16S rRNA gene profiling: Direct and indirect applications for clinical microbiology

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CHAPTER 6

Inter- and intra-patient variability over time of lesional skin microbiota in patients with atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is a common chronic, inflammatory skin disorder associated with *Staphylococcus aureus* colonization and reduced microbiota diversity. The current standard for evaluating the effect of treatment on the skin microbiota is by comparing its composition before and after treatment. The aim of the current evaluation was to determine whether limited sampling is sufficient to capture the full extent of variability in the skin microbiota. To analyze inter- and intra-patient variability of the skin microbiota of 20 patients with mild to moderate AD over a period of 42 days, the coefficient of variation (CoV) was calculated for microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration. The inter-patient variability of microbial diversity was high for lesional skin compared to non-lesional skin (CoVs of 35.5-45.9% vs 16.3-28.0%). For the other test results, high CoVs, in the range 45.3-94.1%, were found for lesional skin. furthermore, a wide range of intra-patient variability was observed for lesional skin compared to non-lesional skin (CoVs of 7.1-173% vs 3.5-29.3%). Based on these data, 3 groups with significantly different microbiological phenotypes were defined. In conclusion, lesional skin microbiota is associated with a large inter- and intra-patient variability. A high sample frequency, e.g. once weekly, yields excellent time-dependent insight into the changes of the variable skin microbiota, which can be used to determine the treatment effect on the lesional skin microbiota in clinical trials.

INTRODUCTION

Atopic dermatitis (AD) is a chronic, inflammatory skin disorder characterized by periodic flares of dry, red itchy skin lesions. AD is a very common skin disorder in developed countries with a prevalence of approximately 20% in children and 3% in adults (1). The pathophysiology of AD is complex and still only partially understood. Current evidence strongly points to a disruption of the skin barrier and subsequent immune dysregulation as the primary pathological drivers in AD (2). The microbiome of the skin is important in maintaining immune homeostasis and preventing the skin from being colonized by pathogens, such as *Staphylococcus aureus*. Approximately 90% of the patients suffering from AD are colonized by *S. aureus* and the relative abundance of *S. aureus* increases during an AD flare due to a reduction in the relative abundance of colonizers of the skin (3, 4). *S. aureus* can produce several molecules with potential to cause inflammation and to promote further immune dysregulation (2). Moreover, the increase in relative abundance of *S. aureus* and the reduction of the microbial diversity of the skin seem to be linked to the severity of the disease, promoting the skin microbiota as a potential biomarker in AD (5). Nonetheless, the potential usefulness of this as an AD biomarker has yet to be defined.

Treatments of AD involve emollients and topical anti-inflammatory corticosteroids. There are limitations to the use of steroids, because of possible skin atrophy and systemic side-effects as well as limited patient tolerance after long-term usage. Currently, more specific treatments are being developed (6, 7). The effects of new treatments are increasingly evaluated using subjective clinical AD scores and the microbiota composition of lesional skin before and after treatment (8-11). The design of the majority of these studies includes the collection of a single sample before and after treatment.

However, healthy skin of each human has a specific microbial 'fingerprint', which depends on the physical and chemical features of the skin as well as on host and environmental factors, including colonization at birth, antibiotic exposure, hygiene, lifestyle, and geographic location (12, 13). The level of variation depends on the topographical diversity of the skin as well as on individual factors (14-16). Lesional skin may also be characterized by large inter- and intra-patient variability of the skin microbiota, implying the need for frequent sampling when the effect of a treatment on the lesional skin microbiota is being investigated. However, data of longitudinal studies analyzing the inter- and intra-patient variability of lesional skin microbiota is lacking.

The aim of the current evaluation was to analyze inter- and intra-patient variability of the skin microbiota of patients with AD over time to determine whether limited sampling is sufficient to capture the full extent of variability in the skin microbiota.

MATERIALS AND METHODS

Source of samples and associated data

Microbiological test results of skin swabs, along with selected clinical data from the placebo group of 2 randomized, double-blind, placebo-controlled mono-centre phase 2 clinical trials conducted at the Centre for Human Drug Research (Leiden, The Netherlands) between June 2015 and December 2017, were used in this evaluation. Both clinical trials were approved by the independent Medical Ethics Committee ('Evaluation of Ethics in Biomedical Research', Assen, The Netherlands) and were designed to assess the pharmacodynamics of omiganan in patients with mild to moderate AD. The Declaration of Helsinki was the guiding principle for trial execution. Written informed consent was obtained from all patients.

