IN-DEPTH REVIEWS

Characterizing the skin and gut microbiome of alopecia areata patients

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ABSTRACT

Background: Alopecia areata (AA) is caused by an autoimmune attack of the hair follicle. The exact pathogenesis is unknown, but hypotheses include innate immunity imbalance, environmental exposures, genetic predisposition, and possibly the microbiome. The objective of this study was to characterize the skin and gut microbiome of AA patients, and compare microbial composition to healthy individuals.

Methods: This was a pilot, case-control study. Scalp and fecal microbiome samples were collected from 25 AA patients, and 25 age, gender, and race-matched healthy controls in Southern California with no significant difference in demographic characteristics. After library preparation and identification of bacterial and fungal taxonomy, multivariant analysis was performed to compare AA and healthy microbiomes.

Results: The AA scalp microbiome was significant for decreased *Clostridia* and *Malasseziomycetes*, and the gut microbiome was significant for decreased *Bacteroidia* and increased *Bacilli* ($p<0.05$) compared to healthy controls.

Conclusions: The composition of the AA bacterial and fungal, scalp and gut microbiome is significantly different than healthy individuals. Future directions include using this data to characterize microbial changes associated with AA patient diet, relating to disease severity, and predicting disease progression, prognosis, and/or therapeutic response.

INTRODUCTION

Alopecia areata (AA) is an inflammatory, autoimmune, non-scarring type of hair loss resulting from immune attack of the hair follicle. Disease severity ranges from characteristic bald patches of the scalp (AA), to the entire scalp (alopecia totalis/AT), and even the entire body (alopecia universalis/AU). AA affects up to 2% of the population, and is associated with high disease burden, decreased patient quality of life, as well as a high rate of psychological

comorbidity including depression and anxiety. The exact pathogenesis of AA remains elusive, however researchers hypothesize that it is a multifactorial disease with components including derangements in innate immunity, environmental exposure, genetic predisposition, and now possibly an altered microbiome.[1]

The microbiome is the community of commensal and pathogenic microorganisms (including bacteria and fungi) that live on and within our bodies, and contributes to human health through host defense mechanisms, inflammatory modulation and homeostasis mechanisms. Current research suggests derangements in resident microbiota leads to inflammatory diseases of the gastrointestinal, cardiovascular, respiratory, endocrine and cutaneous systems. Specific to dermatology and hair biology, recent animal studies suggest that the gut microbiome may play a role in the development of AA.[2,3]

With mounting evidence of the microbiome's importance in various inflammatory disorders, we hypothesize that the skin and gut microbiome of AA patients differ significantly from healthy controls. Understanding the composition of the AA microbiome will help to elucidate the role that commensal and pathogenic microbes may play in disease pathogenesis.

MATERIALS AND METHODS

This project is approved by the University of California, Irvine (UC Irvine) Institutional Review Board. Case-control study design is summarized in Figure 1. We enrolled 25 patients with AA/AT/AU that had not received active treatment (including topicals, intralesional or systemic modalities) for at least four weeks, and 25 healthy, age and race-matched controls for a total of 50 participants. At the time of enrollment, participants could not have active or symptomatic gastrointestinal disease. Enrollment occurred over the period of March to May 2018. Subjects were all recruited from a single, tertiary medical center in Southern California. Demographic data and medical history were collected from each participant. Skin (scalp) and gut (fecal) microbiome samples were collected from each patient for a total of 100 samples.

The skin microbiome was collected by swabbing the entire scalp using Culture Swabs™ EZ Collection and Transport system (Becton Dickinson, Franklin Lakes, NJ, USA) which were soaked in 0.15 M normal saline. The ends of the swabs were cut off and stored in 1.5 mL DNAse/RNAse-free microcentrifuge tubes (ThermoFisher Scientific, Waltham, MA, USA) at -80 ^{O}C until time of sample analysis. Fecal samples were collected by giving each patient a sterile urine container (Starplex Scientific Inc., Etobicoke, ONT, Canada) to collect their stool at their convenience at home. Patients were instructed to store the sample in their home freezer until transport to UC Irvine was possible. Once samples arrived, they were also stored at -80 ^{O}C.

