples to the laboratory daily (Monday through Friday), placed these samples into cryovials under sterile conditions, and froze the samples to -80ºC for long-term storage.

For the current study, patients were included if they received antibiotics for fever and neutropenia and had pre- and post-antibiotic fecal samples available. Pre-antibiotic samples were collected ≤10 days from the start of antibiotics for fever and neutropenia. During-antibiotic samples were collected while the patient was still on antibiotics, and after receiving antibiotics for ≥2 days. Patients were divided into two groups based on antibiotic regimen they received for fever and neutropenia: anaerobic regimen (piperacillin-tazobactam or a carbapenem) and aerobic-only regimen (ceftazidime, cefepime, or aztreonam). Patients were excluded if they had previously received during the study period or were concurrently receiving clindamycin, metronidazole, or other antibiotics with substantial anaerobic activity (e.g. cephamycins). There were 8 patients in the anaerobic antibiotic group and 20 patients in the aerobic-only antibiotic group.

Sequencing and Bioinformatics

For each patient, approximately one fecal sample per week was selected from admission for HSCT through day +35 or hospital discharge, whichever came first. In addition, when available, we selected a fecal sample obtained prior to admission for HSCT and, if the patient was discharged prior to day +100, the final outpatient fecal sample. We isolated total DNA from stool using PowerMag™ Soil DNA Isolation Kits (MO BIO Laboratories, Solana Beach, CA). We performed shotgun metagenomic sequencing using an Illumina NextSeq500 platform and 150 bp paired-end reads. Human DNA reads were removed and MetaPhlAn2 was used to assign sequencing reads to bacterial species.[5] We aligned sequencing reads with publicly available reference databases to identify bacterial genes encoding butyrate biosynthesis enzymes and genes involved in bile acid metabolism.[6,7]

STATISTICAL ANALYSES:

Aim 1:

Outcome measures

To evaluate for changes in microbiome diversity and composition associated with antibiotic exposures, we will use the Shannon diversity index, Chao 1 index, and relative abundances of bacterial genera, families, and orders in paired stool samples from subjects collected before and during antibiotic exposure.

Exposure variable

Antibiotic exposure is a dichotomous variable, determined by type of antibiotic given to subjects for episodes of fever and neutropenia. Antibiotic exposure was either anaerobic (piperacillin-tazobactam or carbapenem) or aerobic-only (cefepime).

Statistical Methods

Given the available sample size and non-normal distribution of observations, we will use non-parametric testing on paired samples. We will use the Wilcoxon signed-rank tests to compare changes in alpha diversity (Shannon diversity and Chao 1 richness) within antibiotic groups. To determine differences in the overall bacterial composition in pre- and during-antibiotic samples, we will use PERMANOVA stratified by subject to account for paired samples. PERMANOVA is a non-parametric method of multivariate analysis of variance based on pairwise distances.[8] We will use the Bray-Curtis method of estimating distances of paired samples for these analyses. The Bray-Curtis dissimilarity measure is a semimetric, non-Euclidean measurement that represents the proportion of abundance not shared between paired samples.[9] The measurement ranges from 0-1, with 0 representing no dissimilarity between paired samples and 1 representing complete dissimilarity. PERMANOVA calculates the pseudo F statistic (formula below) where N = the number of Bray-Curtis dissimilarity measures, i = the pre-antibiotic distance, j = the post-antibiotic distance, and d = the dissimilarity.[10]

$$SS_{\mathrm{T}} = \sum_{i=1}^{(N-1)} \sum_{j=(i+1)}^{N} d_{ij}^2 / N$$

We will then use Wilcoxon signed-rank tests to evaluate for differences in the relative abundances of bacterial orders with antibiotic treatment. For bacterial orders that differ in pre- and during-antibiotic samples within a given antibiotic group, we will use Wilcoxon signed-rank tests to evaluate for a change in the relative abundances of highly abundant genera in pre- and during-antibiotic samples for that specific antibiotic group.

Aim 2:

Outcome measures

The outcome for Aim 2 is the relative abundance of butyrate biosynthesis genes and bile acid metabolism genes, as determined by output from metagenomic sequencing as described above in the methods.

Exposure variable

Here, again, antibiotic exposure is a dichotomous variable, determined by type of antibiotic given to subjects for episodes of fever and neutropenia. Antibiotic exposure was either anaerobic (piperacillin-tazobactam or carbapenem) or aerobic-only (cefepime) in this patient population.

Statistical Methods

We will use Wilcoxon signed-rank tests to evaluate for a change in the relative abundance of genes encoding butyrate biosynthesis enzymes with exposure to anaerobic antibiotics or cefepime. We will similarly use Wilcoxon signed-rank tests to evaluate for a change in the relative abundance of bile acid metabolism genes within antibiotic exposure groups.

Aim 3:

Outcome measures

To evaluate for changes in microbiome diversity and composition associated with antibiotic exposures, we will use the Shannon diversity index, Chao 1 index, and relative abundances of bacterial genera, families, and orders in paired stool samples from subjects collected before and during antibiotic exposure. Additionally, we will measure the relative abundance of butyrate biosynthesis genes and bile acid metabolism genes, as determined by output from metagenomic sequencing as described above in the methods.

Exposure variable

For each patient, the presence of acute gut/liver GVHD is a dichotomous variable, either present or absent. This was determined through chart review of patients up to one year following HSCT.

Statistical Methods

We will use Wilcoxon signed-rank tests to compare changes in alpha diversity (Shannon diversity and Chao 1 richness) according to acute gut/liver GVHD status. To determine differences in the overall bacterial composition in pre- and during-antibiotic samples, we will use PERMANOVA stratified by subject to account for paired samples. We will then use Wilcoxon signed-rank tests to evaluate for differences in the relative abundances of bacterial orders within acute gut/liver GVHD groups. For bacterial orders that differ in pre- and during-antibiotic samples within a given GVHD group, we will use Wilcoxon signed-rank tests to evaluate for a change in the relative abundances of highly abundant genera in pre- and during-antibiotic samples for that specific GVHD group. We will then use Wilcoxon signed-rank tests to evaluate for a change in the relative abundance of genes encoding butyrate biosynthesis enzymes in those who do and do not go on to develop acute gut/liver GVHD. We will similarly use Wilcoxon signed-rank tests to evaluate for a change in the relative abundance of bile acid metabolism genes according within acute gut/liver GVHD groups. These analyses will be done independent of group of antibiotic exposure.

Software Programs

Statistical testing, figures, and images will be performed using R with the RStudio interface (R version 3.6.1 2019-07-05)

References

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