

Association of Disease Severity With Skin Microbiome and Filaggrin Gene Mutations in Adult Atopic Dermatitis

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 Supplemental content

IMPORTANCE Skin microbiome correlates with disease severity for lesional and nonlesional skin, indicating a global influence of atopic dermatitis (AD). A relation between skin microbiome and filaggrin gene (*FLG*) mutations proposes a possible association between skin microbiome and host genetics.

OBJECTIVES To assess skin and nasal microbiome diversity and composition in patients with AD and compare with healthy controls, and to investigate the microbiome in relation to disease severity and *FLG* mutations in patients with AD.

DESIGN, SETTING, AND PARTICIPANTS An observational case-control study of 45 adult healthy controls and 56 adult patients with AD was carried out from January 2015 to June 2015 in a tertiary referral center, Department of Dermatology, Bispebjerg Hospital, Denmark.

EXPOSURES Bacterial swabs were taken from patients with AD (lesional skin, nonlesional skin, and anterior nares) and from healthy controls (nonlesional skin and anterior nares). Eczema severity was assessed and *FLG* mutations noted. Bacterial DNA was extracted from swabs, and V3-V4 16S rDNA regions amplified with PCR. Samples were analyzed at Statens Serum Institut September 2015 to September 2016. Bioinformatics analyses of the microbiome were analyzed using R statistical software (version 3.3.1, R Foundation Inc).

MAIN OUTCOMES AND MEASURES Skin microbiomes were investigated using next-generation sequencing targeting 16S ribosomal RNA.

RESULTS Microbiome alpha diversity was lower in patients with AD compared with healthy controls in nonlesional skin (effect size, 0.710; 95% CI, 0.27-1.15; $P = .002$), lesional skin (effect size, 0.728; 95% CI, 0.35-1.33; $P = .001$), and nose (effect size, 1.111; 95% CI, 0.48-0.94; $P < .001$). Alpha diversity was inversely correlated with disease severity for lesional (effect size, 0.530; 95% CI, 0.23-1.64; $P = .02$) and nonlesional skin (effect size, 0.451; 95% CI, 0.04-2.44; $P = .04$) in patients with AD. Microbiome composition in AD nonlesional skin was linked to *FLG* mutations.

CONCLUSIONS AND RELEVANCE An altered microbiome composition in patients with AD in nonlesional skin, lesional skin, as well as nose, suggests a global influence of AD. Microbiome composition in AD nonlesional skin is associated with *FLG* mutations, proposing a possible association between the skin microbiome and host genetics.

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Research has shown that the human microbiome, ie, the bacteria, viruses, and fungi inhabiting the human body, plays a role in health and disease, and in the development of our immune system.^{1,2} In atopic dermatitis (AD), the skin microbiome is dominated by *Staphylococcus aureus* (*S aureus*) and less diverse than in healthy skin.³ The association between skin microbiome diversity and severity of AD has sparingly been studied in adults and an inverse correlation was reported in children.^{3,4}

Skin microorganisms seem capable of influencing host cells, eg, by affecting the production of endogenous antimicrobial peptides (AMPs),⁴⁻⁶ and contributing to host immunity by cooperating with the innate and adaptive defense system. A balanced interplay between host cells and resident bacteria is important for optimal skin defense and health,⁷ and it is essential to understand how the microbiome interacts with dermatological conditions, and how this eventually can be used in new treatment strategies.

Atopic dermatitis is the most common inflammatory skin disease affecting up to 20% of children and up to 10% of adults worldwide.⁸ Atopic dermatitis is characterized by overactive immune response, impaired skin barrier, and skin colonization with *S aureus*, which is present in up to 90% of patients with AD.⁹ Colonization with *S aureus* contributes to frequent flare-ups and disease worsening,¹⁰ and *S aureus* colonisation is a considerable problem in the treatment of AD. Recent studies have revealed how *S aureus* plays a role in skin barrier dysfunction by increasing inflammation,¹¹ worsening barrier impairment,¹² and penetrating the epidermis in the presence of increased Th2 cytokines,¹³ which are found in AD.

