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ORIGINAL ARTICLE

Atopic Dermatitis, Urticaria and Skin Disease

Microbial and transcriptional differences elucidate atopic dermatitis heterogeneity across skin sites

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Abstract

It is well established that different sites in healthy human skin are colonized by distinct microbial communities due to different physiological conditions. However, few studies have explored microbial heterogeneity between skin sites in diseased skin,

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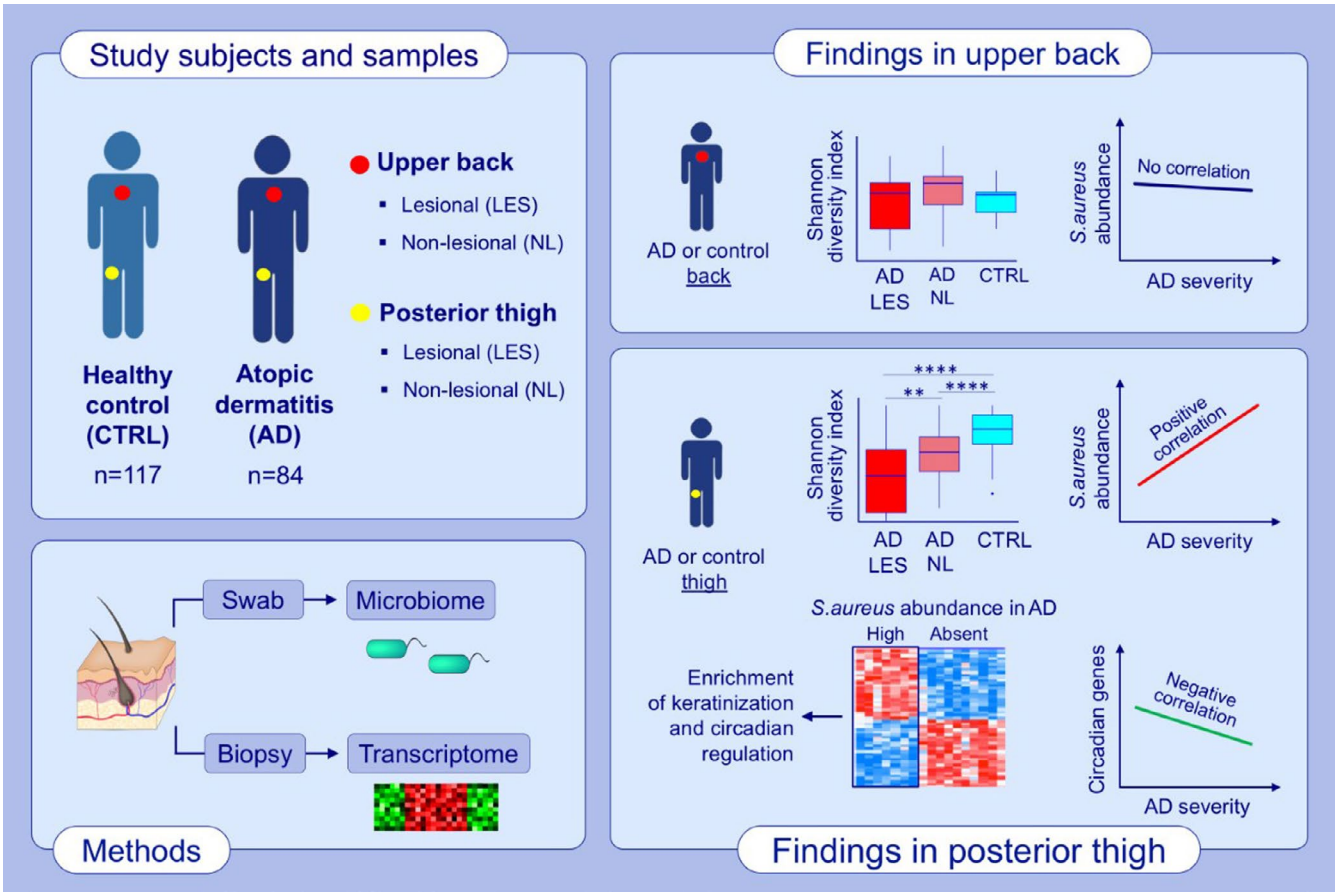
such as atopic dermatitis (AD) lesions. To address this issue, we carried out deep analysis of the microbiome and transcriptome in the skin of a large cohort of AD patients and healthy volunteers, comparing two physiologically different sites: upper back and posterior thigh. Microbiome samples and biopsies were obtained from both lesional and nonlesional skin to identify changes related to the disease process. Transcriptome analysis revealed distinct disease-related gene expression profiles depending on anatomical location, with keratinization dominating the transcriptomic signatures in posterior thigh, and lipid metabolism in the upper back. Moreover, we show that relative abundance of *Staphylococcus aureus* is associated with disease severity in the posterior thigh, but not in the upper back. Our results suggest that AD may select for similar microbes in different anatomical locations—an "AD-like microbiome," but distinct microbial dynamics can still be observed when comparing posterior thigh to upper back. This study highlights the importance of considering the variability across skin sites when studying the development of skin inflammation.

KEYWORDS
atopic dermatitis, inflammation, microbiome



GRAPHICAL ABSTRACT
Distinct disease-related gene expression profiles depend on anatomical location—keratinization is dominating in the posterior thigh and lipid metabolism in the upper back. Relative abundance of *Staphylococcus aureus* is associated with disease severity in the posterior thigh, but not in the upper back. The abundance of *S aureus* in posterior thigh is associated with keratinization and circadian rhythm regulating genes

1 | INTRODUCTION

Skin forms a life-sustaining interface between the human body and the environment, and recent research reveals the importance of cutaneous microbial communities in health and disease. The healthy skin microbiota varies widely between individuals and changes with age, gender, genetic variation, health status, and living environment.[1-3] It is also clear that the skin microbiota is not one single community, but a collection of commensal communities where each community adapts to and modulates the physiological and immunological environment of the sites they inhabit.[4] Typically, the healthy human skin microbiome is mostly stable within an individual over time.[5] The maintenance of skin homeostasis relies on a finely tuned balance between the host and its microbes at each site, and a disruption of this balance likely contributes to inflammatory skin diseases such as atopic dermatitis (AD).[6,7]

AD is a highly heterogeneous disease characterized by chronic itchy rashes, inflammation and frequent skin infections, and it is currently the most common chronic inflammatory skin disease in industrialized countries.[8] AD lesions can extend from the typical sites of predilection, such as body folds, to most skin surfaces,[8] and manifestations vary extensively by age, severity, age of onset,[9] and ethnicity.[10] However, at present, the precise mechanisms driving the onset and course of AD are insufficiently understood and the optimal type and time-point for intervention are unknown.[11] Investigating the role of the microbiome and its interaction with the skin in AD will likely reveal important clues regarding disease mechanisms and improved biomarkers to stratify patients into disease subtypes. This will also stimulate future research on innovative treatments directed at endotype components.[12]

We and others have reported previously that the AD skin microbiota is perturbed, including a decrease in microbial diversity in comparison with healthy subjects.[13,14] In many cases, *Staphylococcus aureus* colonizes the skin, with a prevalence of 70% on lesional skin and 39% on nonlesional skin of AD patients according to culture-based methods.[15] Culture-independent studies have shown that the relative abundance of staphylococci, *S aureus* and *S epidermidis* in particular, positively correlates with AD severity during flares.[14,16] A recent culture-based study supported these findings by demonstrating that AD patients colonized with *S aureus* have more severe disease symptoms as well as a greater degree of type 2 immune deviation and barrier disruption compared to patients not colonized with *S aureus*.[17] From the host perspective, several studies of the human skin transcriptome have found an induction of type 2 immune pathways, and disturbances in lipid metabolism and barrier function.[18-27] However, most of these studies have been limited by small sample sizes.[22,26,27] Our recent study combined analyses of the skin microbiota and host transcriptome analyses from the same subjects with a greater sample number to elucidate microbe-host interactions in AD.[13]

Only a few microbiome and transcriptome studies have explored AD heterogeneity between skin sites. Most studies have focused on a single site or pooled samples from different body sites for statistical analyses. Since the anatomical location is known to be a strong determinant of the microbial composition in healthy individuals,[28] local skin physiology could determine the role of the microbiota in AD in a skin site-dependent fashion. A recent study analyzed the effect of different skin sites and their physiological properties such as lipid profile at the microbiome level in patients with AD.[29] Epidermal lipid composition correlated strongly with bacterial diversity and composition at AD predilection sites. Higher levels of long-chain unsaturated fatty acids were found to be associated with increased abundance of the lipophilic Propionibacteria and Corynebacteria.[29]

In the present study, we aimed to further explore the interaction between host and skin microbiome in AD by examining two physiologically distinct body sites: posterior thigh and upper back. The skin microbiota was determined using 16S rRNA gene sequencing and the host transcriptome by DNA microarrays. Integration of these results revealed site-specific characteristics of AD pathophysiology which have relevance for understanding both disease mechanisms and treatment targets.

