ABSTRACT

Purpose: We aimed to investigate the effects of dupilumab on 1) the permeability and antimicrobial barrier, 2) the composition of the skin microbiome, and 3) the correlation between changes in skin barrier properties and microbiota in atopic dermatitis (AD) patients.

Methods: Ten patients with severe AD were treated with dupilumab for 12 weeks. Disease severity was assessed using the Eczema Area and Severity Index (EASI). Skin barrier function was evaluated by measuring transepidermal water loss, stratum corneum (SC) hydration, and pH. The following parameters were analyzed in the pre- and post-treatment SC samples; 1) skin microbiota using 16S rRNA gene sequencing, 2) lipid composition using mass spectrometry, and 3) human \( \beta \)-defensin 2 (hBD-2) expression using quantitative reverse transcription polymerase chain reaction.

Results: SC hydration levels in the lesional and non-lesional skin increased after 12-week dupilumab therapy (24.2%, \( P < 0.001 \) and 59.9%, \( P < 0.001 \), respectively, vs. baseline) and correlated with EASI improvement (\( r = 0.90, \ P < 0.001 \) and \( r = 0.85, \ P = 0.003 \), respectively). Dupilumab increased the long-chain ceramide levels in atopic skin (118.4%, \( P = 0.028 \) vs. baseline) that correlated with changes in SC hydration (\( r = 0.81, \ P = 0.007 \)) and reduced the elevated hBD-2 messenger RNA levels (−15.4%, \( P = 0.005 \) vs. baseline) in the lesional skin. Dupilumab decreased the abundance of Staphylococcus aureus. In contrast, the microbial diversity and the abundance of Cutibacterium and Corynebacterium species increased, which were correlated with an increase in SC hydration levels (Shannon diversity, \( r = 0.71, \ P = 0.027 \); Cutibacterium, \( r = 0.73, \ P = 0.017 \); Corynebacterium, \( r = 0.75, \ P = 0.012 \)). Increased abundance of Cutibacterium species was also correlated with EASI improvement (\( r = 0.68, \ P = 0.032 \)).

Conclusions: Th2 blockade-induced normalization of skin microbiome in AD patients is associated with increased SC hydration.

Keywords: Atopic dermatitis; dupilumab; stratum corneum hydration; microbiota; ceramides; Staphylococcus aureus; Cutibacterium
INTRODUCTION

Impaired skin barrier function, strong type 2 inflammation, and microbial dysbiosis are hallmarks of atopic dermatitis (AD) and their mutual interactions contribute to the onset and progression of disease.1

Dupilumab, a monoclonal antibody that selectively inhibits interleukin (IL)-4 receptor-α (IL-4Rα) demonstrates clinical improvements in moderate-to-severe AD patients. Inhibition of the IL-4/IL-13 signaling targets pathways in both immune cells and epithelial cells that are related to the pathogenesis of AD. Indeed, dupilumab has been shown to reduce epidermal hyperplasia and upregulate the expression of epidermal differentiation- and lipid metabolism-related genes in atopic skin.2 Moreover, recent studies reported a rapid reduction in transepidermal water loss (TEWL) in the lesional skin after dupilumab treatment3,4 and its correlation with Th2 biomarker thymus and activation-regulated chemokine.3 Despite the improvement in dryness in AD patients who received dupilumab treatment clinically, previous studies have reported that dupilumab does not increase skin hydration.3,4

In addition to improving inflammatory and barrier impairments, a recent study reported that IL-4Rα blockade increases microbial diversity and decreases Staphylococcus aureus colonization in the skin of patients with AD.5 This amelioration of cutaneous dysbiosis has been shown to correlate with clinical improvement. A systematic review and meta-analysis of 8 studies also demonstrated an association of dupilumab treatment with a decreased incidence of skin infections and eczema herpeticum in moderate-to-severe AD patients.5 However, it is unclear how IL-4/IL-13 blockade results in normalization of dysbiosis and reduction of S. aureus in patients with AD.