The filaggrin protein is a key protein in the structure of the stratum corneum, ensuring a resilient and tight skin barrier, and filaggrin gene (*FLG*) mutations are the strongest identified genetic risk factor for the development of AD.¹⁴ The link between *FLG* mutations and AD has highlighted the importance of skin barrier function in the pathogenesis of AD, and the presence of *FLG* mutations may even have implications for the skin microbiota, since *FLG* mutations were recently reported to be linked to a certain *S aureus* clonal type in patients with AD.¹⁵

The relationship between eczema severity and the skin microbiome in adult patients with AD is sparsely characterized, and the interplay between host barrier impairment (*FLG* mutations) and the skin microbiome in AD is unknown. This exploratory study aimed to investigate microbiome composition and diversity in adult patients with AD, and compare with healthy controls. Furthermore, to investigate differences in the microbiome in relation to disease severity and *FLG* mutations.

Methods

Study Participants

Patients with AD from the outpatient clinic of the Department of Dermatology, Bispebjerg Hospital, January 2015 to June 2015 were invited to participate in the study. Inclusion criteria were age 18 years or older and presence of AD according to UK criteria¹⁶ when examined at inclusion. Exclusion criteria were

Key Points

Question Is the skin microbiome in patients with atopic dermatitis (AD) associated with disease severity and filaggrin gene mutations?

Findings In this case-control study, bacterial diversity (alpha diversity) of the skin microbiome in patients with AD was inversely correlated with disease severity for both lesional and nonlesional skin. Skin microbiome composition (beta diversity) in nonlesional skin of patients with AD was linked to the presence of filaggrin gene mutations.

Meaning The findings of this study suggest that the severity of AD influences the skin microbiome globally, even in nonlesional skin. Furthermore, our findings propose a possible association between skin microbiome and host genetics.

pregnancy, breastfeeding, and ultraviolet light therapy within the past 2 months. Patients were characterized by demographic data, and *FLG* mutations common among whites, R501X, 2282del4, and R2447X.¹⁷ Disease severity was assessed by Severity Scoring of Atopic Dermatitis (SCORAD)¹⁸ and grouped into 3 groups: mild (SCORAD <25), moderate (SCORAD 25-50), and severe (SCORAD >50).¹⁹ Patients were included regardless of therapy; however, all types of treatment were registered (topical treatment, systemic treatment, antibiotics). Patients were previously described with regard to *S aureus* colonization, *FLG* mutations, and *S aureus* clonal complex (CC)/spa-type.¹⁵

Healthy controls, recruited from the hospital office staff and employees at Statens Serum Institute, Denmark, were included in the study. Inclusion criteria were age 18 years or older. Exclusion criteria were current or previous atopic dermatitis, as well as other major skin diseases including psoriasis and rosacea. Information on other diseases, use of medication and antibiotics during or before sampling was registered. Ethnicity was assessed in all participants by the investigator (Table).

The study was approved by the National Ethical Committee as well as the Danish Data protection Agency. All patients provided written informed consent. They were not compensated.

Microbiome Sampling

Swabs were taken from each patient with AD (lesional skin, nonlesional skin, and nose) and control (nonlesional skin and nose) using e-Swab (Copan). Skin samples were collected by rubbing the skin for 30 seconds, and nasal samples by rotating the swab 3 times in one of the anterior nares. All nonlesional samples (patients with AD and controls) were collected from the antecubital crease; however, in patients with visible eczema in the antecubital crease, samples were taken from the volar forearm. Samples from AD lesional skin were collected depending on location of eczema, primarily from the arms.