2 | METHODS

2.1 | Subject recruitment and sampling

Adult patients (18-83 years) with moderate-to-severe chronic AD (SCORAD score > 25, n = 91), as well as healthy volunteers (n = 126), were recruited to the study. Microbiome skin swab samples and skin biopsies were obtained from areas with active disease in the upper back or posterior thigh (from above poplitea to bottom of buttocks) in AD patients. Healthy volunteers were sampled in corresponding skin areas. All clinical procedures were approved by the appropriate local Institutional Review Boards (UH, Dnro 91/13/03/00/2011; HHU, 3647/2011; KINGS, 11/H0802/6), and all subjects provided written informed consent before participation. A detailed description of the recruitment and sampling is available in Fyhrquist *et al*.[13] Figure S1 in the supplementary material describes the patient and control numbers, sample numbers per anatomical location, and study method used. All affymetrix analyzed samples are matched with the 16S analyzed samples.

2.2 | 16S amplicon processing and data analysis

Library preparation and sequence preprocessing were performed as described in Fyhrquist *et al*.[13] Briefly, DNA was extracted from the microbiome samples, barcoded, regions V3-V4 amplified using primers 341F/805R, and sequenced with 454 FLX titanium. Sequences were preprocessed with Qiime, and operational taxonomic units (OTUs) were picked at 99.3% identity.

Alpha diversity measures were calculated with vegan package in R,[30] tested with Mann-Whitney U test and FDR corrected with Benjamini-Hochberg (BH). Beta diversity was calculated using the

Bray-Curtis dissimilarity, and ordination was performed with PCoA and UMAP. Permanova test for differences within groups was implemented with the adonis function from the vegan R package. Differential abundance testing of clades was carried out using ANCOM from the python scikit-bio skbio.stats.composition.ancom package version 0.4.2.[31] The test was run with default parameters and "holm-bonferroni" multiple comparisons correction. All analyses should be interpreted as exploratory.

2.3 | Microarray data processing and data analysis

Total RNA was extracted from skin tissue samples using the RNeasy Fibrous Tissue Mini kit (Qiagen), amplified according to Affymetrix protocols, and hybridized to array plates.

Normalization, batch correction, and differential analysis were performed in the eUTOPIA[32] platform (https://github.com/Greco-Lab/eUTOPIA). In short, the affymetrix arrays were normalized and technical batch effects originating from sample preparation were removed (SVA-package with Combat function). For identification of differentially expressed genes, a linear model (R package Limma) was fitted to the data (using age, gender, clinical center as covariates), and pairwise comparisons were done using the empirical Bayes method. Transcriptomes were defined based on a fold change of 1.5 or greater and a Benjamini-Hochberg–adjusted *P*-value <0.05.

Network analysis was generated using the INfORM[33] (https://github.com/Greco-Lab/INfORMhttps://github.com/Greco-Lab/INfORM) by Greco-Lab with default parameters. Functional enrichment analyses were performed using web-based tools Enrichr (http://amp.pharm.mssm.edu/Enrichr/) and the PANTHER Gene

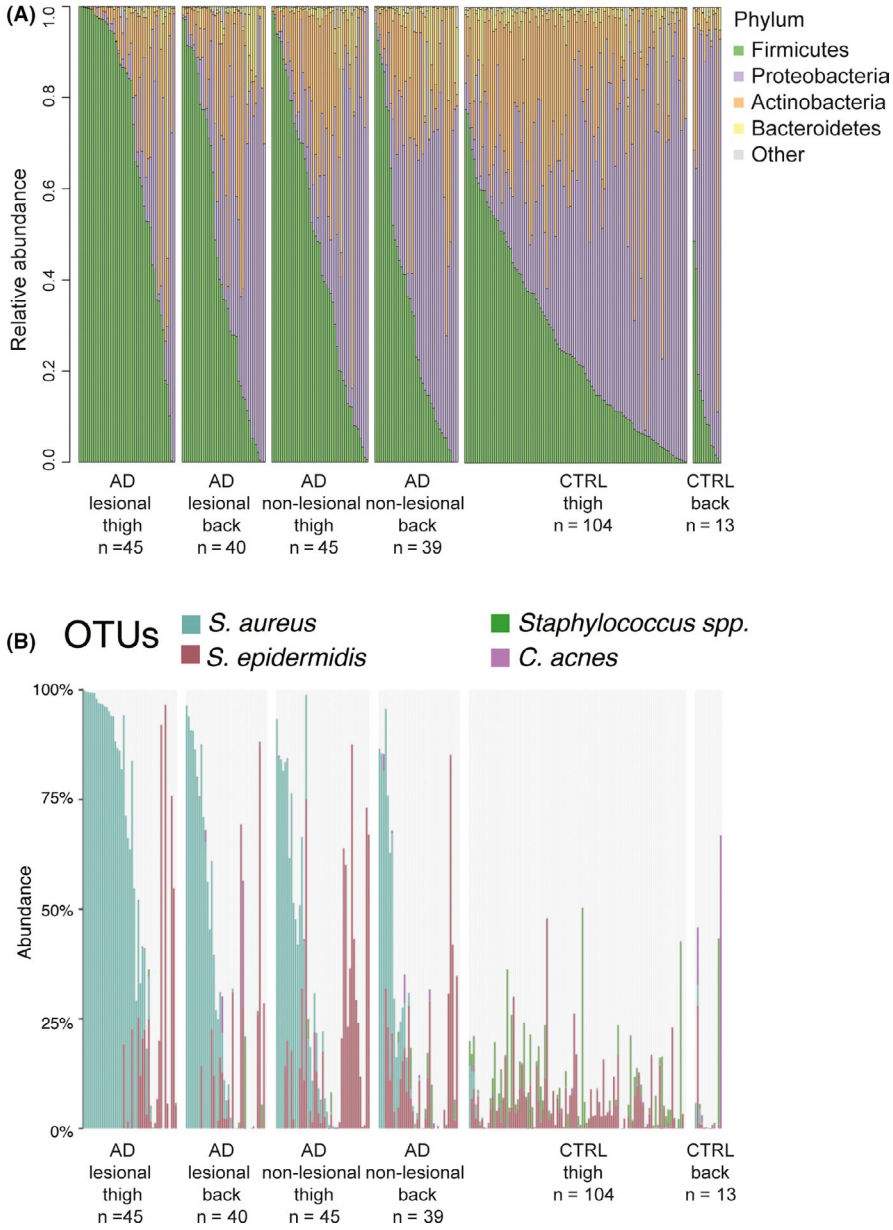


FIGURE 1 Microbial composition of the samples. A, Phyla-level barplot, highlighting the 4 most prevalent phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. B, OTU-level barplot of relative abundances, highlighting *S aureus* and *Staphylococcus spp.* which were differentially abundant between groups by ANCOM analysis. *C acnes* and *S epidermidis* are included for reference

Ontology version 13.1 and the Ingenuity Pathway Analysis by Qiagen. Heatmaps were generated with Perseus.