IL-4 and IL-13 promote S. aureus adhesion by enhancing fibronectin and fibrinogen synthesis6 and inhibit antimicrobial peptide (AMP) production by keratinocytes in vitro.8,9 These findings suggest that Th2 cytokine blockade might reduce S. aureus colonization via modulating fibronectin and AMPs production by the skin. However, despite the induction of AMPs in the lesional atopic skin, S. aureus is highly colonized, suggesting that altered AMPs expression cannot fully explain S. aureus colonization on the skin of AD patients.

Recent evidence indicates that permeability barrier properties also impact the cutaneous microbiome.10,11 A mouse model for cutaneous colonization demonstrated that skin barrier defects facilitate S. aureus skin colonization and persistence.12 Filaggrin-deficient skin also showed decreased bacterial diversity and commensal bacteria, and increased Firmicutes abundance, resembling the AD-related pattern.13,14 Moreover, epidermal lipid composition has been shown to correlate with bacterial composition.15 These findings support the hypothesis that the defective skin barrier promotes colonization of S. aureus and dupilumab improves skin barrier functions by inhibiting Th2 inflammation, thereby reducing the abundance of S. aureus. To verify this hypothesis, it is necessary to investigate the correlation between the improvement of skin barrier properties and microbial dysbiosis during dupilumab treatment, but there are few reports investigating their interrelationship.

In this study, we investigated the effects IL-4Rα blockade by dupilumab on the epidermal barrier function, stratum corneum (SC) lipid composition, AMP expression, and cutaneous microbiome in patients with AD. We also examined the correlation between changes in skin barrier properties and microbial composition during dupilumab treatment.

Disclosure

There are no financial or other issues that might lead to conflict of interest.
MATERIALS AND METHODS

Study design and sample collection
All methods were approved by the Institutional Review Board (IRB No.3-2018-0270) of Yonsei University College of Medicine and were performed in compliance with the Declaration of Helsinki and Good Clinical Practice. Prior to inclusion, written informed consents were obtained from all participants. Ten healthy subjects (mean age, 28.3 years) and ten patients with severe AD (mean age, 34.2 years and mean Eczema Area and Severity Index [EASI] score, 33.3) were enrolled. All patients initially received 600 mg of dupilumab followed by 300 mg every other week. Skin barrier function was measured and SC samples were obtained at baseline and after 12 weeks of treatment. Subjects were instructed to avoid topical medication and moisturizer application 24 hours prior to sampling. For SC sampling, a total of 25 consecutive D-squame tape strips (22 mm diameter; CuDerm, Dallas, TX, USA) were collected from the lesional skin of the antecubital fossa. The non-lesional skin samples were obtained from the upper inner arm of the same side. Tape strips were stored at −80°C until use. The first two tape strips were discarded. Five strips were used to extract DNA for 16S sequencing (tape strips 3–7), five strips were used for lipidomic analysis (tape strips 8–12), and ten strips (tape strips 13–22) were used to extract RNA for real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Measurement of skin barrier function
Basal TEWL, SC hydration and skin surface pH were evaluated at baseline and week 12. Basal TEWL was measured with a Tewameter (TM210 apparatus; Courage and Khazaka, Cologne, Germany). SC hydration was assessed via capacitance with a Corneometer (CM820 device; Courage and Khazaka). Skin surface pH was measured using a pH meter (PH 905; Courage and Khazaka). All measurements were performed by the same trained physician in the same room at 20°C-22°C and 20%-40% humidity conditions after 15-30 minutes acclimatization.