Processing and analysis of microbiome samples was completed in conjunction with the UC Irvine Microbiome Initiative. DNA isolation was completed using the ZymoBIOMICS™ Mini DNA kit HT (ZymoResearch, Irvine, CA, USA). 16S rRNA amplicon polymerase chain reaction (PCR) was performed targeting the full V5 region using the Earth Microbiome Project (EMP) primers barcoded 515 forward (515F) and 926 reverse (926R).[4] The internal transcribed spacer (ITS) 2 region for fungal community composition was amplified using the ITS9f as a forward primer and the ITS4 as a barcoded reverse primer in a staggered design.[5,6] The

two libraries were combined and sequenced at the UC Irvine Genomics High Throughput Facility using a MiSeq Version 3 chemistry (Illumina, San Diego, CA, USA) with a paired end 300 base pair (PE300) sequencing length. Sequencing resulted in 24 million reads passing filter (of which 34% were PhiX Control Version 3 adapter-ligated library; Ilumina, San Diego, CA, USA).

Final sequencing resulted in 94 bacterial and 65 fungal samples with reads. Operational taxonomic units (OTUs) of bacterial sequences were identified and assigned taxonomic classification using the May 2013 GreenGenes database[7] trained with original primer pairs used to amplify the 16S region. Fungal sequences were assigned taxonomic classification using the Unite database[8] training the classifier with the whole database since ITS genes can be very variable.

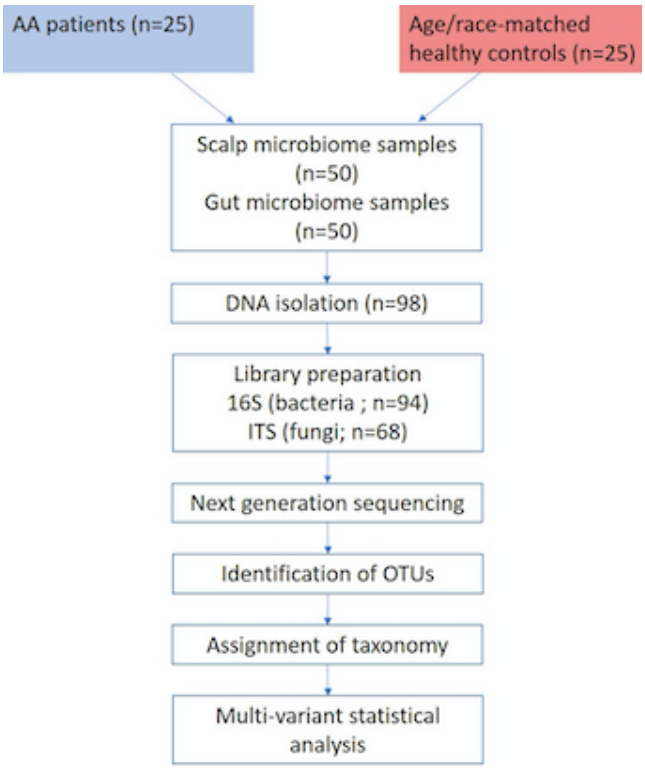
After barcode removal, de-multiplexing, and denoising, samples were imported into Quantitative Insights Into Microbial Ecology (QIIME) Version 2.0.[9] Data interpretation and multi-variant statistical analysis was completed to analyze α- and β-diversity and determine significant differences between AA and healthy skin and gut microbiomes by two authors (SC and MJ). For each taxonomic level of bacterial and fungal data sets, the student *t*-test was performed to determine significant differences in the relative abundances of microbial content between the AA and healthy skin and gut microbiomes.