2.4 | Association with severity

Association between AD severity and *S aureus* abundance was performed using the Spearman correlation. Two measures of severity were used: (a) objective SCORAD as a measure of global AD severity, and (b) local SCORAD, a study-specific measure of severity of the sampled lesions, based on the objective SCORAD criteria (see ref. 13).

2.5 | Leukocyte deconvolution and module detection

To evaluate the relative cell fraction of involved leukocyte subsets in all studied skin sites, the CIBERSORT (CS) algorithm was used to estimate cell proportions of 22 individual immune cells utilizing the "L22" validated gene signature matrix.[34] Estimations are based on 1000 permutations. No significance filter has been applied to the estimated cell fractions to include all samples for further analysis (Figure S11).

Weighted Gene Co-Expression Analysis (WGCNA) was used to integrate the relation of relative abundance of *S aureus* and *S epidermidis* as well as estimated cell fraction of the CS algorithm in lesional skin of the posterior thigh.[35] The parameters used were minCorKME = 0.5 and minKMEtoStay = 0.3 and minModuleSize = 50, resulting in a total of 15 modules. Generated module eigengenes were correlated with microbial and cellular traits of interest (Figure S12). Modules with a correlation coefficient > 0.5 and p-value < 0.001 were further analyzed for gene enrichment and pathway analysis using clusterProfiler.[36]

3 | RESULTS

3.1 | Differences in microbial dynamics of AD skin between thigh and upper back are characterized by *S aureus*

The skin microbiota of both AD patients (n = 84) and healthy subjects (n = 117) was dominated by the phyla Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (Figure 1A) as expected.[37] Of these, Firmicutes and Proteobacteria showed differences in abundance between the clinical groups (ANCOM[31] analysis). Firmicutes were increased in both AD lesional and nonlesional skin compared with healthy controls, while Proteobacteria were decreased in AD lesional samples compared with both nonlesional and controls (Figure S1).

At the genus level, *Staphylococcus* was the most common genus observed in AD samples, with *S aureus* as the most prevalent species (Figure 1B). *S aureus* (OTU 883 806) showed significant differential abundance between the groups. *S aureus* was increased in AD lesions and nonlesional samples compared with controls (Figure S2), and it was differentially abundant between AD and healthy controls in the thigh samples (n = 45), but the evidence in back samples was inconclusive (n = 39) (Figure S3).

To investigate the skin site differences further, we determined the microbial alpha diversity (in this case the Shannon index), which measures species richness and distribution within a population (Figure 2A). In posterior thigh, the alpha diversity was progressively reduced from healthy skin to nonlesional AD skin and further to lesional AD skin. A closer look at the microbial composition of the samples suggested that a gradual increase in *S aureus* from healthy person to nonlesional AD and further to lesional AD skin is an important driver of the reduction in alpha diversity. In contrast, alpha diversity did not differ between any of the studied groups in upper back samples. Of note, microbial diversity was lower in healthy back compared with healthy posterior thigh (n = 92; Figure S4A), consistent with previous studies.[38] We obtained similar results by using the Simpson index (Figure E4B,C). It should be noted, however, that low number of samples from the back of healthy control (n = 13) subjects limits the power of the microbiome analysis.

Analysis of beta diversity, measuring the degree of similarity between sample groups, was also carried out. AD lesional and nonlesional samples from both skin sites were found to form an AD sample cluster, while the thigh healthy skin cluster showed some overlap with AD samples, and upper back healthy controls formed a more separate group (Figure 2B). These results indicated that there is an "AD-like microbiome" which is skin site-independent.

Further analysis revealed additional skin site-specific dynamics in AD, as indicated by the alpha diversity measures (Figure 2B and Figure S5). In thigh samples, healthy, nonlesional and lesional samples formed clear but partially overlapping clusters. *S aureus* and *Staphylococcus* spp. were found to be differentially abundant between the three conditions (Figure S3A). In contrast, upper back healthy samples formed a separate cluster from the corresponding AD samples, but the lesional and nonlesional samples clustered more closely than the corresponding thigh samples. Two OTUs, labeled as *Herbaspirillum* spp. and *Curvibacter* spp., were differentially abundant in upper back AD compared with controls, but these have been identified as common kit contaminants[39,40] (Figure S3B). We also identified a group of samples with a distinct bacterial profile. These samples contained OTUs from the Burkholderiaceae, Enterobacteriaceae, Microbacteriaceae, and Oxalobacteraceae families, with *Cryocola* spp. and *Halomonas phoceae* as the most abundant taxa (Figure S6).

3.2 | The nonlesional skin transcriptome in AD differs from that of healthy skin and is strongly influenced by anatomical site

To identify skin processes altered in AD in the two sampled anatomical locations, we compared the transcriptional profile of nonlesional

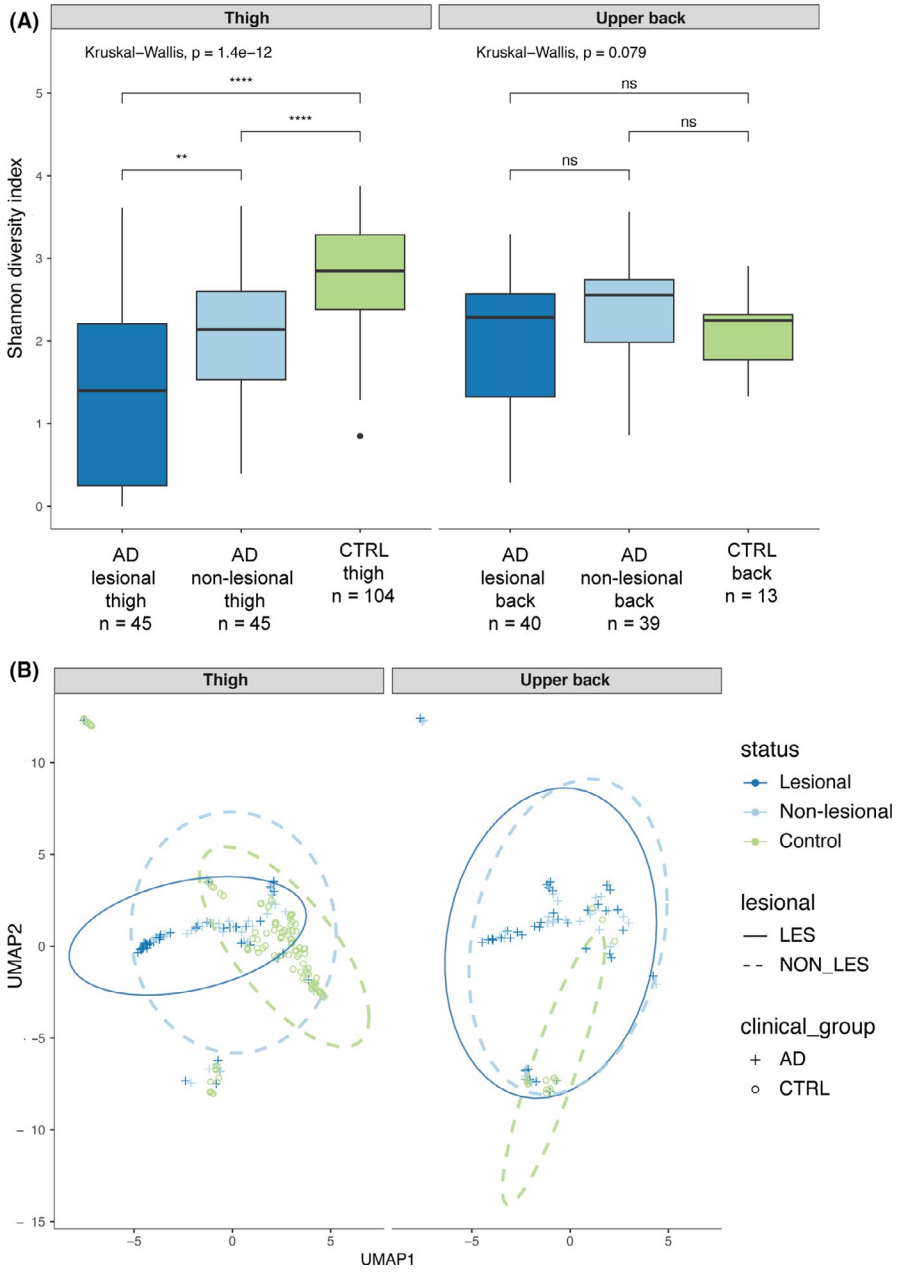
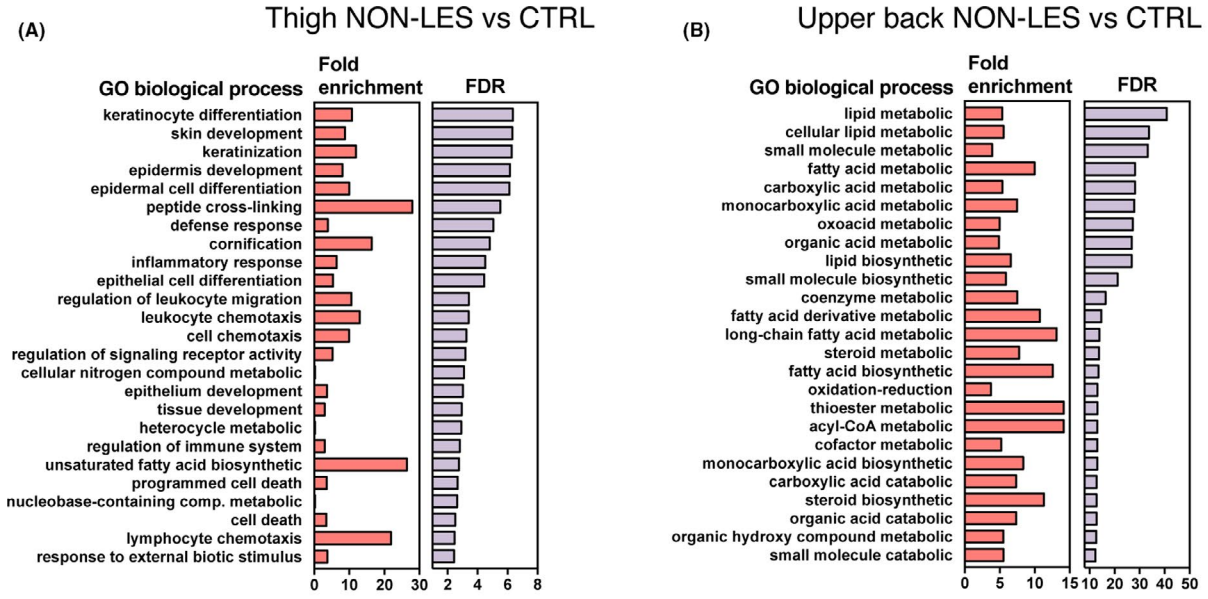