Data from 250 samples obtained in the initial clinical trial (ClinicalTrials.gov: NCT03091426) were used to determine the variability of the skin microbiota. In this trial, the placebo group (n = 20) consisted of 11 (55%) females and 9 (45%) males with a mean ± standard deviation age of 24 ± 5 years and clinical AD score (objective-scoring atopic dermatitis: oSCORAD) of 21.1 ± 5.6. Briefly, each patient administered the vehicle gel (hydroxyethyl cellulose, sodium benzoate, glycerin, purified water) without the active compound twice daily for 28 consecutive days on all AD lesions. At the start of this treatment period (Day 0), the severity of the lesional skin was assessed clinically. Two skin swabs were collected for bacterial culture and molecular methods using an ESwab and a sterile cotton swab (Puritan, Guilford, ME, USA), respectively. Swabs were dipped in a NaCl-Tween solution, before rubbing the tip of the swab firmly over 4 cm^2 of the target lesion for 5 times. Hereafter the swab material was placed in a vial containing 1 mL NaCl-Tween solution. The skin swabs were obtained from a predefined part of an AD lesion (preferably the antecubital fossa) and from a predefined part of non-lesional skin (preferably the contralateral site). Both clinical assessment and sample collection were repeated each week during a period of 42 days. During the treatment period, patients were allowed to use bland emollients (unguentum leniens) as maintenance therapy. The patients were not allowed to wash the selected sites 6 h prior to the clinical assessment and sample collection and had to avoid prolonged exposure of their involved skin to sunlight during the complete study period. Incomplete datasets or data of samples obtained after concomitant use of corticosteroids were excluded from the analysis.

Data from 76 skin swabs obtained in a separate clinical trial with a comparable study population (ClinicalTrials.gov: NCT02456480) were used for verification purposes. In this trial, the placebo group (n = 12) consisted of 8 (67%) females and 4 (33%) males with an age of 25 ± 11 years and clinical AD score of 19.0 ± 7.4. This clinical trial differed in study design as: (i) the vehicle gel without the active compound was administered once daily on only the predefined AD lesion on the antecubital fossa; (ii) only lesional skin was sampled each week; (iii) clinical assessment of lesional skin was not measured at day 35 and 42; and (iv) bacterial culture was not performed.

Clinical assessment of lesional skin

The severity of the lesional skin was assessed clinically based on the oSCORAD system, calculated as: A/5+7B/2 (17). 'A' in the calculation was defined as the extent of AD, which was assessed as a percentage of each defined body area and reported as the sum of all areas, with a maximum score of 100%. 'B' in the calculation was defined as the severity of 6 specific symptoms of AD (erythema, excoriation, swelling, oozing/crusting, lichenification and dryness), which were scored 0-3 and reported as the sum of all symptoms, with a maximum score of 18. A total score of 0-7.9 was categorized as clear skin, 8.0-23.9 as mild AD, 24.0-37.9 as moderate AD, and 38.0-83.0 as severe AD.

Bacterial culture

Skin swabs were inoculated on blood agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and incubated at 35 °C in a 5% CO_2 incubator for 24 h. Species identification was performed by MALDI-TOF (Bruker Corp., Billerica, MA, USA) and colony-forming units (CFU) were calculated for *S. aureus* after dilution if necessary.

DNA extraction

Each skin swab was diluted by addition of 50 µL 10x phosphate-buffered saline (PBS) to 450 µL swab in NaCL-Tween solution. DNA was extracted and eluted in a final volume of 100 µL with the MagNA Pure 96 instrument using the MagNA Pure 96 DNA and Viral NA Large Volume Kit and the Pathogen universal 500 protocol (Roche Diagnostics, Meylan, France).