DNA Extraction and Sequencing

DNA was extracted using a MagNa lyser instrument (Roche, Mannheim, Germany) and FastDNA SPIN Kit for Soil (MP Biomedicals). Custom made primers (341F: 5'-ACTCCTAYGG-GRBGCASCAG-3'; 806R: 5'-AGCGTGGACTACNNGGGTATC-TAAT-3') were used to amplify the V3-V4 16S rDNA.²⁰ Initial

amplification of the target site was followed by a secondary PCR where adaptors containing indexes and sequencing primer sites were attached (PCR programs: 95°C for 2 minutes; 20 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds; 72°C for 7 minutes). DNA fragments shorter (<300 nt) or longer (>1000 nt) than the expected amplicon target site were removed using Agencourt AMPure XP bead (Beckman Coulter Inc) purification. Paired-end sequences (2×250) were obtained using a MiSeq instrument (Illumina Inc). Sequences can be found at <http://www.ebi.ac.uk/ena/data/view/PRJEB21789>.

Operational Taxonomic Unit Picking

BION (<https://app.box.com/bion>) was used for operational taxonomic unit (OTU) picking.²⁰ The same parameters as in the study by Ring et al²⁰ were used, with a few exceptions: (1) Chimera filtering precluster was set to 96% and a minimum score of 15 was used. (2) Sequences were clustered by 100% stringency, (3) reference matching was performed with a k-mer length of 8, and step size of 4. The RDP database (RDP 11.5)²¹ was used as query sequences, and (4) individual profile tables were written for family, genus, and species.

Statistical Analyses

Statistical analysis and visualizations were performed using R statistical software²² (version 3.3.1, R Foundation Inc). Species known to be contaminants were manually removed from analyses (list of contaminants: eTable 1 in the [Supplement](#)). Isolates with less than 20 000 counts after removal of contaminants were removed from further analysis. Only complete sample sets were investigated (lesional, nonlesional, and nose for patients, and skin and nose for controls).

The R package phyloseq²³ was used for microbiome analysis (version 1.16.2) and visualized with the ggplot2 package (version 2.2.1).²⁴ Alpha diversity was measured using Shannon diversity through the phyloseq package on raw data. Shannon diversity takes into account both the richness (number of different species) and evenness (how evenly the species are distributed) of the bacterial community. Differences in Shannon diversity between groups were tested with Mann-Whitney tests for unpaired, and Wilcoxon signed rank test for paired samples. To compare overall differences between groups, principal coordinate analysis (PCoA) plots were used for visualization together with permutational multivariable analysis of variance using distance matrices (PERMANOVA) tests for statistical analysis. The PCoA plots and PERMANOVA tests were performed on rarefied data (rarefied to 20 000 counts). The PERMANOVA tests were run through the Adonis function on Bray-Curtis distances (using the vegan package, version 2.4.2)²⁵ and setting number of permutations to 1000. The PCoA plots and PERMANOVA tests attempt to summarize interobject dissimilarity, providing information on the overall composition of the microbiome. Both rarefying and PERMANOVA functions use randomization of numbers, the set.seed function was used (with seed = 42) to obtain reproducible results. For barplots, all OTUs were merged to genus level, except for OTUs containing *Staphylococcus*; these were kept on species level. Sixteen percent of the sequences identified as *Staphylococcus*

Table. Demographic and Clinical Data of 101 Participants

Characteristic	Atopic Dermatitis	Healthy Controls
Patients analyzed, No.	56	45
Age, mean (range), y	38 (18-77)	45 (26-69)
Female:male ratio	28:28	26:19
Ethnicity, No.		
White:Asian ratio	53:3	45:0
Sites analyzed	LS, NLS, nose	NLS, nose
SCORAD score		NA
Mean (range)	30 (6-65)	NA
Mild:Moderate:Severe	19:32:5	NA
Filaggrin Mutation:Wt:Unknown	17:33:6	Unknown
Treatment, No.		
Steroid	44	0
Antibiotics (topical:systemic)	6:12	0:4
Atopy, No.		Unknown
Family disposition	23	
Asthma	16	
Allergy	39	
Hay fever	36	
<i>Staphylococcus aureus</i> positive, No.	41	Unknown
Nose	31	
Nonlesional	15	
Lesional	32	