FIGURE 2 Alpha and beta diversity of skin samples. A, Shannon diversity index of lesional, nonlesional and healthy control samples divided by skin site. Significant differences between groups were found in thigh (Kruskal-Wallis, $P < .001$) but not in upper back ($P > .05$). B, UMAP-based ordination of the samples using Bray-Curtis dissimilarity. Thigh and upper back samples were ordinated together; the visualization was split into two identical axes to highlight the site = specific dynamics. Other visualizations of the same ordination can be seen in Figure S5

AD skin with healthy controls. In posterior thigh, 108 genes (Table S2) were differentially expressed (fold change (FC)> 1.5, adj. *P*-value < 0.05) between nonlesional AD and healthy skin samples, while the same comparison in upper back resulted in 371 (Table S4) differentially expressed genes (DEGs) (Figure S7).

Analysis of the identified gene signatures for functional enrichment based on gene ontology (GO) categories revealed clear differences between the anatomical locations. Nonlesional AD in posterior thigh showed an enrichment in biological processes related to cornification and epidermal cell differentiation, as well as lymphocyte chemotaxis, compared to healthy thigh controls (Figure 3A). In upper back, the same comparison revealed mainly the enrichment of lipid, small molecule, cofactor, and organic acid metabolism, lacking any immune components (Figure 3B). Ingenuity pathway analysis (IPA) suggested that functions related to inflammatory responses, cellular movement and immune cell trafficking were activated in thigh nonlesional AD sites in comparison with healthy control samples (Figure 3C). In contrast, in nonlesional AD sites in upper back, small-molecule biochemistry and lipid metabolism were strongly down-regulated compared with control samples (Figure 3D).

FIGURE 3 Gene ontology-based analysis of functional enrichment in (A) thigh nonlesional (n = 41) vs control sites (n = 99), and B, back nonlesional (n = 36) vs control sites (n = 13). Downstream effects analysis in IPA was used to visualize, via color-coded heatmaps, putative biological and disease trends in (C) thigh nonlesional vs control sites, and (D) back nonlesional vs control sites. The color intensity of the squares in the heatmaps reflects the strength of the absolute z-score for predictions (orange = positive, blue = negative). The size of the squares reflects the B-H–adjusted p-values