Microbiota analysis

Microbiota analysis was performed as described elsewhere (18). Briefly, a fragment of approximately 464 bp of the V3–V4 regions of the 16S ribosomal RNA (rRNA) gene was amplified and sequenced with the MiSeq desktop sequencer (Illumina, San Diego, CA, USA). Sequencing data was processed using the QIIME pipeline and a pre-clustered version of the Augustus 2013 GreenGenes database. High-quality sequences (> 100 bp in length; quality score > 20) were clustered into operational taxonomic units (OTUs) using an open reference-based approach that implements reference-based clustering following by *de novo* clustering at a 97% similarity level. No low abundance filtering was used. For the bar charts, a limited number of genera were selected, representing the microbiota composition of each sample. Only genera with a relative abundance ≥ 1% of the total reads were included. The remaining genera formed the other genera category.

Quantitative real-time PCRs

S. aureus was detected by quantitative real-time PCRs (qPCRs) aimed at the *nuc* gene, using primers and a probe described elsewhere (19). The total bacterial DNA load (16S rRNA gene) was established using a primer set (Fw 5'-CGAAAGCGTGGGGAGCAAA-3', Rv1

5'-CCGTACTCCCCAGGCGG-3' and Rv2 5'-GTCGTACTCCCCAGGCGG-3') based on Bogaert et al. (20) and 20x EVA green (Biotium, Inc., Fremont, CA, USA). Both qPCRs were carried out in a total volume of 10 µL, containing 5 µL (2x) LC480 Probes Master mix (Roche) and 2 µL of extracted DNA. Amplification reactions were performed using a LightCycler 480 II Instrument (Roche) under the following conditions: 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 50 s and 72 °C for 1 s (*nuc* gene) or 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 sec and 72 °C for 1 s (16S rRNA gene). For quantification, a 10-fold dilution series of a plasmid was included in each run and the second derivative analysis method was used for data analysis.

The total load of human DNA (RNaseP gene) was determined using primers and a probe described elsewhere (21). Each qPCR was carried out in a total volume of 25 µL, containing 12.5 µL (2x) IQ Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) and 5 µL of extracted DNA. Amplification reactions were performed using a CFX96 instrument (Bio-Rad Laboratories Inc.) under the following conditions: 3 min at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 50 s. For quantification, a 10-fold dilution series of MOLT cell line DNA was included in each run. For data analysis, the threshold was set on 850 relative fluorescence units.

Statistical analysis

The statistical software package SPSS was used for statistical analysis. To characterize the microbiota of lesional and non-lesional skin over time, paired sample t-tests and unstructured linear mixed models were performed on the first set of samples. The paired-samples t-test was used to compare microbial diversity (Shannon diversity index) and the relative abundance of *Staphylococcus* spp. of lesional and non-lesional skin at baseline. Unstructured linear mixed model with time as repeated factor was used to compare clinical AD score (oSCORAD), microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration (culture and qPCR) of lesional and non-lesional skin at baseline with data obtained 7, 14, 21, 28, 35 and 42 days later.

To analyze the inter- and intra-patient variability of the lesional and non-lesional skin microbiota, the coefficient of variation (CoV) was calculated for the microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration by dividing the standard deviation by the mean. This was performed for the first and second set of samples. For inter-patient variability, the CoV was calculated per time-point and for intra-patient variability per patient over time. A CoV ≤ 25% has been considered as an acceptable level of variation for analytical methods (22, 23). Clinical data for patient groups were compared using the 1-way analysis of variance (ANOVA) and chi-square tests.

RESULTS

Comparison of lesional and non-lesional skin microbiota

To characterize the microbiota of lesional and non-lesional skin over time, microbiota composition was first compared at baseline. A significant lower microbial diversity of 3.8 ± 1.7 was observed for lesional skin compared to 5.1 ± 1.0 for non-lesional skin (p = 0.011; **Figure 1a**). The lower microbial diversity of the lesional skin was due to the presence of a lower number of OTUs and the relatively high abundance of the genus *Staphylococcus* (**Figure 1b**). Subsequent detection and quantification of *S. aureus* showed a correlation between the relative abundance of the genus *Staphylococcus* and the concentration of *S. aureus* (**Figure 1c, d**). This confirms that the relative abundance of *S. aureus* was higher on lesional skin compared to non-lesional skin as expected.