RESULTS

Demographics

The average AA patient was female (72%), 40.28+/-14.68 years and Caucasian (60%). There was no statistical differences in mean age, as well as gender or race/ethnicity distribution between the alopecia and healthy control groups. AA patients reported an increased prevalence of allergic rhinitis/seasonal allergies (40%), thyroid disease (including hyper- and hypothyroidism; 32%), gastrointestinal disease (including irritable bowel syndrome, inflammatory bowel disease, gluten sensitivity, lactose intolerance, constipation or gastroesophageal reflux disease; 28%), and psychologic comorbidities (including depression and anxiety; 20%) compared to healthy controls. Five patients with AA reported a history of “dermatitis”, including atopic dermatitis and contact dermatitis (Table 1).

Figure 1. Schematic representing the design of this case-control study.



The majority of alopecia patients reported initially having AA subtype (88%) with their first episode 16±14 years prior to presentation at our clinical site. Patients reported their most current alopecia episode started 7±11 years prior to presentation and

was also of the AA subtype (56%). The most common prior treatments for alopecia included intralesional steroid injections (52%), topical minoxidil (24%), topical squaric acid (20%), and topical clobetasol (16%) (Table 2).

Table 1. Demographic data of alopecia patients (n=25) and healthy controls (n=25).

ALOPECIA AREATA			HEALTHY CONTROLS	
F M	72% 28%	**GENDER**	F M	68% 32%
40.28+/-14.68		**AGE (years)**	35.88+/-14.65	
White Asian Hispanic Black	60% 20% 12% 8%	**RACE**	White Asian Hispanic Black	64% 16% 16% 4%
Allergic rhinitis/seasonal allergies Thyroid disease Gastrointestinal disease Psychologic disorder Asthma Acne Localized morphea	40% 32% 28% 20% 12% 8% 4%	**COMORBID DISEASE**	Asthma Gastrointestinal disease Allergic rhinitis/seasonal allergies Non-allergic rhinitis	12% 12% 8% 4%

Table 2. Alopecia areata disease characterization (n=25) including a summary of previous treatment(s) used. It is important to note that patients had to discontinue treatment for at least four weeks prior to enrollment.

ALOPECIA AREATA DISEASE PRESENTATION	
Initial onset (years)	16+/-14
Type of alopecia	
AA	88%
AT/AU	8%
AO	4%
Current episode (years)	7+/-11
Type of alopecia	
AA	56%
AT/AU	44%
AO	0%
Previous treatments	
Intralesional triamcinolone injections	52%
Topical minoxidil	24%
Topical squaric acid	20%
Topical clobetasol	16%
Investigational oral Janus kinase inhibitor	12%
Oral tofacitinib	12%
Complementary and alternative medicine	8%
Topical diphenylcyclopropenone	8%
Topical ketoconazole shampoo	8%
Oral corticosteroid	8%
Platelet-rich plasma	4%
Topical calcineurin inhibitor (eyebrows only)	4%
Unknown systemic immunosuppressant	4%

The AA microbiome is significantly different than healthy individuals

Analysis of the relative abundance of bacterial classes showed the AA scalp was significant for decreased class *Clostridia* (p=0.03287), while the gut was significant for increased *Bacilli* (p=0.007512). There was a trend to decreased *Bacteroidia* in the AA gut, however significance was not reached (p=0.07645). Prior results from animal models describe a significant increase in *Lactobacillales*, an order in the class *Bacilli*. *Lactobacillales* was significantly increased in the AA gut microbiome compared to healthy (p=0.03262). There were no significant differences in the abundance of the other orders, *Bacillales* or *Turicibacterales* (p>0.05), in this class. The family *Lactobacillaceae* and the genus *Lactobacillus* were not significantly increased in the AA gut microbiome, which may be due to the large number of bacterial families and genera present in our small patient sample. The overall α- and β-bacterial diversity was similar between AA patients and controls for both the skin and gut microbiome (Figure 2).

There was a significant decrease in relative abundance of fungal class *Malasseziomycetes* (p=0.03506) on the AA scalp, with a non-significant decrease in fungal genus *Malassezia* (p=0.0801). In addition, a non-significant increase in the class *Eurotiomycetes* was also noted (p=0.0696). There were no significant changes in overall fungal diversity or relative abundance of fecal fungal classes. In contrast, the AA scalp fungal microbiome was significant for decreased α-diversity evenness and β-diversity compared to healthy (α-diversity p=0.049; β-diversity p=0.026), and α-diversity richness trended to significance (p=0.0567) (Figure 3).