(A)
Thigh NON-LES vs CTRL
GO biological process
Fold enrichment
FDR
keratinocyte differentiation
skin development
keratinization
epidermis development
epidermal cell differentiation
peptide cross-linking
defense response
cornification
inflammatory response
epithelial cell differentiation
regulation of leukocyte migration
leukocyte chemotaxis
cell chemotaxis
regulation of signaling receptor activity
cellular nitrogen compound metabolic
epithelium development
tissue development
heterocycle metabolic
regulation of immune system
unsaturated fatty acid biosynthetic
programmed cell death
nucleobase-containing comp. metabolic
cell death
lymphocyte chemotaxis
response to external biotic stimulus
0 10 20 30
2 4 6 8
(B)
Upper back NON-LES vs CTRL
GO biological process
Fold enrichment
FDR
lipid metabolic
cellular lipid metabolic
small molecule metabolic
fatty acid metabolic
carboxylic acid metabolic
monocarboxylic acid metabolic
oxoacid metabolic
organic acid metabolic
lipid biosynthetic
small molecule biosynthetic
coenzyme metabolic
fatty acid derivative metabolic
long-chain fatty acid metabolic
steroid metabolic
fatty acid biosynthetic
oxidation-reduction
thioester metabolic
acyl-CoA metabolic
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organic acid catabolic
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small molecule catabolic
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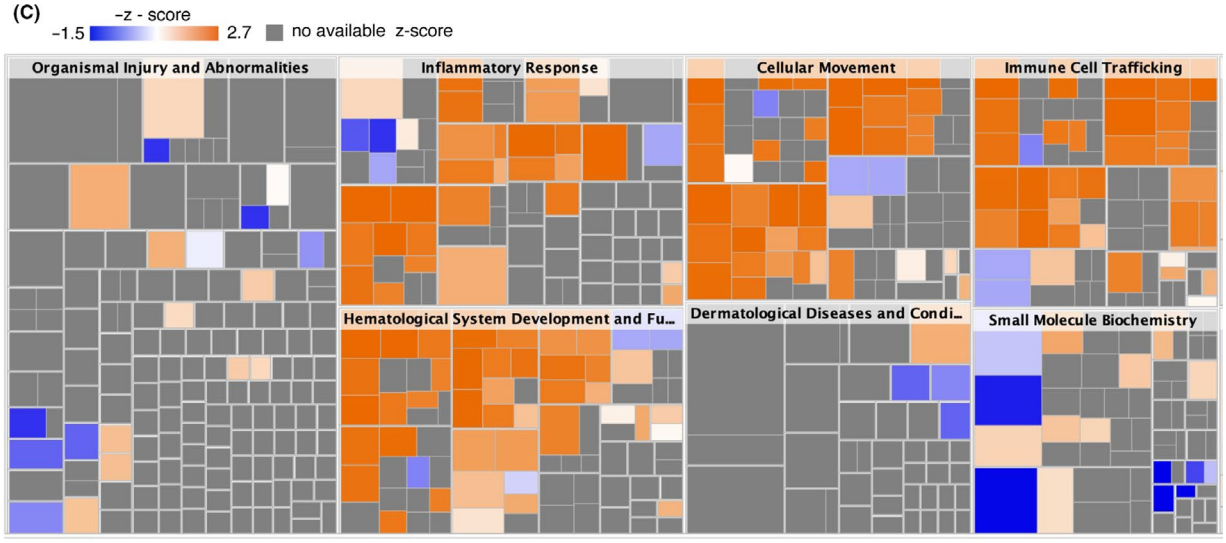
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Dermatological Diseases and Condi...
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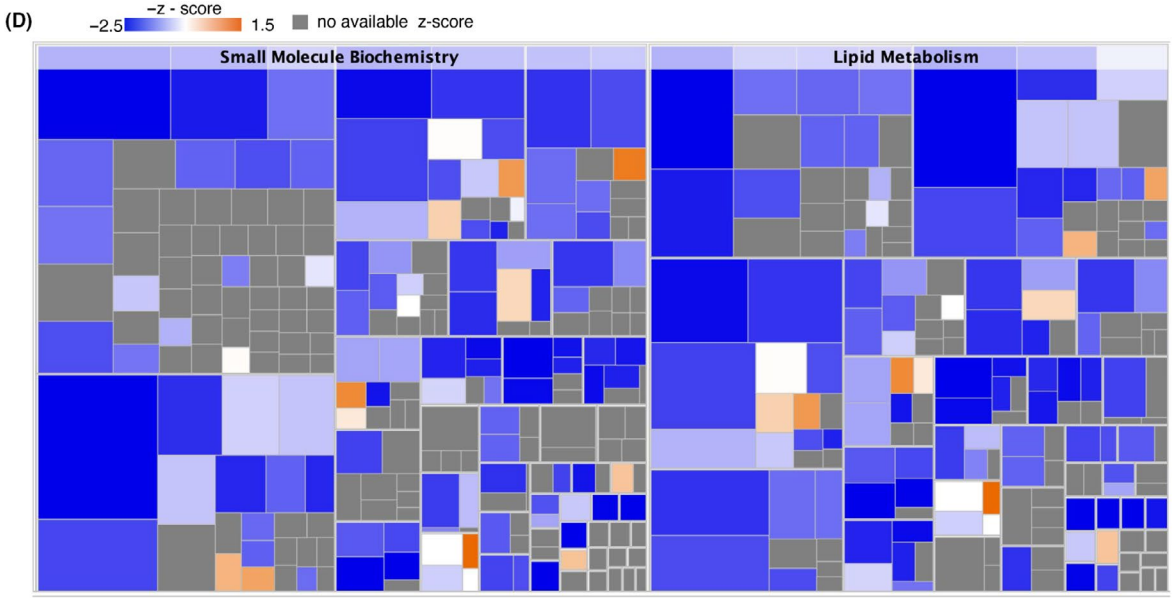
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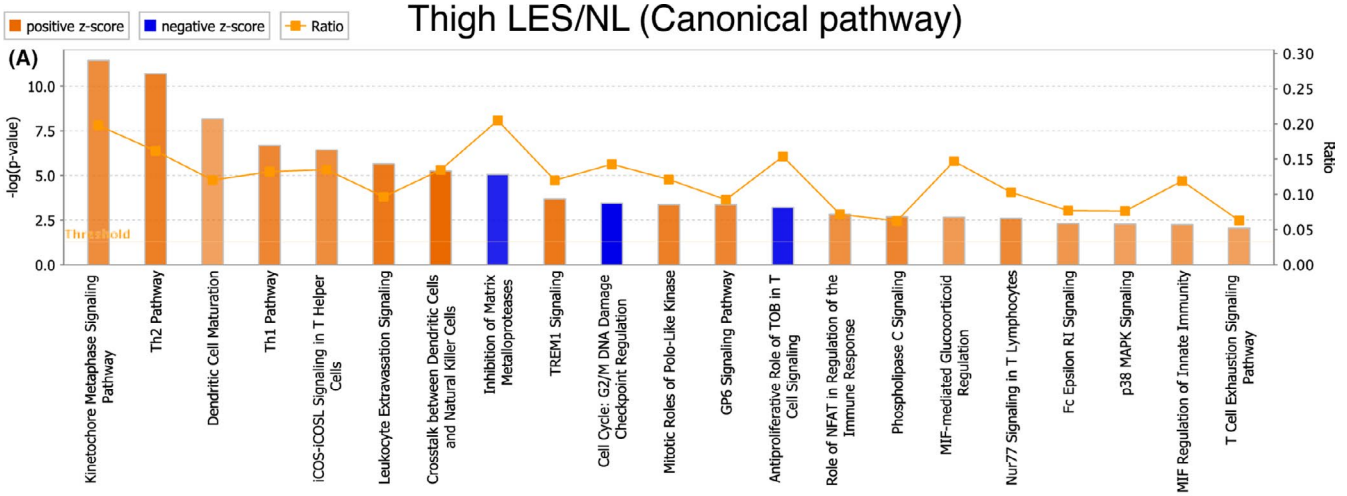
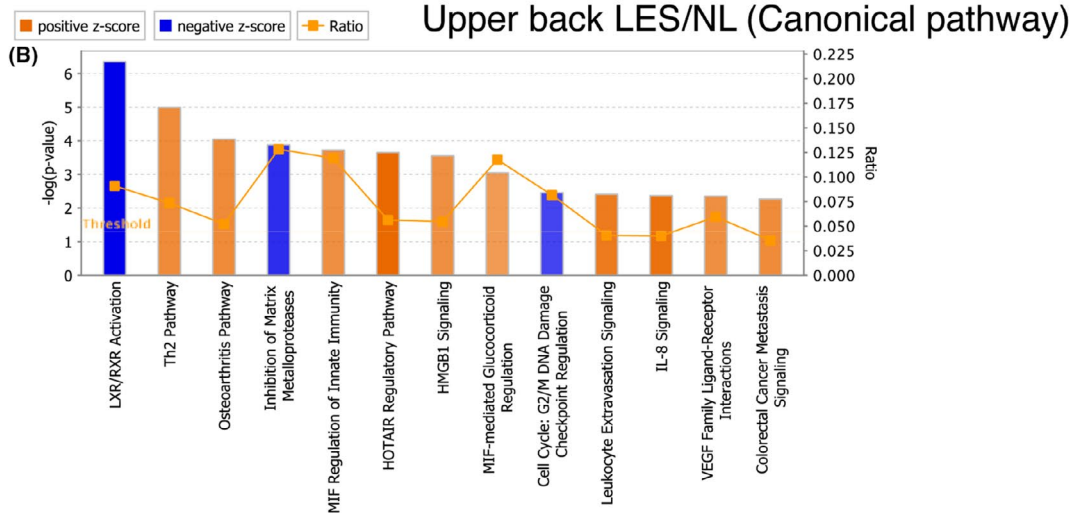
Small Molecule Biochemistry
Lipid Metabolism





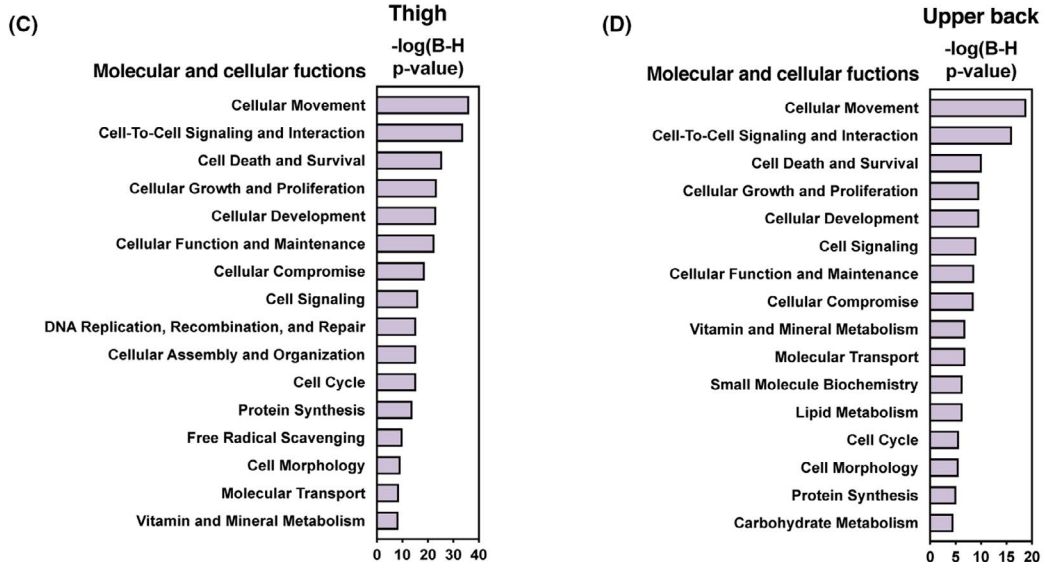


FIGURE 4 Ingenuity canonic pathway analysis (IPA) of differentially expressed genes between (A) lesional (n = 43) and nonlesional (n = 41) samples in thigh and B, lesional (n = 37) and nonlesional (n = 36) samples in back. Top molecular and cellular functions (IPA) between (C) thigh lesional and nonlesional samples, and (D) back lesional and nonlesional samples