DNA extraction and 16S rRNA gene sequencing
SC samples were incubated in a lytic enzymatic mixture of lysozyme, mutanolysin, proteinase K, and lysostaphin along with 0.1-mm sterile silica beads to homogenize the samples. DNA cleanup was performed with a fecal DNA extraction kit (Zymogen, Irvine, CA, USA). The V3V4 variable region of the 16S rRNA gene was PCR-amplified from purified DNA and sequenced using the Illumina HiSeq 250-bp paired-reads platform (Illumina Inc., Cambridge, UK) as previously reported.17

Metagenomic data analysis
The raw FASTQ files for all samples were demultiplexed based on the barcode. Low-quality, merged, and chimeric reads were excluded. The trimmed reads were then merged using FLASH v1.2.11 with default parameters. The remainder of the reads (≤97%) were clustered de novo into operational taxonomic units (OTUs; using a CD-HIT-EST-based OTU analysis program [CD-HIT-OTU]),18 followed by taxonomical classification using the BLASTN v2.4.0 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).19 Only a top BLAST hit with alignment that spanned >85% of the original query sequence was assigned. The OTU taxonomies from phylum to species were determined based on the NCBI database. QIIME v1.9 was used to analyze 3.33 million 16S rRNA gene reads, resulting in the identification of 17,725 OTUs at a 97% identity level.20 After the removal of rare OTUs, 2,186 OTUs were analyzed. The microbial Shannon diversity index was computed in QIIME v1.9 through the whole tree phylogenetic diversity
Bacterial community profiles at each taxonomic level, from phylum to species, were compared using the MetaStats 2.0 package.

**Quantification of ceramide N-lignoceroyl-D-erythro-sphingosine (NS)**

Human skin tissues collected by the D-squame tape were lysed in RIPA buffer followed by extraction of sphingolipids, as we reported previously. The extracted lipids dried using a vacuum system (Vision, Seoul, Korea) were re-dissolved in methanol and analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS, API 3200 QTRAP mass; AB/SCIEX, Framingham, MA, USA) by selective ion monitoring mode. The ceramide MS/MS transitions (m/z) were 510 → 264 for C14-ceramide, 538 → 264 for C16-ceramide, 552 → 264 for C17-ceramide, 566 → 264 for C18-ceramide, 594 → 264 for C20-ceramide, 648 → 264 for C24:1-ceramide, 676 → 264 for C26:1-ceramide, and 678 → 264 for C26-ceramide respectively. All data were acquired using Analyst 1.5.1 software (Applied Biosystems, Foster City, CA, USA).

**Real-time qRT-PCR**

Collected tape strips 13-22 from human skin were sequentially scraped into the RLT buffer (Qiagen, Mississauga, Canada) on the day of collection and frozen at −80°C. RNA was isolated from skin tape strips using RNeasy Micro Kits (Qiagen) according to the manufacturer’s protocol. After quantification of RNA with a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA), complementary DNA (cDNA) was synthesized using the cDNA Synthesis kit (Thermo Fisher Scientific). TaqMan real-time PCR assays (Applied Biosystems) were performed to analyze the messenger RNA (mRNA) levels. A TaqMan probe for hBD-2 (DEFB4A) (Thermo Fisher Scientific) was used. The relative gene expression was normalized to the mean level of glyceraldehyde 3-phosphate dehydrogenase mRNA (Thermo Fisher Scientific).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A paired *t*-test was used for analysis of qRT-PCR data. Mann-Whitney or Wilcoxon rank-sum test were used for analysis of barrier parameters and to determine differences in microbial alpha diversity and abundance of individual taxa between pre- and post-treatment skin. We calculated the Spearman correlation of percentage change from baseline to week 12 in EASI scores or C26 ceramide amounts with percentage change from baseline in SC hydration levels at week 12 and evaluated the correlation of percentage change from baseline to week 12 in EASI scores or SC hydration levels with change in Shannon diversity index or the relative abundance of individual bacterial taxa from baseline to week 12 by using Spearman correlation coefficients. *P* < 0.05 was considered significant.