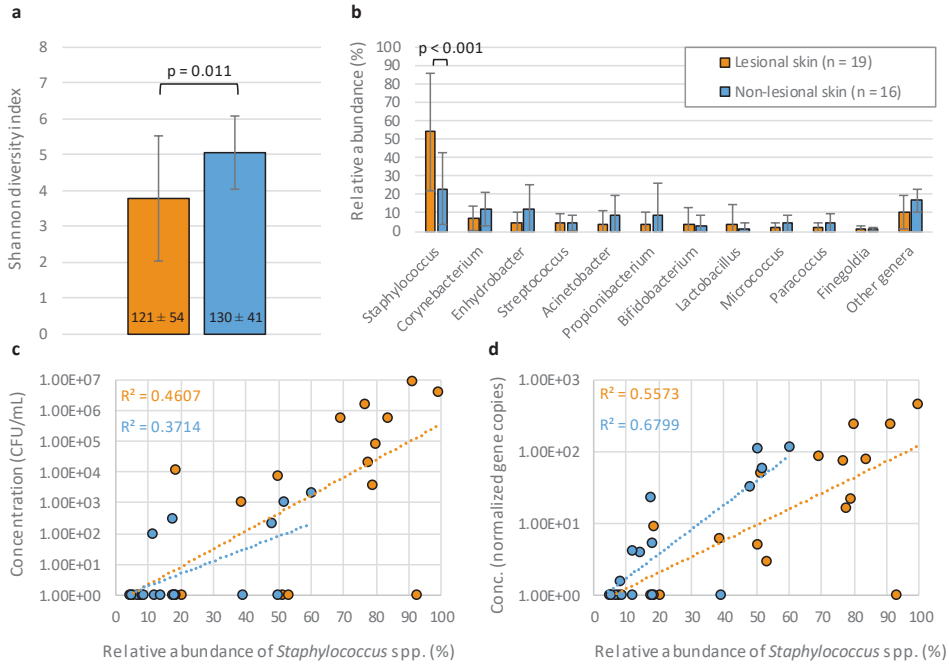

Figure 1. Baseline characteristics of lesional (*orange*) and non-lesional (*blue*) skin microbiota in terms of (**a**) microbial diversity, (**b**) microbiota composition, (**c, d**) *Staphylococcus aureus* concentration based on culture or quantitative real-time PCR (qPCR) in relation to the relative abundance of *Staphylococcus* spp. determined by microbiota analysis. For microbiota diversity, means ± standard deviations of operational taxonomic units are indicated in the bars. The *S. aureus* concentration based on qPCR is normalized by calculating the *nuc* gene copies per 1000 16S rRNA gene copies. All p-values are based on a paired-sampled t-test.

During the following 42 days, the mean clinical AD score of lesional skin was significantly lower ($p \leq 0.036$) compared with the baseline scores (**Figure 2a**). During these days, the mean clinical AD score ranged between 16.1 ± 5.6 and 19.3 ± 4.9, still corresponding with mild to moderate AD. In comparison with the clinical AD score, there was no significant difference in the mean microbial diversity, mean relative abundance of *Staphylococcus* spp. and the mean *S. aureus* concentration determined by qPCR over time (**Figure 2b-e**). The mean microbiota composition of non-lesional skin remained relatively stable over time.

