Understanding the Gut Microbiota in Pediatric Patients with Alopecia Areata and their Siblings: A Pilot Study

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A cross-sectional study of 41 children aged 4–17 years with alopecia areata and 41 of their siblings without alopecia areata was conducted. A total of 51% had the Severity of Alopecia Tool scores in the range of 0–25%, 12% had scores between 26% and 49%, and 36% had scores between 75% and 100%. The fecal microbiome was characterized using shotgun metagenomic sequencing. A comparison of alpha and beta diversity yielded a small but statistically significant difference on the basis of Jaccard distance, which measures species presence and absence between samples. However, a follow-up analysis did not reveal the particular species that were present more often in one group. The relative abundance of one species, Ruminococcus biocellars, was decreased in patients with alopecia areata relative to that in their sibling controls. An analysis of gene ortholog abundance identified 20 orthologs that were different between groups, including spore germination genes and genes for metal transportation. The associations reported in this study support a view of pediatric alopecia areata as a systemic disease that has effects on hair but also leads to internal changes, including differences in the gut microbiome.

INTRODUCTION

Alopecia areata (AA) is a T-cell–mediated autoimmune disease (AID) with unknown pathogenesis and no approved therapies (Gilhar et al., 2012; Rajabi et al., 2018). Genetic profiling of AA has shown deficiencies in the mechanisms of both peripheral and central tolerance (Coda and Sinha, 2011; Jabbari et al., 2016; Suárez-Farinás et al., 2015). In other autoimmune disorders, such as rheumatoid arthritis and inflammatory bowel disease, many genetic and mechanistic parallels exist, and there is a growing body of data about the role of microbiota in onset and flares. AA risk genes are shared with many other AIDs such as rheumatoid arthritis, type 1 diabetes, celiac disease, systemic lupus erythematosus, multiple sclerosis, and psoriasis (Petukhova et al., 2010). The current off-label immune-suppressive therapies can put children at short-term risk for infection and long-term risk for malignancy. In similar autoimmune diseases, there has been increasing evidence that altering the bacteria of the gastrointestinal tract may mitigate the disease. Fecal microbiome transplantation has been successfully applied to control recurrent Clostridiodes difficile infection and can be beneficial for inducing the remission of inflammatory bowel disease (Weingarden and Vaughn, 2017). Owing to its effectiveness in AIDs, there are ongoing randomized clinical trials of fecal microbiome transplantation in patients with rheumatoid arthritis (Zhang, 2019). In 2017, a case study reported hair growth in two young adults with alopecia universalis (and inflammatory bowel disease) treated with fecal transportation for secondary C. difficile infections. Both subjects previously were refractory to standard therapy (Rebello et al., 2017). The gut microbiota has been analyzed in adults with AA but not in pediatric populations. In this study, we address the knowledge gap by conducting a cross-sectional study evaluating the microbiome of 41 children aged 4–17 years with AA and their siblings aged 4–17 years without AA as control subjects.

RESULTS

Of 41 children with AA, 11 were males, and 30 were females. Ages ranged from 4–17 years, with 22% of them aged 4–7 years, 27% of them aged 8–11 years, 34% of them aged 12–15 years, and 17% of them aged 16–17 years. Of them, 29 of 41 (71%) subjects identified as Caucasian. AA severity ranged from mild to severe: 51% had Severity of Alopecia Tool scores in the range of 0–25% (mild), 12% had scores between 26% and 49%, and 36% had scores between 75% and 100% (severe). The most common comorbidity among subjects with AA was atopy (26.8%), including eczema, seasonal allergies, food allergies, and asthma. The diet was predominantly Western/meat eaters (83%), but a small portion was vegan (2%), vegetarian (2%), gluten free (5%), or dairy free (7%) (Table 1).

We characterized the fecal microbiome of all subjects using shotgun metagenomics and recovered 2.7 million read
Table 1. Patient Characteristics (N = 41)

| Characteristics          | Value          |
|--------------------------|----------------|
| Sex assigned at birth, n (%) |                |
| Male                     | 11 (26.8)      |
| Female                   | 30 (73.2)      |
| Age, y, n (%)            |                |
| 4–7                      | 9 (22.0)       |
| 8–11                     | 11 (26.8)      |
| 12–15                    | 14 (34.1)      |
| 16–17                    | 7 (17.1)       |
| Race, n (%)              |                |
| American Indian or Alaska Native | 0 (0)        |
| Asian                    | 5 (12.2)       |
| Black or African American| 2 (4.9)        |
| Indian                   | 1 (2.4)        |
| Native Hawaiian or Other Pacific Islander | 0 (0) |
| White/Caucasian          | 29 (70.7)      |
| Other                    | 3 (7.3)        |
| Refused                  | 1 (2.4)        |
| Diet, n (%)              |                |
| Western                  | 34 (82.9)      |
| Vegan                    | 1 (2.4)        |
| Gluten free              | 2 (4.9)        |
| Dairy free               | 3 (7.3)        |
| Vegetarian               | 1 (2.4)        |
| Comorbidities, n (%)     |                |
| Yes                      | 26 (63.4)      |
| No                       | 15 (36.6)      |
| Conditions reported, n (%) |            |
| Belly pain               | 6 (14.6)       |
| Food allergy             | 6 (14.6)       |
| Constipation             | 3 (7.3)        |
| Eczema                   | 2 (4.9)        |
| Asthma                   | 2 (4.9)        |
| Down syndrome            | 2 (4.9)        |
| Seasonal allergy         | 1 (2.4)        |
| Diarrhea                 | 1 (2.4)        |
| Lactose intolerant       | 1 (2.4)        |
| GERD                     | 1 (2.4)        |
| Autism                   | 1 (2.4)        |
| Headaches                | 1 (2.4)        |
| Sarcoidosis              | 1 (2.4)        |
| Cerebral palsy           | 1 (2.4)        |
| SALT score, n (%)        |                |
| 0–25                     | 21 (51.2)      |
| 26–49                    | 5 (12.2)       |
| 50–74                    | 0 (0)          |
| 75–100                   | 15 (36.6)      |