**RESULTS**

**Dupilumab increases SC hydration and long-chain ceramide (C26) levels in patients with AD**

We first assessed the effect of dupilumab on epidermal permeability barrier function. SC hydration levels in both lesional and non-lesional skin of patients with AD significantly increased from baseline to week 12 (24.2%, *P* < 0.001 and 59.9%, *P* < 0.001, respectively, vs. baseline) (**Fig. 1A**). Decreases in basal TEWL was observed in the lesional (~26%, *P* = 0.006, vs. baseline) but not in the non-lesional skin by week 12. Skin surface pH in the lesional and the non-lesional skin did not significantly change from baseline at week 12 (**Fig. 1A**). We also
analyzed changes in SC ceramide subclasses in dupilumab-treated patients with AD using high-performance LC-ESI-MS/MS. Remarkably, the amount of C26 in the lesional and the non-lesional skin significantly increased at week 12 (118.4%, $P = 0.029$ and 25%, $P = 0.043$, respectively, vs. baseline) (Fig. 1D).

We then evaluated whether changes in SC hydration and C26 at week 12 correlate with the clinical response to dupilumab. The mean EASI score decreased by 72.5% at week 12 ($P < 0.001$, vs. baseline) (Fig. 1B). Percentage change in SC hydration levels in both lesional ($r = 0.9$, $P < 0.001$) and non-lesional ($r = 0.851$, $P = 0.003$) skin to week 12 was highly correlated with percentage change in EASI scores from baseline to week 12 (Fig. 1C). However, the percentage changes in basal TEWL in the lesional skin and C26 ceramide amounts in the lesional and the non-lesional skin from baseline to week 12 were not significantly correlated.
with the EASI reduction. Percentage change in C26 ceramide amounts from baseline to week 12 was significantly correlated with percentage change in SC hydration levels from baseline to week 12 in the lesional skin ($r = 0.806, P = 0.007$) (Fig. 1E).

**Human β-defensin 2 (hBD-2) mRNA levels in SC are upregulated in the lesional AD skin and decreased after dupilumab treatment**

hBD-2 mRNA expression levels in the lesional and the non-lesional SC samples of patients with AD were quantified by using real-time qRT-PCR before and after dupilumab treatment. At baseline, the mRNA expression levels of hBD-2 were higher in the lesional skin than in the non-lesional skin and the normal skin of healthy controls (HC) (fold change 1.32 vs. non-lesional skin and fold change 1.56 vs. HC, all $P < 0.05$). After treatment, hBD-2 levels in the lesional skin decreased from baseline to week 12 ($-15.4\%, P = 0.005$), while hBD-2 levels in the non-lesional skin showed no significant change (Fig. 2).

**Dupilumab alters the microbiota composition in atopic skin**

We next investigated dupilumab-induced changes in cutaneous microbiome profiles. Tape strips were collected from the lesional and the non-lesional skin from patients with AD before and after 12 weeks of dupilumab treatment. At baseline, the lesional and the non-lesional skin showed a reduced microbial diversity (alpha diversity), assessed by Shannon diversity index (lesional skin, 2.9; non-lesional skin, 3.34; HC, 4.49; $P = 0.008$ and 0.01 for the lesional skin vs. HC and the non-lesional skin vs. HC, respectively). Dupilumab significantly increased bacterial diversity in both lesional (baseline, 2.9 vs. week 12, 4.4, $P = 0.005$) and non-lesional (baseline, 3.34 vs. week 12, 4.6, $P = 0.004$) skin at week 12 to levels comparable with those of the HC (Fig. 3).

We then examined the changes in relative abundance of individual bacterial taxa at the phylum and genus levels following treatment. The abundance of *Staphylococcus* spp. was higher in the lesional and the non-lesional skin, while that of certain skin commensals,
including *Cutibacterium* spp. and *Lactobacillus* spp. was lower in the lesional skin than in the healthy skin at baseline (Fig. 4A). The relative abundance of *S. aureus* significantly decreased from baseline to week 12 in the lesional (33.4% vs. 13.3%, *P* = 0.049) and the non-lesional (29.7% vs. 10.3%, *P* = 0.016) skin following treatment (Fig. 4B and C). Furthermore, significant increases in the relative abundance of *Lactobacillus* spp. (0.08% vs. 0.59%, *P* = 0.003) and major skin commensals including *Cutibacterium* spp. (1.1% vs. 3.58%, *P* = 0.005) and *Corynebacterium* spp. (5.9% vs. 11.6%, *P* = 0.005) were seen at week 12 in the lesional skin (Fig. 4B).