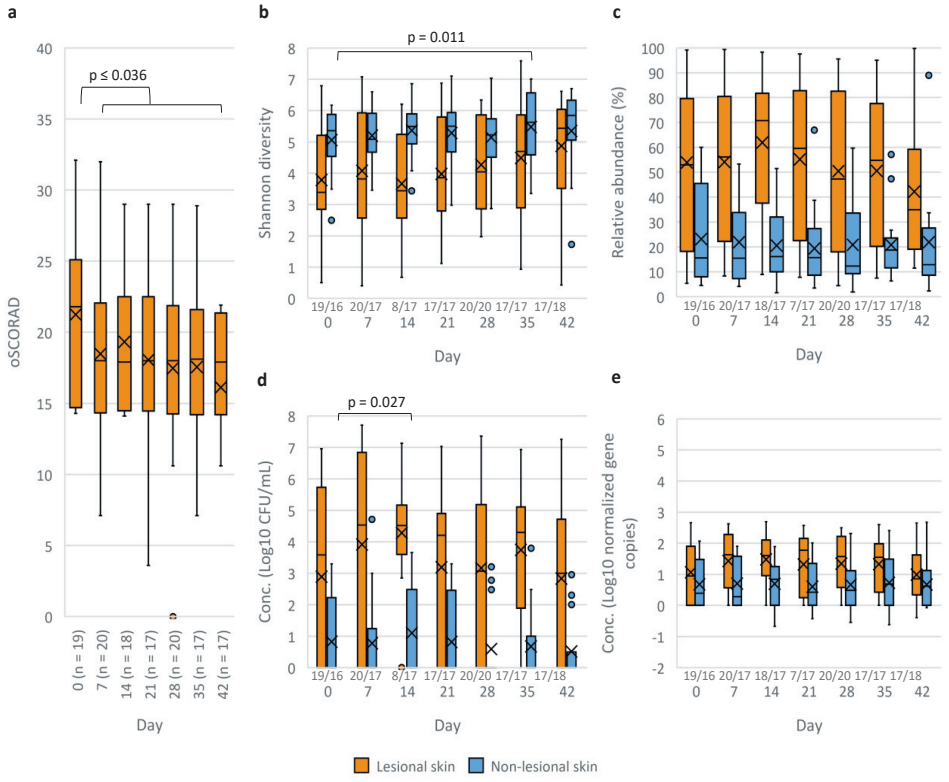

Figure 2. Analysis of lesional (*orange*) and non-lesional (*blue*) skin microbiota during a period of 42 days in terms of (**a**) clinical AD score, (**b**) microbial diversity, (**c**) relative abundance of *Staphylococcus* spp., and (d, e) *Staphylococcus aureus* concentration based on culture or qPCR. The *S. aureus* concentration based on qPCR is normalized by calculating the *nuc* gene copies per 1000 16S rRNA gene copies. Mean values are indicated by crosses and outliers by dots. Number of samples are indicated below the bars. All p-values are based on an unstructured linear mixed model.

Inter-patient variability at each time-point

To quantify the extent of inter-patient variability of the skin microbiota, the CoV was calculated at each time-point for all test results. For lesional skin, high CoVs were observed, in the range 35.5-45.9% for microbial diversity, 46.9-65.2% for relative abundance of *Staphylococcus* spp., and 45.3-94.1% for *S. aureus* concentration. For microbial diversity of non-lesional skin, low CoVs, in the range 16.3-28.0%, were found. These data strongly indicate that there was considerable variation in lesional skin microbiota between patients.

Intra-patient variability over time

To analyze the skin microbiota variability within an individual patient over time, the CoV for microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration was calculated per patient. For all test results of lesional skin, CoVs ranging between 7.1% and 173% were observed. For microbial diversity of non-lesional skin, low CoVs, ranging between 3.5% and 29.3%, were found. These data indicate that there was a wide range of intra-patient variability for lesional skin.

Defining microbiological phenotypes

The patient population could be divided into 3 groups with different microbiological phenotypes, as shown by 3 representative patients in **Figure 3.** The lesional skin microbiota of group I (n = 7; *orange*) and II (n = 8; *blue*) were dominated by *Staphylococcus* spp., resulting in a different profile compared to their non-lesional skin microbiota. These groups differed in variability, as the lesional skin microbiota of group II was relatively unstable (**Supplementary Figure S1**). The lesional skin microbiota of group III (n = 5; *red*) was not dominated by *Staphylococcus* spp. Its composition and variability were similar to their non-lesional skin microbiota. This group had a significantly higher microbial diversity ($p < 0.001$), lower relative abundance of *Staphylococcus* spp. ($p < 0.001$), lower *S. aureus* concentration ($p < 0.001$) and lower clinical AD score ($p = 0.032$) compared with group I and II. There was no significant difference between the 3 groups in age, sex, Fitzpatrick skin type, season of participation, target area for sample collection or total bacterial load.