Figure 2. The AA bacterial microbiome. There are no significant differences noted in α- and β-diversity of the skin and fecal microbiome of AA patients compared to healthy controls. The AA scalp microbiome was significant for decreased *Clostridia*, and the AA gut microbiome was significant for increased *Bacilli*, most notably due to an increase in *Lactobacillales*.

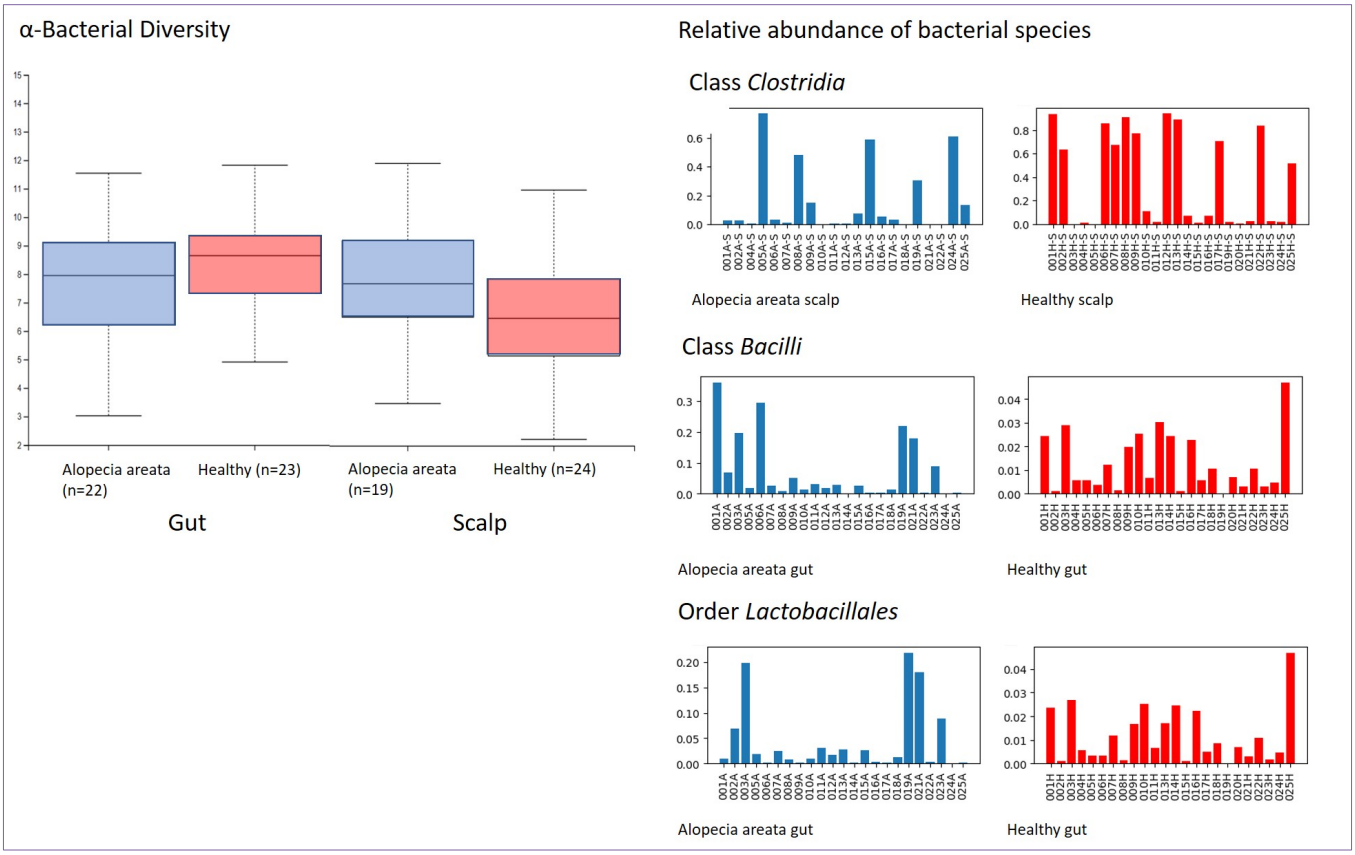
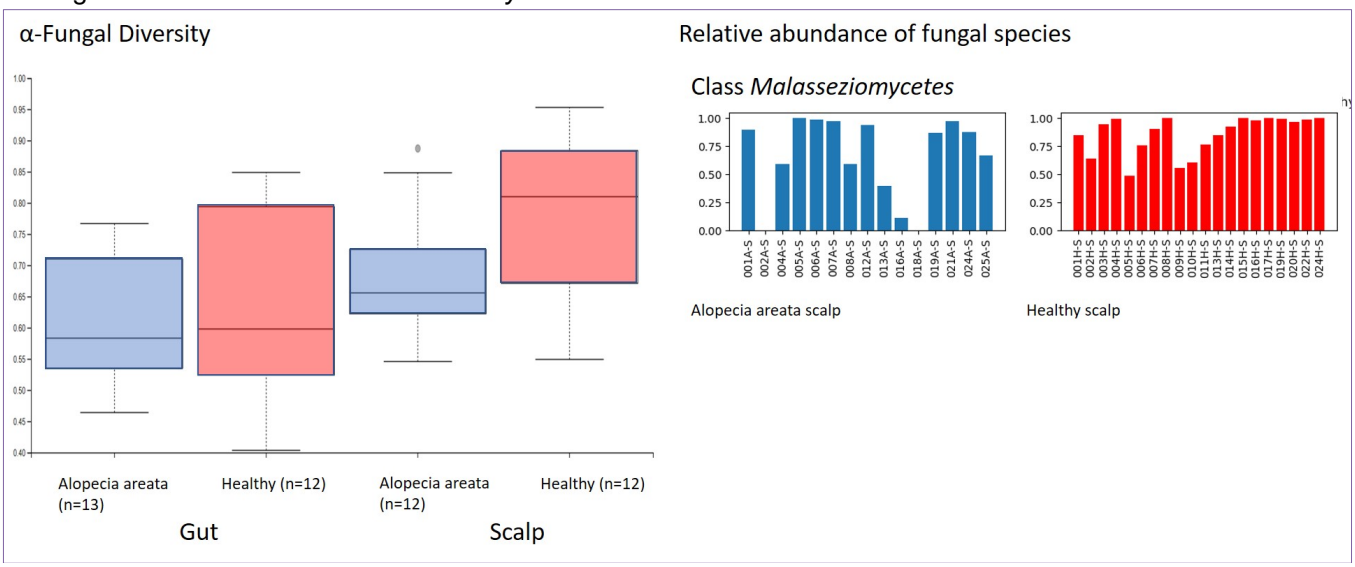