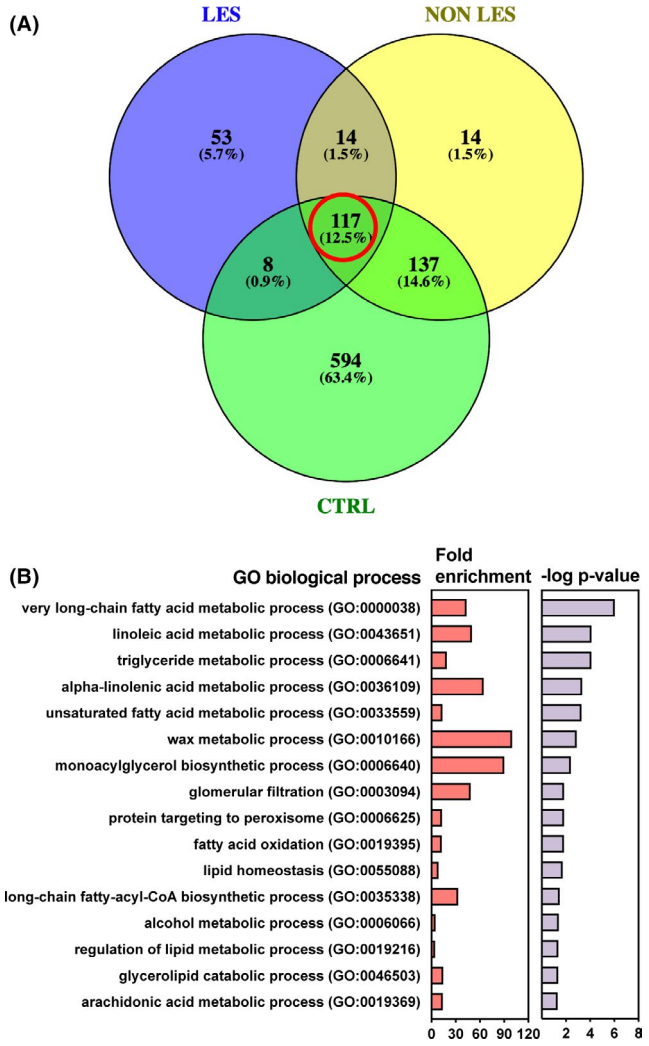


FIGURE 5 Analysis of differentially expressed genes in thigh vs back contrast. A, Venn diagram summarizing the differentially expressed genes between thigh and upper back on the different sample types: lesional AD, nonlesional AD and healthy controls. B, Gene Ontology enrichment analysis of the 117 genes differentially expressed in all three conditions

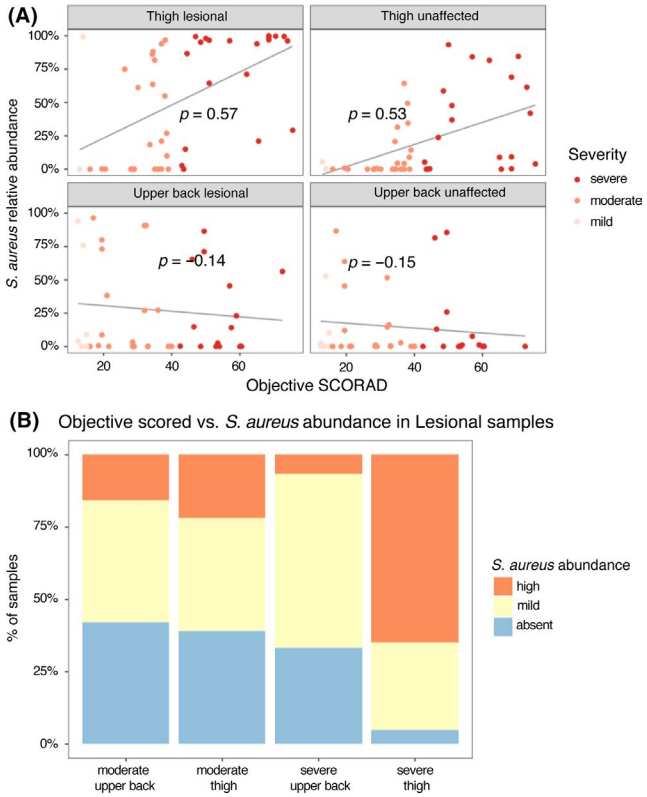


FIGURE 6 S. aureus—objective SCORAD association. A, Measure of AD severity (objective SCORAD as a measure of global AD severity) correlates (Spearman) with *S aureus* relative abundance in lesional (n = 45; rho = 0.57) and nonlesional (n = 45; rho = 0.53) skin from the thigh but the association is not observed in the back (n = 40). (B) Dividing samples high (>80%), mid (1% to 80%), and absent (<1%) *S aureus* abundance reveals that individuals with severe AD are more likely to have *S aureus*-dominated thigh lesions with respect to moderate AD patients, while no difference in *S aureus* colonization trends for lesions is observed for upper back between severe and moderate patients