**Increases in SC hydration correlate with increases in the microbiota diversity and proportion of major commensal microbes following dupilumab treatment**

Then, we assessed the correlation between changes in microbial composition, barrier properties, and disease severity from baseline to week 12 (Fig. 5A). An increase in the Shannon diversity index and decrease in the relative abundance of *S. aureus* in the lesional skin were not significantly correlated with EASI improvement. Instead, the increased relative abundance of *Cutibacterium* spp. to week 12 was significantly correlated with the decline in EASI score following treatment (r = 0.677, *P* = 0.032) (Fig. 5B).

Remarkably, we observed a significant positive correlation between the change in SC hydration levels and the increase in Shannon diversity index from baseline to week 12 in the lesional skin (r = 0.709, *P* = 0.027). An increase in SC hydration levels from baseline to week 12 was also significantly correlated with the increase in the relative abundance of *Cutibacterium* spp. (r = 0.729, *P* = 0.017) and *Corynebacterium* spp. (r = 0.754, *P* = 0.012) to week 12 in the lesional skin (Fig. 5C). However, there was no significant correlation between changes in *S. aureus* abundance and SC hydration. Moreover, the increase in the relative abundance of *Cutibacterium* spp. (r = 0.746, *P* = 0.017) and *Corynebacterium* spp. (r = 0.770, *P* = 0.013) in the lesional skin from baseline to week 12 was significantly correlated with an increase in Shannon diversity index (Fig. 5D).

There was no significant correlation between change in the amounts of C26 ceramide with either change in bacterial diversity or relative abundance of individual taxa following treatment.

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**Fig. 3.** Changes in microbiota α-diversity (Shannon diversity index) in atopic lesional and non-lesional skin before and after 12 weeks of dupilumab treatment. Data are expressed as mean ± standard error of mean. HC, healthy control; L, lesional; NL, non-lesional; AD, atopic dermatitis. *P* < 0.05; †*P* < 0.01.
DISCUSSION

We demonstrated that IL-4/IL-13 blockade with dupilumab increased SC hydration in both lesional and non-lesional skin in patients with AD. Moreover, this increase in SC hydration levels was significantly correlated with improvement in disease severity. At the lesional skin, dupilumab also reduced TEWL at week 12, however, there was no significant change of skin surface pH from baseline. These findings suggest that SC hydration is an important biophysical parameter of skin barrier function to predict or monitor the response to anti-IL-4/IL-13 therapy in patients with AD. Decreased levels of the filaggrin breakdown products and reduced production of ceramides in SC cause dry skin in patients with AD.23-25 Notably, dupilumab increased C26 content in atopic skin, in line with a previous report showing that dupilumab upregulates ELOVL3 gene expression.22 In addition, change in C26 contents at
SC Hydration and Microbial Changes After Dupilumab

Fig. 5. Correlation between changes in EASI, SC hydration, bacterial diversity and relative abundance of major taxa following dupilumab treatment. (A) Heat map of the overall correlation matrix. Colors display the strength of correlation based on Spearman's correlation coefficient in each pair of biomarkers. Significant correlations (corrected $P < 0.05$) are indicated with yellow boxes. (B) Correlation between the percentage change in EASI scores from baseline to week 12 and changes in Shannon diversity index, relative abundance (%) of S. aureus, and relative abundance (%) of Cutibacterium in lesional skin following 12 weeks of dupilumab treatment. (C) Correlation between the percentage change in SC hydration levels from baseline to week 12 and changes in Shannon diversity index, relative abundance (%) of Cutibacterium and Corynebacterium in lesional skin following 12 weeks of dupilumab treatment. The linear regression curve fit, Spearman’s correlation coefficient $r$, and $P$ values are shown for each graph. EASI, eczema area and severity index; SC, stratum corneum; AD, atopic dermatitis.
week 12 significantly correlated with change in SC hydration levels at week 12 in the lesional skin. These findings suggest that the increased level of C26 is a potential mechanism by which dupilumab increases SC hydration. Importantly, dupilumab increased SC hydration and long-chain ceramide content not only in the lesional skin, but also in the non-lesional skin, suggesting that Th2 blockade can prevent the recurrence or progression of AD by enhancing permeability barrier function in the non-lesional skin.