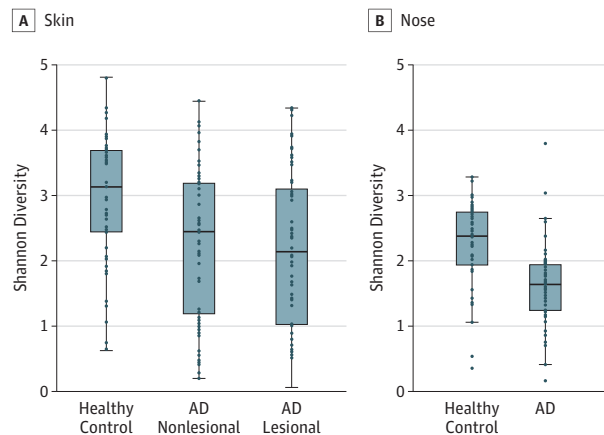
Abbreviations: FLG, filaggrin gene; LS, lesional skin; NA, not applicable; NLS, nonlesional skin; SCORAD, Severity Scoring Atopic Dermatitis; Wt, wildtype.

were unclassified at species level (ie, strains with no discriminatory nucleotides in the amplicon region). Samples were made comparable by transforming to percentage, and the 10 species with the highest percentage over all samples were plotted. Clusters were made using hierarchical clustering, with the Ward method for clustering on Bray-Curtis distance. The number of optimal clusters was decided by visual inspection.

Results

A total of 56 patients with AD (28 women and 28 men), aged 18 to 77 years, were included in the study. Mean SCORAD was 30 (25th-75th quartile: 18.1-40.6). Topical corticosteroids (TCS) were used daily or weekly by 44 of the patients, systemic antibiotics had been used within 3 months by 12 patients, and topical antibiotics (Fucicort) had been used within 2 weeks by 6 patients. There were *FLG* mutations present in 17 patients with AD, of whom 14 were heterozygotes, 2 were homozygotes, and 1 was compound heterozygote. Forty-five healthy controls (aged 26-69 years), 26 women and 19 men, were included in the study. Within the past 3 months none had used topical corticosteroid, whereas systemic antibiotics had been used by 4 (Table). Sex distribution for patients and controls did not differ (effect size, 0.313; 95% CI, 0.39-1.16; Fisher test, $P = .55$). Distribution of age differed between patients with AD and controls, however age did not significantly influence the skin microbiome composition (eFigure 2 in the [Supplement](#)).

Figure 1. Shannon Diversity Index for Skin Samples and Nose Samples of Patients With AD and Healthy Controls



Shannon diversity index, which considers both the richness (number of different species) and evenness (how evenly the species are distributed), is shown for skin samples of patients with AD (nonlesional skin and lesional skin) (A) and healthy controls, as well as nose samples (B). Differences in microbiome diversity were found between patients with AD and healthy controls for both skin and nose. Boxes indicate the 25th percentile, median, and 75th percentile. Dots represent each individual sample. Whiskers show the minimum and maximum ranges. AD indicates atopic dermatitis.

Nonlesional skin samples of patients with AD were primarily collected from the antecubital crease (moist skin); however, 9 samples were collected from the volar forearm owing to visible eczema in the antecubital crease (dry skin). Sample location had no impact on the composition of the skin microbiome, with no significant difference between the 2 sample sites (eFigure 3 in the [Supplement](#)).

Skin and Nasal Microbiome in Patients With AD Compared With Healthy Controls

Initial exploration of the microbiome found differences in composition (beta diversity) between AD and controls, both for skin and nose samples. Furthermore, differences in composition were found between AD lesional skin and AD nonlesional skin (eFigure 4 in the [Supplement](#)).

Bacterial diversity (alpha diversity) of the microbiome was investigated using Shannon diversity analysis (**Figure 1**). Control skin had the greatest diversity, compared with both AD nonlesional skin and AD lesional skin (effect size, 0.710; 95% CI, 0.27-1.15; $P = .002$ and effect size, 0.728; 95% CI, 0.35-1.33; $P = .001$, respectively). No difference was found in bacterial diversity between AD nonlesional skin and AD lesional skin (effect size, 0.128; 95% CI, -0.36 to 0.59; $P = .57$). In nasal samples, controls had greater diversity than patients with AD (effect size, 1.111; 95% CI, 0.48-0.94; $P < .001$).