Figure 3. The AA fungal microbiome. There is a significant decrease in AA scalp microbiome α- and β-diversity compared to healthy controls. No differences were noted in AA gut microbiome diversity. The AA scalp microbiome was significant for decreased *Malasseziomycetes*.



DISCUSSION

Our research describes the components of the human AA microbiome and how it may differ from a healthy individual. The data demonstrates that there is significant dysbiosis in the AA scalp and fecal microbiome compared to healthy controls. Significantly increased gut *Lactobacillales* in AA patients compared to healthy controls mimics results previously described in mouse models.[10,11]

The microbiome is the "new frontier" of medicine with evidence that commensal and pathogenic microbes living on and within our bodies play a role in the development of multiple inflammatory conditions. The healthy bacterial skin microbiome is made of multiple species including *Actinobacteria, Bacteroides, Firmicutes,* and *Proteobacteria*. With inflammatory skin disorders, such as atopic dermatitis, it has been shown that microbial diversity decreases, with increased colonization by *Staphylococcus* species.[12-14] We did not account for the diagnoses of atopic dermatitis and how this may have affected our AA skin microbiome. However, given that atopic dermatitis rarely affects the scalp, we believe that our microbiome results are not greatly affected.

Our cohort of AA patients demonstrated a significant decrease in fungal class *Malasseziomycetes* on the scalp compared to healthy controls, of which the genus *Malassezia* belongs. Only 8% of AA patients were previously treated with topical ketoconazole, and it is unlikely that this affected results given that patients remained off treatment for at least four weeks before microbiome collection. The role of the skin microbiome has not been elucidated in AA. In male androgenetic alopecia (AGA), *Malassezia* overgrowth has been described in areas affected by hair loss on the scalp vertex. This increase in *Malassezia* burden causes expression of oxidative stress genes, which may be related to AGA disease pathogenesis.[15] It is possible that changes in *Malassezia* abundance, either increase or decrease, causes exogenous inflammation around and within the hair follicle, thus contributing to alopecia development. However, it is also possible that due to lack of a hair shaft within the follicle, AA patients may inherently have a decreased *Malassezia* population due to an altered follicular environment.

Changes in the gut microbiome have been associated with increased prevalence of autoimmune diseases in developed countries. Currently it is unknown what causes these microbiome changes, however hypotheses include environmental exposures, hygiene hypothesis, and the "Western" diet high in inflammation-inducing fats and processed sugars. Mice exposed to a Western diet demonstrate changes in their gut microbial composition as soon as one day, with an increase in *Firmicutes* and decrease in *Bacteroidetes* phyla, with accompanying changes in metabolic pathways and gene expression leading to increased adiposity by two weeks. Decreased *Bacteroidetes* abundance leads to reduced production of anti-inflammatory short-chain fatty acids, dysregulation of colonic T-regulatory cells, as well as downstream effects on innate immune activation and tolerance. In humans, gut microbiota differences have been noted in patients with rheumatoid arthritis (decreased *Bifidobacterium* and *Bacteroides*) and atopic dermatitis (decreased *Lactobacillus*) compared to healthy controls.[2,16]

A recent case series, supports our findings that the human microbiome plays a role in pathogenesis of AA. Scalp and body hair regrowth was noted in two AU patients after

fecal microbiota transplant for recurrent *Clostridium difficile* colitis. The long-term persistence of the transplant-induced hair regrowth suggests a fundamental change in the composition of the gut microbiome, and that prior gut dysbiosis may have contributed to the autoimmune alopecia.[3] Along those lines, gut monocolonization by *Lactobacillus murinus* has been hypothesized as a contributing factor for AA in two mouse models. C5BL/6 mice were treated with vancomycin leading to increased abundance of gut *L. murinus* leading to a biotin-deficient alopecia phenotype. C3H mice prone to AA demonstrate increased baseline gut *Lactobacillus*, causing AA development in healthy mouse skin grafts. After treatment with broad-spectrum antibiotics (ampicillin, neomycin, metronidazole and vancomycin), *Lactobacillus* abundance was decreased and grafts did not develop AA.[10,11]

Limitations of this study include small sample sizes. The restricted geographical location to Southern California provided for a well-controlled study. Previous studies have shown that there are geographical differences in microbiome samples and our results may not be generalizable throughout all geographic areas.

CONCLUSIONS

The results from a cohort of AA patients located in Southern California demonstrates significant dysbiosis of the skin and gut microbiome of AA patients compared to healthy controls. The cause(s) of dysbiosis in AA patients are not known, however it is believed that both environmental and genetic factors play a role in microbiome composition. Changes in abundance of skin and gut bacteria and/or fungi may lead to inappropriate activation of the innate immune response, changes in tolerance, and ultimately systemic inflammation in predisposed individuals. This novel data may be used in the future to determine skin and gut microbiome changes associated with AA patient diet, disease severity, disease prognosis and progression, as well as response to targeted therapies.

Keywords: alopecia, alopecia areata, microbiome, bacteria, fungi, dysbiosis, inflammation

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