While our results are consistent with previous data demonstrating a reduction of TEWL following dupilumab treatment in the lesional skin,3,4 our findings differ from a recent study by Furuhashi et al.4 in that the SC hydration was transiently increased at head and neck area within week 14, but was not increased across diverse body sites during 24 weeks of dupilumab treatment in Japanese patients with AD. This difference may be partially due to the fact that our study measured barrier function only at the initial time point (week 12). In addition, barrier impairment in AD patients is related to inherited FLG mutations as well as Th2 inflammation. The frequency of FLG mutations in Koreans (14%-16%) with AD is lower than that in other Asian countries (up to 27%) and Europe (up to 50%),26,27 suggesting that barrier defects in Korean AD patients are more likely to be caused by Th2 inflammation rather than inherited FLG mutations. Therefore, improvements of barrier defects by Th2 blockade are more likely to occur in Korean AD patients than in other populations with a high frequency of FLG mutations. To clarify these issues, a long-term study of more patients, along with information on the FLG mutation is needed.

Cutaneous dysbiosis that is characterized by the colonization of S. aureus and a loss of other commensal microbes such as Staphylococcus epidermidis and Corynebacterium spp. affects the onset and progression of AD, and vice versa.28,29 We observed a decreased colonization of S. aureus and increased bacterial diversity in both lesional and non-lesional skin of dupilumab-treated AD patients, as reported previously.5 This normalization of cutaneous dysbiosis following IL-4/IL-13 blockade may result from several mechanisms: alteration in epidermal permeability or antimicrobial barrier or direct effect of Th2 blockade on the S. aureus adhesion. To understand how Th2 blockade decreases S. aureus colonization, we assessed change in hBD-2 expression in dupilumab-treated skin of patients with AD. Consistent with previous studies,10-13 SC in the lesional skin had higher hBD-2 levels than the non-lesional and the control skin. After treatment, a significant reduction in hBD-2 levels was observed in the lesional skin. This result is inconsistent with the previous findings that IL-4 and IL-13 inhibit hBD-2 upregulation in TNF-α- and IFN-γ-stimulated keratinocytes in vitro.34 However, the regulation of hBD-2 in atopic skin in vivo is more complex because IL-17 and IL-22, the potent AMP inducers in keratinocytes, are also increased in atopic skin.36-37 Moreover, a previous study reported that dupilumab downregulated Th17- and Th22-regulated genes in the lesional atopic skin.2 Together, our data suggest that elevated hBD-2 levels in the lesional SC result from atopic skin inflammation, and the reduction in hBD-2 by dupilumab is possibly due to its anti-inflammatory effects beyond the known Th2-driven inflammation. These findings also suggest that the dupilumab-induced decrease in S. aureus colonization in the lesional skin is not due to upregulation of hBD-2, one of the most efficient AMPs.