Bacterial Composition in Patients With AD Compared With Healthy Controls

Hierarchical clustering of the samples was performed according to similarity of the microbiome. Among skin samples 5 different clusters were identified, and among nasal samples another 5 clusters were identified (**Figure 2**) (eFigure 5 in the [Supplement](#)).

In skin samples, cluster I was dominated by *S aureus*, cluster II was dominated by *Staphylococcus caprae*, cluster III by *Staphylococcus epidermidis*, cluster IV by *Staphylococcus hominis*, and cluster V showed great variation with presence of *Propionibacterium* species. Cluster V was the most dominant cluster for all skin types (>55%). For cluster distribution, please see supplementary files (**Figure 2**) (eTable 6 in the [Supplement](#)).

In nasal samples, cluster A was dominated by *Moraxella*, cluster B by *S aureus* and *Dolosigranulum*, cluster C by *Corynebacterium*, cluster D by *S epidermidis*, and cluster E by *Propionibacterium spp* and *S epidermidis* (eFigure 5 in the [Supplement](#)). The cluster distribution was rather even in nasal samples, and there was no dominant cluster across the 2 groups, as seen in skin samples.

Microbiome Diversity and Disease Severity

Association between microbiome composition (beta diversity) and disease severity (SCORAD) was investigated and visualized with PCoA plots. Beta diversity was associated with SCORAD for AD nonlesional skin, lesional skin, as well as for AD nose samples (eFigure 7 in the [Supplement](#)).

Alpha diversity was investigated in relation to disease severity (SCORAD), and for all AD skin samples an inverse relation between bacterial diversity and disease severity was found (**Figure 3**). For AD nonlesional skin, mild or moderate AD showed greater bacterial diversity than severe AD and for AD lesional skin, mild AD showed greater bacterial diversity than moderate and severe AD (**Figure 3**).

In AD lesional skin, patients with severe AD predominantly belonged to cluster I, whereas patients with mild AD predominantly belonged to cluster V; however, no clear pattern was found between disease severity and cluster distribution (**Figure 2**). For AD nonlesional skin, clusters I, II, and III were dominated by moderate and severe AD, whereas patients with mild AD belonged mostly to clusters IV and V (**Figure 2**).