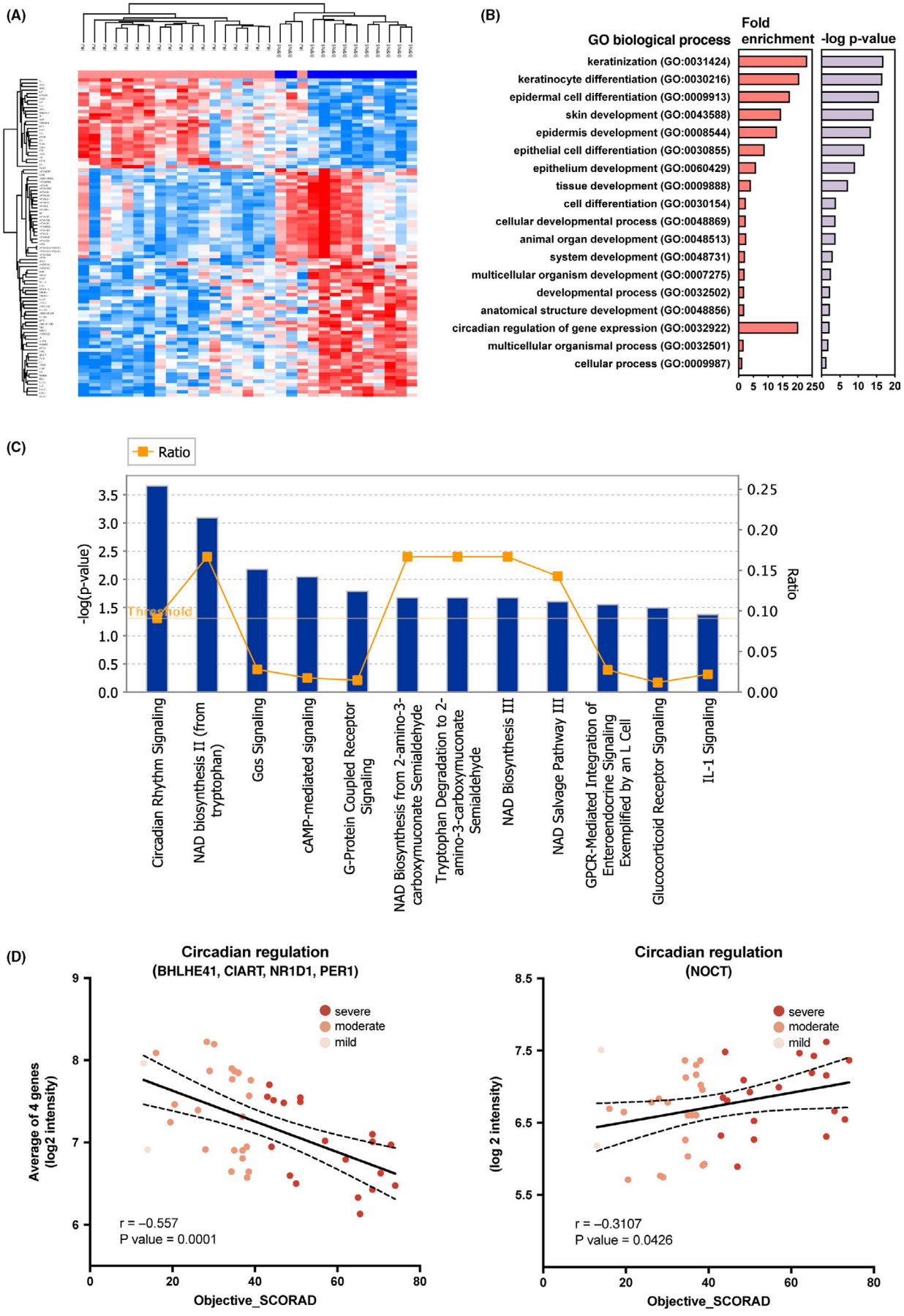
3.3 | Lesional AD skin is characterized by increased Th2-type immune responses regardless of the anatomical location

The skin transcriptional profiles of lesional and nonlesional sites of AD patients were also compared. We found 709 DEGs (Table S1) between thigh lesional and nonlesional samples, whereas 328 genes (Table S3) were found to differ in expression between back lesional and nonlesional samples (Figure S7). Functional characterization of the differentially expressed genes using IPA revealed that many of the same pathways (eg, *Th2 pathway*, *MIF regulation of innate immunity*, *Leukocyte extravasation signaling*) were activated in lesional sites in both anatomical regions. However, there were also important differences between the two different sites (Figure 4A, B). In posterior thigh, the *Th1 pathway* and *iCOS-iCOSL signaling in T helper cells* were among the most significantly activated pathways (Figure 4A). In the upper back, *LXR/RXR activation*, involved in the regulation of lipid metabolism and inflammation, was strongly suppressed in the lesional sites in comparison with nonlesional skin (Figure 4B).

IPA analysis of cellular and molecular functions of DEGs between lesional and nonlesional AD samples also showed a high degree of similarity between thigh and back (Figure 4C, D). In both sites, functions associated with *Cellular movement*, *Immune cell trafficking*, *Cell-to-cell signaling and interaction*, and *Hematological system function* were the most significantly enriched.

3.4 | Transcriptional differences between thigh and back are defined by major variations in lipid metabolism

To understand the molecular basis behind the physiological differences between posterior thigh and upper back, we compared the gene expression across these sites for both AD patients and


(A)
(B)
GO biological process
Fold enrichment
-log p-value
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keratinocyte differentiation (GO:0030216)
epidermal cell differentiation (GO:0009913)
skin development (GO:0043588)
epidermis development (GO:0008544)
epithelial cell differentiation (GO:0030855)
epithelium development (GO:0060429)
tissue development (GO:0009888)
cell differentiation (GO:0030154)
cellular developmental process (GO:0048869)
animal organ development (GO:0048513)
system development (GO:0048731)
multicellular organism development (GO:0007275)
developmental process (GO:0032502)
anatomical structure development (GO:0048856)
circadian regulation of gene expression (GO:0032922)
multicellular organismal process (GO:0032501)
cellular process (GO:0009987)
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0 5 10 15 20
(C)
Ratio
-log(p-value)
Threshold
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
0.00 0.05 0.10 0.15 0.20 0.25
Circadian Rhythm Signaling
NAD biosynthesis II (from tryptophan)
Gαs Signaling
cAMP-mediated signaling
G-Protein Coupled Receptor Signaling
NAD Biosynthesis from 2-amino-3-carboxymuconate Semialdehyde
Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde
NAD Biosynthesis III
NAD Salvage Pathway III
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell
Glucocorticoid Receptor Signaling
IL-1 Signaling
(D)
Circadian regulation
(BHLHE41, CIART, NR1D1, PER1)
severe
moderate
mild
Average of 4 genes (log2 intensity)
6 7 8 9
r = –0.557
P value = 0.0001
0 20 40 60 80
Objective_SCORAD
Circadian regulation
(NOCT)
severe
moderate
mild
(log 2 intensity)
5.5 6.5 7.5 8.5
r = –0.3107
P value = 0.0426
0 20 40 60 80
Objective_SCORAD

FIGURE 7 A, Heatmap of DEGs in *S aureus* "high" (n = 19) vs "absent" (n = 12) contrast in lesional thigh samples. B, GO analysis of functional enrichment of DEGs in *S aureus* "high" vs "absent" contrast in biological processes. C, Enrichment of canonical pathways in IPA analysis of DEGs in *S aureus* "high" vs "absent" contrast. D, Correlation of circadian regulation-associated genes with disease severity SCORAD (n = 43)

healthy controls. We found 117 genes that were differentially expressed between thigh and upper back in all three clinical conditions (Table S6), that is, nonlesional and lesional AD and healthy controls. (Figure 5A). Functions such as long-chain fatty acid metabolism and unsaturated fatty acid biosynthesis were highly enriched in upper back samples compared with posterior thigh, regardless of the clinical condition (Figure 5B). Furthermore, the metabolism of linoleic, alpha-linolenic, and arachidonic acid was also induced in upper back samples.

3.5 | Association between *S aureus* abundance and disease severity is dependent on the skin site

As several previous studies have linked *S aureus* abundance in lesional and nonlesional skin to AD severity,[14,16,17,41] we examined whether this association was present in the two different skin sites. In our dataset, the abundance of *S aureus* in both thigh lesional and nonlesional samples correlated positively with the objective SCORAD (as a measure of global AD severity), whereas no such correlation was found in either sample type in upper back samples (Figure 6A). Similar results were obtained when assessing the correlation between *S aureus* and sample-specific local severity measures (Figure S8).

A closer inspection of the association between *S aureus* and objective SCORAD levels revealed that severe lesions (objective SCORAD > 40) in the thigh region are more often densely colonized by *S aureus*, as opposed to severe lesions in the upper back region. In Figure 6B, thigh and back lesional samples were partitioned into three groups based on the relative abundance of *S aureus*: Samples with 1% or less *S aureus* were labeled "absent," samples with 80% or more *S aureus* were labeled "high," and the samples in between were labeled "mid."

3.6 | The abundance of *S aureus* in posterior thigh associates with keratinization and circadian rhythm regulating genes

To investigate links between *S aureus* colonization and transcriptional profiles in lesional skin samples, we compared gene expression profiles between thigh lesional samples which were abundantly colonized by S *aureus* (referred as *S aureus* "high" samples), and those lacking *S aureus* (referred as *S aureus* "absent" samples). This resulted in the identification of 99 DEGs (Table S5), which clearly separated the samples into two categories based on hierarchical clustering analyses (Figure 7A). Functional enrichment analyses revealed overrepresentations of biological processes such as keratinization and epidermal cell differentiation, as well as circadian regulation of gene expression (Figure 7B). IPA analysis further identified *Circadian rhythm signaling* as the most significantly enriched pathway in this set of genes (Figure 7C). Inference of networks based on the level of co-expression, importance and centrality of the genes, supported these findings further, revealing three clusters, enriched for keratinization, circadian regulation of gene expression, and cell division, respectively (Figure S9).