Abbreviations: GERD, gastroesophageal reflux disease; SALT, Severity of Alopecia Tool.
improved understanding of environmental triggers such as diet and antibiotic exposure in pediatric AA. *R. bicirculans* has been reported as decreased in other autoimmune diseases (Bibbo et al., 2020; Forbes et al., 2016) and has the selective capacity to aid with uptake of nutrients from polysaccharides (Dassa et al., 2014). Consequently, we might hypothesize that this observation is diet associated. We know that dietary patterns play a role in genetically susceptible hosts, but this might provide evidence for why some are more likely to develop the disease. As for the differences in bacterial gene abundance, we identified genes that were associated with spore germination and multidrug resistance. Most members of the Clostridia family are spore-forming bacteria, including *R. bicirculans*. Thus, the result is broadly compatible with our taxonomic results. Our observation of increased abundance among multidrug-resistant genes might suggest a microbial response to antibiotic exposure. Future studies may explore this association by collecting long-term antibiotic exposure data in pediatric subjects with AA.

**MATERIALS AND METHODS**

The study was approved by the Children’s Hospital of Philadelphia (PA) Institutional Review Board (#18-01550), and parents/guardians gave their written informed consent to participate, and children provided assent. Patients with AA aged 4–17 years were identified through medical record review. Children were excluded if antibiotics were taken in the last 6 months. Eligible subjects were provided with home stool collection kits with instructions for putting a tray on the toilet and then transferring to a sterile container and package.
Figure 3. Candidate species. Candidate species were identified as present (black square) or absent (white square) more often in the gut microbiome of children with AA (uncorrected $P < 0.05$, corrected $P > 0.05$ for all species shown). AA, alopecia areata.

Figure 4. Species and gene ortholog abundance in the gut microbiome. (a) Relative abundance of Ruminococcus bicirculans in patients with AA and their sibling controls. Lines connect samples from sibling pairs. (b) The estimated effect size for relative abundance differences in 20 gene orthologs where statistically significant relative abundance differences were identified. Effect size estimates were determined from linear mixed models of log-scaled gene ortholog abundance versus age and disease status. Positive estimates correspond to genes that are higher in affected patients. Error bars extend to one standard error above and below the estimate. AA, alopecia areata.
Stool kits were mailed to Children's Hospital of Philadelphia and were then processed in the Children's Hospital of Philadelphia Microbiome Center.

Shotgun metagenomic sequencing was carried out in the Children's Hospital of Philadelphia Microbiome Center. The DNeasy PowerSoil Kit (Qiagen, Germantown, MD) was used for DNA extraction. The NexteraXT DNA Library Preparation Kit (Illumina, San Diego, CA) was used to generate DNA libraries for shotgun metagenomic sequencing. DNA sequencing was carried out on a HiSeq 2500 instrument, producing 125 base pair paired-end sequence reads. Additional samples of DNA-free water and DNA extraction blanks were processed in parallel with the experimental samples to assess reagent and laboratory contamination.

Bioinformatics analysis was conducted with the Sunbeam metagenomics pipeline (Clark et al., 2019). Taxonomic assignments were generated with Kraken, version 2.1.1. (Wood et al., 2019). The Kyoto Encyclopedia of Genes and Genomes database was used to assign and classify gene orthologs (Kanehisa and Goto, 2000). Species richness or the number of bacterial species per sample was estimated using a sequencing depth of 10,000 reads per sample. Shannon diversity or the abundance-weighted species diversity was calculated using a natural logarithm. Community-level differences between sample groups were assessed using Bray–Curtis distance and Jaccard distance between samples. Sample—sample distances were tested with permutational ANOVA, where permutations were restricted to randomize samples within sibling pairs (Anderson, 2001). When multiple tests were conducted, P-values were corrected using the method of Benjamini and Hochberg (1995) to control for a false discovery rate of 5%.

Data availability statement
The data that support the findings of this study are available on request from the corresponding author, LCS. The data are not publicly available because they contain information that could compromise the privacy of research participants.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
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