Filaggrin and the Skin Microbiome

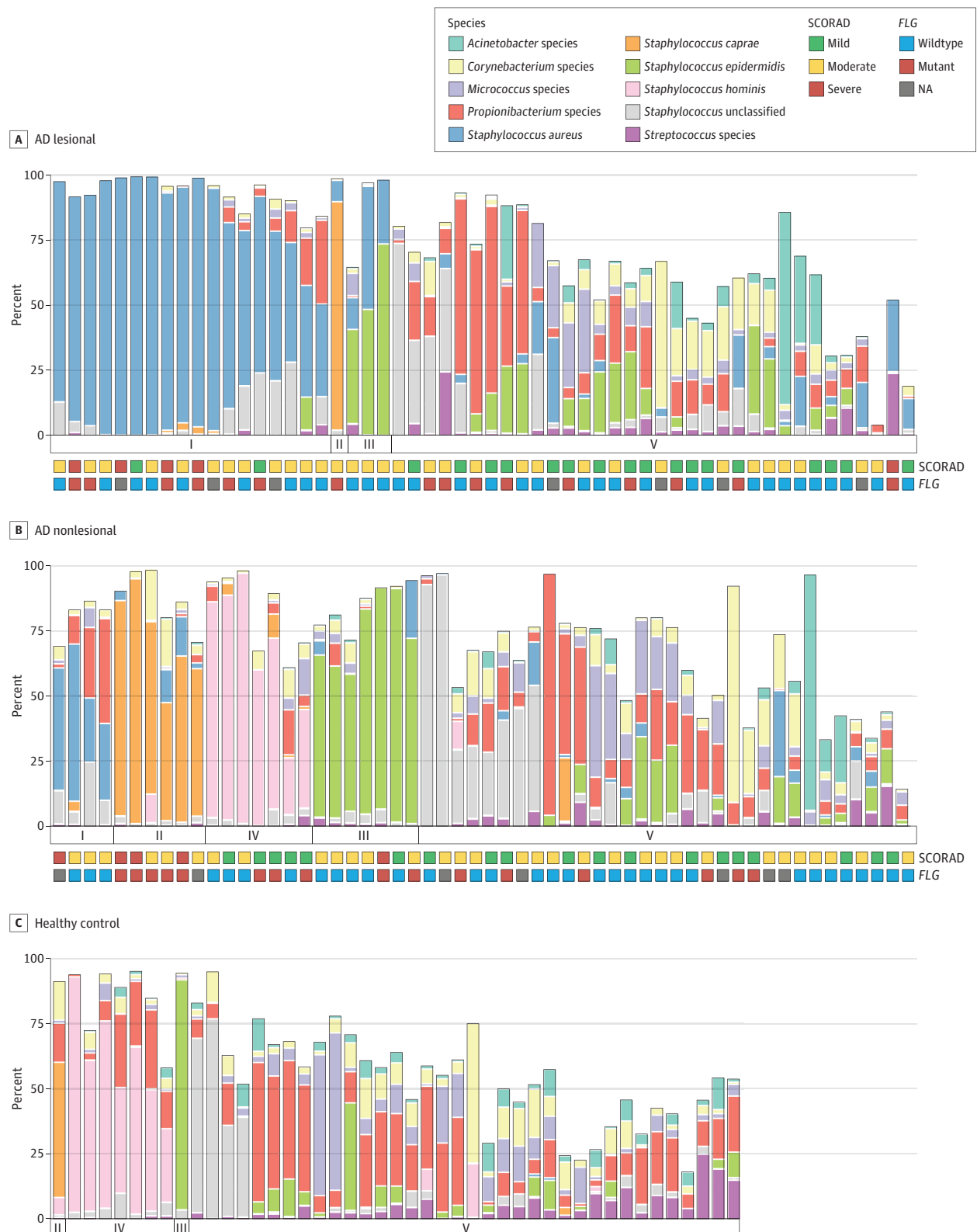
An association between *FLG* mutations and the overall microbiome composition (beta diversity) was tested for and visualized by PCoA plots. A difference in the microbiome composition between patients with AD who had *FLG* mutations and patients with AD without *FLG* mutations was found for nonlesional skin, but not for lesional skin, or nose (**Figure 4**).

Alpha diversity showed no difference between patients with AD who had *FLG* mutations and patients with AD without *FLG* mutations for neither lesional nor nonlesional skin. There was no obvious relation between clusters and *FLG* mutations; however, *S caprae* was significantly more frequent in patients with *FLG* mutations compared with patients without *FLG* mutations ($P = .007$).

Discussion

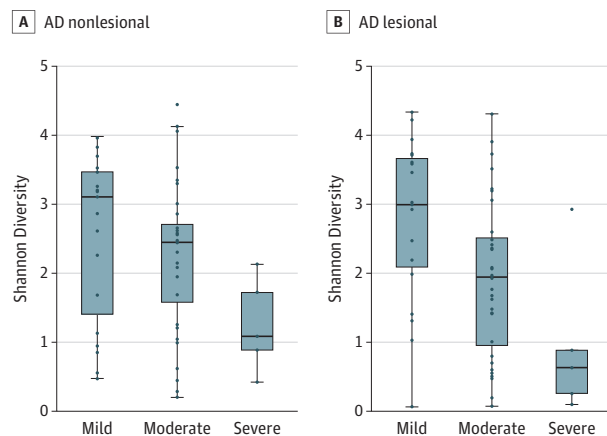
The most important and new findings in this study are the association between microbiome diversity and disease severity in lesional and nonlesional AD skin, and the

Figure 2. Barplots of Bacterial Species in Skin Samples of Patients With Atopic Dermatitis (AD) and Healthy Controls



Barplots of bacterial species in skin samples of patients with AD and healthy controls, performed at genus level, except for *Staphylococcus*, which was kept at species level. Hierarchical clustering was performed according to similarity of the microbiome, and 5 different clusters were identified (I-V). Barplots were performed for AD lesional skin, AD nonlesional skin, and healthy control skin. Disease severity (SCORAD) and FLG mutation status are shown for patients with AD.