Confirmation of large inter- and intra-patient variability for lesional skin microbiota

The large inter- and intra-patient variability for lesional skin microbiota was confirmed by data of the second sample set obtained from an independent but comparable study population (**Supplementary Tables S1, S2**). For lesional skin, the CoV for microbial diversity, the relative abundance of *Staphylococcus* spp. and the *S. aureus* concentration at each time-point was between 27.0% and 68.8%. The variability of all test results within an individual patient in time ranged between a CoV of 1.3% and 161.3%. This second sample set also confirmed the existence of 3 different microbiological phenotypes (**Supplementary Table S3, Figure S2**).

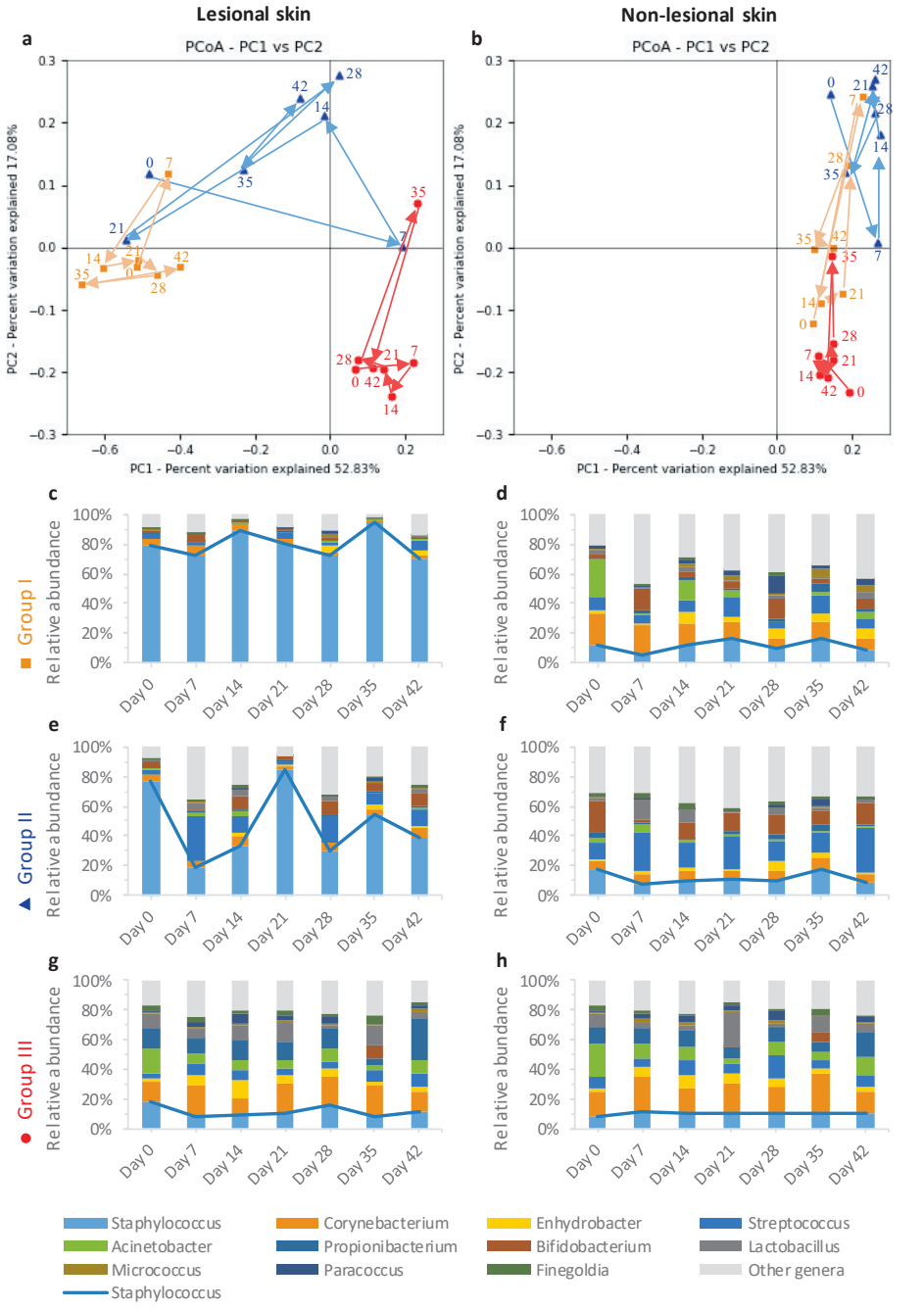

Figure 3. Lesional and non-lesional skin microbiota of 3 selected patients representing 3 groups of patients with different microbiological phenotypes shown in (**a, b**) principal coordinates analysis (PCOA) plots and (**c-h**) bar charts. In the PCOA plots, the arrows combined with the day numbers show how the microbiota composition changed over time.