Instead, we found an increase in the relative abundance of major skin commensals in dupilumab-treated lesional skin, which has not been observed in previous studies. Remarkably, an increase in Cutibacterium abundance was strongly correlated with clinical improvement. Growing evidence suggests that commensal bacteria can inhibit pathogenic
S. aureus by producing antimicrobial substances or activating host defense mechanisms.\textsuperscript{11,38-41} Cutibacterium spp. produce thiopeptides that are homologous to berninamycin and have antibacterial activity against Staphylococcus.\textsuperscript{42} Certain strains of Corynebacterium spp. release free fatty acids with antibacterial activity from the skin surface lipids.\textsuperscript{43} Moreover, S. aureus was inversely correlated with the abundance of Cutibacterium spp. and Corynebacterium spp. in atopic skin.\textsuperscript{44-46} Additionally, dupilumab elevated the Lactobacillus spp. abundance in this study. The probiotic Lactobacillus spp. regulates the gut microbial population and function. Despite its low abundance in the skin, a recent study showed that Lactobacillus spp. inhibits S. aureus growth \textit{in vitro} and topical application of live Lactobacilli on the skin of patients with acne reduces \textit{Staphylococcus} colonization.\textsuperscript{47} These findings suggest that increased abundance of the commensals is a possible mechanism contributing to the observed decrease in S. aureus abundance following IL-4/IL-13 blockade. Although we did not observe a negative correlation between dupilumab-induced changes in the abundance of S. aureus and these commensals, increases in Cutibacterium spp. and Corynebacterium spp. were significantly correlated with increased bacterial diversity. Whether and how these commensals control S. aureus colonization \textit{in vivo} needs to be analyzed further.

We also found that increases in Shannon diversity and the relative abundance of Cutibacterium spp. and Corynebacterium spp. were highly correlated with increases in SC hydration. These findings suggest that dupilumab-induced increase in SC hydration levels affect bacterial composition, leading to increased abundance of major commensals and bacterial diversity. However, the significant correlations between SC hydration and improvements of skin dysbiosis could be results of improvements of skin Th2 inflammation by dupilumab treatment (epiphenomenon). The possibility of a causal relationship between changes in SC hydration and microbiome composition should be explored in future studies.

Unlike change in SC hydration levels, change in C26 contents did not significantly correlate with change in microbiome configuration. However, we found a significant correlation between the change in C26 ceramides with the change in SC hydration levels during dupilumab treatment, suggesting that the increase of C26 by Th2 blockade is associated with the increase in SC hydration, but the increase of C26 ceramide alone could not directly affect the improvement of dysbiosis. Epidermal lipid composition is known to affect bacterial community configuration,\textsuperscript{48} but it is presumed that lipid components other than long-chain ceramides are also involved in microbiome configuration in a complex manner. Further studies with more subjects are needed to more accurately understand the relationship between changes in lipid profile and changes in the microbiome following dupilumab treatment.

The limitation of our study is the small sample size. Nevertheless, we could identify significant improvement in skin barrier properties, microbiome composition, and a significant correlation between changes in SC hydration and microbiota following dupilumab treatment. Another potential limitation of our study was the lack of age- and sex-matched placebo control group in AD patients when evaluating changes in skin microbiome by dupilumab. Longitudinal data from AD patients receiving dupilumab and placebo would provide more accurate characterization of the changes in skin microbiota associated with dupilumab treatment. Finally, we were unable to analyze various AMPs other than hBD-2 with potent antimicrobial properties in relation to S. aureus because the SC samples were not sufficient for analysis. It would be valuable to analyze other AMPs such as hBD-3 and cathelicidin in patients with AD during dupilumab treatment.
In conclusion, IL-4Rα blockade increases SC hydration levels and long-chain ceramides in the lesional and the non-lesional skin of AD patients. We also found a decreased abundance of *S. aureus*, while increases in skin commensals in dupilumab-treated atopic skin. Our correlation data suggest that dupilumab-induced improvement in dysbiosis with decreased *S. aureus* colonization is associated with increased SC hydration and proportions of major skin commensals, but not with changes in hBD-2 production. Further research is needed on the causal relationship between changes in SC hydration and microbiome composition and the effects of increased skin commensal populations (*Cutibacterium* and *Corynebacterium* spp.) on improving AD during dupilumab treatment.

**ACKNOWLEDGMENTS**

We appreciate the patients for granting permission to publish this information.

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