Figure 3. Shannon Diversity Index in Relation to Disease Severity (SCORAD)



Shannon diversity index in relation to the Severity Scoring of Atopic Dermatitis (SCORAD) results for both AD lesional and nonlesional skin. SCORAD was divided into 3 groups: mild, moderate, severe. Microbiome diversity was associated with SCORAD in AD lesional skin but also in AD nonlesional skin. The group for severe AD had few patients; however, a clear trend for the association with SCORAD on microbiome diversity is visible for both lesional skin and nonlesional skin. Boxes indicate the 25th percentile, median, and 75th percentile. Dots represent each individual sample. Whiskers show the minimum and maximum ranges. AD indicates atopic dermatitis.

difference in the microbiome composition between patients with and without *FLG* mutations.

The inverse relationship between microbiome diversity and disease severity (SCORAD) is particularly important because the finding was significant for AD lesional as well as AD nonlesional skin. This association was reported previously only in children^{3,26} for lesional skin; however, the fact that skin without any visible eczema also harbours a different microbiome from healthy control skin suggests a globally affected cutaneous microbiome in patients with AD. Recently it was shown that AD treatment changed the skin microbiota of AD lesional skin to resemble AD nonlesional skin, but not healthy skin.²⁶ Taken together with previous findings of altered skin barrier properties in AD nonlesional skin with increased transepidermal water loss, changes in epidermal terminal differentiation, lipids, and skin pH²⁷⁻²⁹ all points toward AD affecting the skin more globally—beyond what is clinically visible.

Differences in the microbiome composition in nonlesional skin between patients with and without *FLG* mutations in the present study are important because the alpha diversity (Shannon diversity) of the skin microbiome was not associated with *FLG* mutations. Hence the number of different species and the evenness of how these species are distributed, cannot account for the difference. However, the presence of different species could be an explanation, and we found increased number of *S caprae* in relation to *FLG* mutations in AD nonlesional skin. Previously, similar findings were reported for patients with ichthyosis vulgaris, with microbiome beta diversity significantly different in patients with *FLG* mutations owing to decreased gram-positive anaerobe cocci, yet no difference in alpha diversity.³⁰ The great dominance of *S aureus* in AD lesional skin as well as intense inflammation

in the skin may hinder finding a possible link between microbiome composition and *FLG* mutation status in lesional skin. To further validate a possible link between the skin microbiome composition and *FLG* mutations, studies with larger patient numbers are needed.

In this study we confirm^{3,26,31} that the skin microbiome is significantly different in patients with AD compared with healthy controls with a decreased diversity in AD samples. Interestingly, a difference in the microbiome composition was also found in patients with AD, with decreased diversity in lesional skin compared with nonlesional skin. This is likely owing to AD lesional skin being more frequently colonized by *S aureus* (Figure 2) than nonlesional skin. We found a difference in clusters between lesional and nonlesional skin, with *S hominis* and *S caprae* more frequent in nonlesional skin and scarcely present in lesional skin, supporting an altered microbiome for AD nonlesional skin, different from both lesional AD skin and healthy skin. The most diverse cluster (cluster V) included more patients treated with TCS than did cluster I, supporting a previous finding that intermittent treatment with TCS increases microbiome diversity in lesional skin.³