DISCUSSION

To our knowledge, this is the first longitudinal analysis of inter- and intra-patient variability of skin microbiota of patients with mild to moderate AD. While the sampling method was strictly standardized, large inter- and intra-patient variability for lesional skin microbiota were found. The large inter-patient variability originated from variable *S. aureus* abundance and environmental factors that vary significantly among humans (12, 13). The wide range of intra-patient variability indicated that the skin microbiota of some individuals varied more than others. Based on these data, three patient groups with different microbiological phenotypes were defined. The microbiological phenotype for group I and II can be described as high *Staphylococcal* bioburden, low microbial diversity and either microbiologically stable, or unstable, respectively. The observation that the variability within each of these two groups is consistent within subjects across longitudinal samples, as well as concordant in multiple microbiological assessments, suggests that this difference is not caused by variable sample quality. This difference might be caused by the same unidentified individual (genetic) factors that determine the degree of variability of healthy skin microbiota (15, 16). However, influences of uncontrolled factors (e.g. number of showers, washing with soap, direct UV-exposure) on the stability of the microbiota cannot be excluded. Group III was characterized by a significantly different lesional microbiota compared to group I and II. It could be described as low *Staphylococcal* bioburden and high microbial diversity. The relative lack of dysbiosis was associated with lower clinical AD score.

The data presented in this evaluation suggest that without intervention the individual microbiota composition of the lesional skin can change considerably over a period of 42 days, in particular in patients with a microbiological phenotype of group II. Because the variability over time can be high, single samples collected before and after treatment may not be sufficient to determine the effect of the treatment on an individual's lesional skin microbiota. High sample frequency and statistical analyses methods, which utilize repeated measures across more than one end-of-study time-point, may reduce the effect of the variability in the analyses of clinical trials. The ability to objectively classify subjects to the microbiological phenotypes could be useful in the analyses and interpretation of microbiota data in future clinical trials with larger sample sizes.

The limitation of the presented evaluation is that the sample sets are from patients involved in a clinical trial administering a vehicle gel on AD lesions. Although the vehicle gel did not contain the active compound, this could have had an influence on the lesional skin microbiota as it contains the preservative sodium benzoate. However, this was considered to be minimal because (i) the concentration was far below the minimal inhibitory concentration for *S. aureus* and (ii) the diversity increased under treatment (data not shown). In this evaluation, administration of the vehicle gel had no significant effect on the microbial diversity or relative abundance of *Staphylococcus* spp. However, a significant difference in clinical AS score was

observed after administration of the bland emollient or vehicle gel. Since only one sample for each subject was available prior to initiation of treatment in the clinical trial, we were unable to undertake analyses to evaluate any method to define pre-treatment microbiological stability which could serve as a covariate in statistical analyses or from which to stratify randomization. Another limitation is the small patient group and the omission of including patients of younger age. A larger and more diverse population is required to study the microbiological phenotype classifications and generalize more broadly. Lastly, the limited variation in anatomic target areas and disease states at baseline as inter- and intra-patient variability of lesional skin microbiota might be different for patients e.g. with severe AD located at their dorsal neck.

In conclusion, this evaluation shows that lesional skin microbiota of patient with mild to moderate AD is characterized by large inter- and intra-patient variability, reflecting a highly individual profile. Based on these data, lesional skin microbiota remains a potential target engagement biomarker in AD. A high sample frequency, e.g. once weekly, yields excellent time-dependent insight into the changes of the variable skin microbiota, which can be used to determine the treatment effect on the lesional skin microbiota in clinical trials.

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SUPPLEMENTAL APPENDIX

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