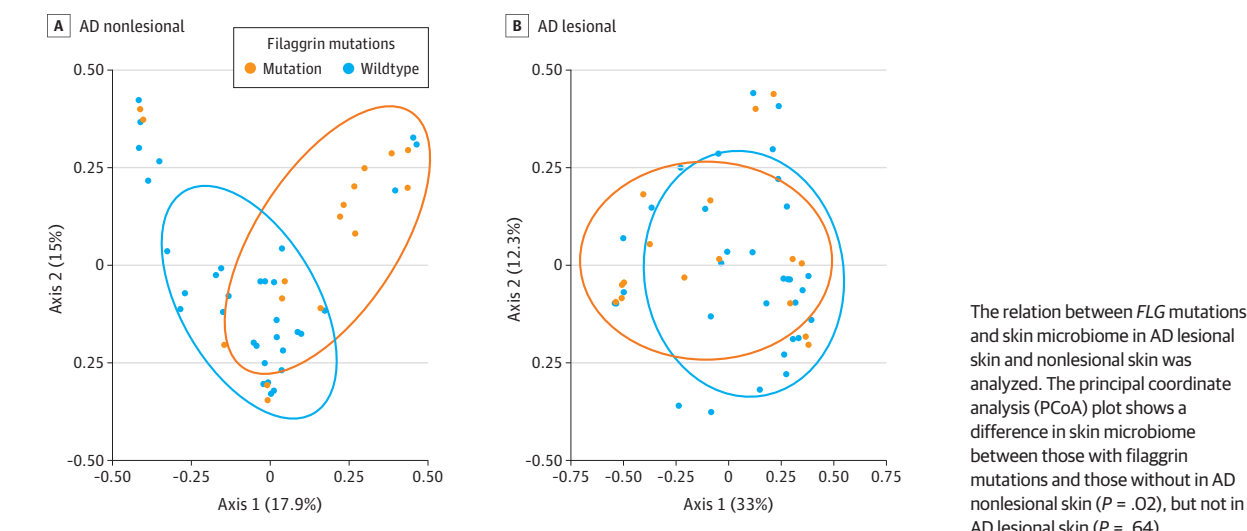
Importantly, nasal samples from AD patients revealed lower bacterial diversity than nasal samples from healthy controls. This could indicate intrapersonal transfer of bacteria from skin to nose, with skin bacteria dominating nasal colonisation; however, it also supports the finding of a microbiome in AD patients distinctly different from healthy controls, regardless of location.

Recent years' focus on the human microbiota and its relevance for health and disease, including development of our immune system, has encouraged the interest in the microbiota's role in inflammatory skin diseases like AD. Studies showing reduced microbial diversity in the gut before development of atopic diseases^{32,33} and findings that early skin colonisation during infancy with commensal bacteria may act as a protective factor for development of AD³⁴ suggest a close link to the immunological development in connection with AD.

Limitations

A limitation to this study is the use of topical treatment for most patients, which may have affected the microbiome³; however, our cohort reflects the typical skin environment of most patients with AD. Samples from both dry and moist skin in patients with AD were included, and though we found no effect of skin type on the microbiome, skin type has previously been shown to influence microbiome composition.³⁵ However, it could be that skin type has less of an influence on the microbiome in patients with AD owing to more influence from immunological parameters. Sample size was large enough to present robust microbiome data, although too small to conclude on any influence from specific treatments. We find a relationship between skin microbiome composition and *FLG* mutations; however, these patients were predominantly heterozygote, hence we cannot comment on any possible effect of homozygosity/heterozygosity. Because nasal swabs were taken at the transitional mucosae, the lack of filaggrin in nasal mucosa³⁶ might explain why no association between *FLG* and nasal microbiome was found.

Figure 4. Skin Microbiome in Relation to FLG Mutations



Conclusions

Knowledge of the association between the microbiota and inflammatory skin diseases, and the interplay between host and microbe is important for a better understanding of the role of microbes in skin diseases.

We believe this is the first study reporting a link between microbiome composition and *FLG* mutations in adult patients with AD. More research into the functional mechanisms of the microbiome will help us understand if microbes can cause or alter skin diseases like AD, and how modulation of the microbiome may have a role in new treatment strategies.

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Study concept and design: Clausen, Agner, Edslev, Andersen.

Acquisition, analysis, or interpretation of data:

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Drafting of the manuscript: Clausen, Agner, Edslev, Andersen.

Critical revision of the manuscript for important intellectual content: All authors.

Statistical analysis: Lilje, Edslev, Johannesen.

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