We looked further into the specific genes regulating circadian rhythms and observed that in lesional thigh samples, four genes (*BHLHE41*, *CIART*, *NR1D1*, and *PER1*) were negatively correlated with objective SCORAD, while *NOCT* was positively correlated (Figure 7D). Moreover, genes related to keratinocyte differentiation were negatively correlated with objective SCORAD (Figure S10). Using the same approach comparing lesional and nonlesional samples in the upper back region did not reveal any significant associations between the abundance of *S aureus* and severity. To conclude, our results show links between disease severity, the expression of disease relevant genes, and the abundance of *S aureus*, but in a site-specific manner.

3.7 | Modular immune responses in lesional skin of the posterior thigh are associated with *S aureus* and *S epidermidis* abundancies

The relative abundances of *S aureus* and *S epidermidis* were inversely correlated ($R = -0.52$; $P < .001$) in lesional skin in the posterior thigh area. To assess the transcriptional impact of these microbes and their relation on lesional skin, we employed leukocyte deconvolution as well as weighted gene co-expression analysis (WGCNA) to detect associated modules within this specific skin site. First, we sought to identify major leukocyte changes within the affected skin using a transcriptome-wide leukocyte deconvolution algorithm. Lesional skin exhibited major changes in leukocyte composition, with significantly higher proportions of dendritic cells (resting and activated) and macrophages as well as significantly lower resting mast cells and activated natural killer cells compared with nonlesional skin and healthy controls (Figure S11). Thus, the microbial relation and estimated relative cell fractions were further used as traits in the WGCNA. Hence, we identified 2 modules associated with the relation of relative abundance of *S aureus* and *S epidermidis*; 1 positively (tan module, further referred as *S aureus* module (SAMod)) and 1 negatively (brown module, further referred as *S epidermidis* module (SEMod)) correlating module (Figure 8A). The SAMod displayed enrichment for extracellular matrix organization, complement activation, angiogenesis, and leukocyte migration. Additionally, KEGG pathway enrichment identified *S aureus* infection and complement and coagulation cascades as highly enriched terms. However, this module was not highly correlating ($P < .001$) with any immune cell type. In contrast, SEMod exhibited GO BP enrichment for fat cell differentiation and

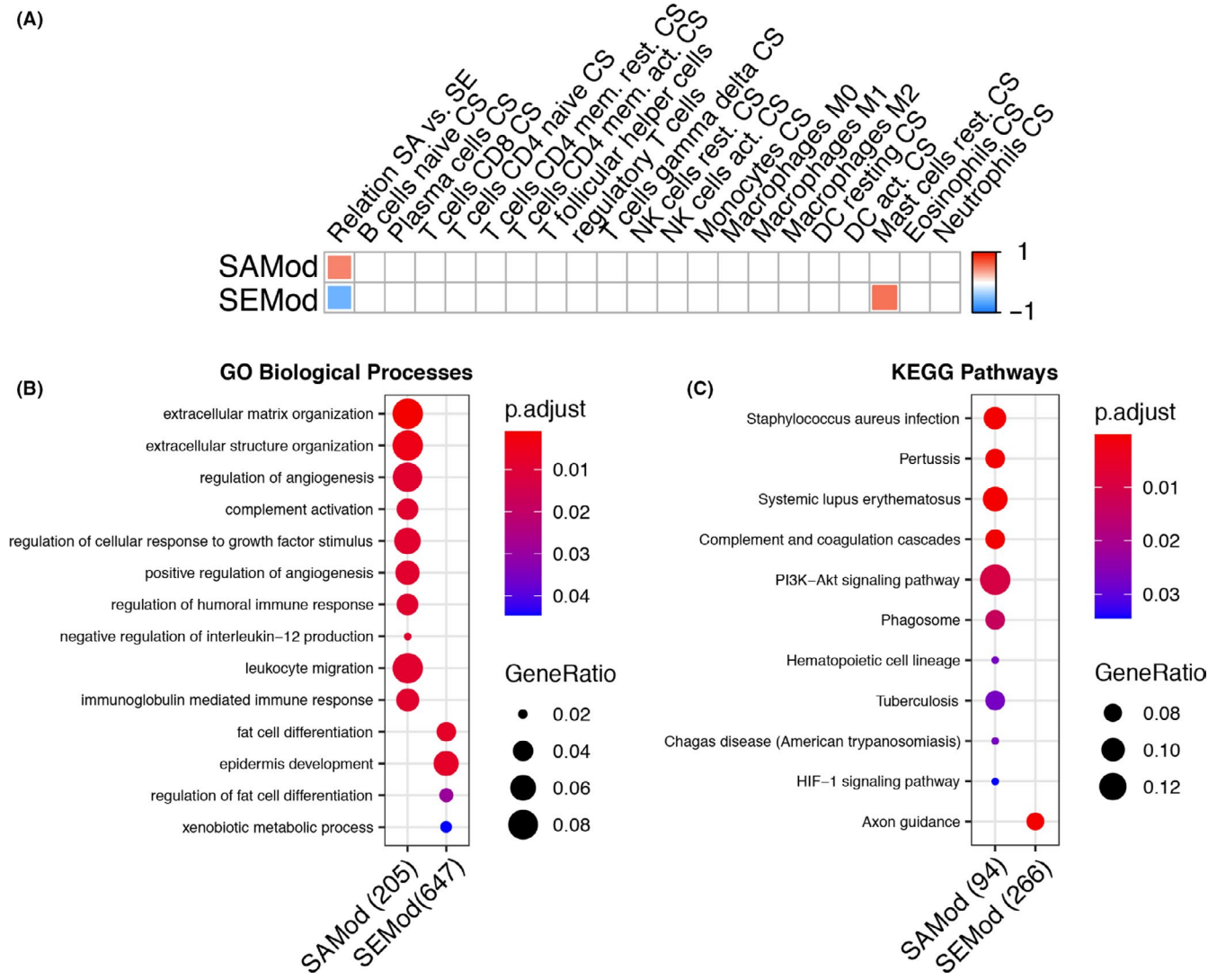

FIGURE 8 Co-expression networks identify response modules of posterior thigh lesional skin associated with relative abundances of *S aureus* and *S epidermidis*. Weighted gene co-expression analysis (WGCNA) was used to determine modules associated with the relation of relative abundances of *S aureus* and *S epidermidis*. Furthermore, the leukocyte deconvolution algorithm Cibersort (CS) was applied on transcriptomics data to estimate the relative cell fractions within the affected skin to link modules toward cell fractions. Modules with a correlation coefficient > 0.5 and a *P*-value < 0.001 of the phenotypic trait "Relation SA vs. SE" were further analyzed. Two modules were associated with the relation of the relative abundance of *S aureus* and *S epidermidis*: 1 positively (referred as *S aureus* module (SAMod)) and 1 negatively (referred as *S epidermidis* module (SEMod)) correlating module a). SEMod was also positively associated with estimated mast cell fraction (A). ClusterProfiler was used for GO biological processes (B) and KEGG pathway (C) enrichment

epidermis development. Intriguingly, this module was highly associated with the estimated mast cell fractions (Figure 8B,C).

4 | DISCUSSION

Our study highlights the importance of the ecological skin niches in defining microbe-host interaction, and this needs to be taken into account when studying AD etiology and progression. By analyzing a large cohort of AD patients and healthy controls and focusing on two anatomical locations: the upper back and posterior thigh, we were able to show that although AD selects for similar microbes across anatomical locations—an "AD-like microbiome," microbial dynamics in AD differ significantly between the different sites. Moreover, we show that the abundance of *S aureus* is associated with disease severity only in the posterior thigh region, and not in the upper back region. Transcriptome analysis revealed distinct disease-related processes depending on the anatomical location, with keratinization playing a major role in posterior thigh, and lipid metabolism dominating in the back, suggesting possible links between the skin microenvironment and microbial dynamics. Thus, our results emphasize the concept of ecological niches that define microbe-host interaction in AD: The host transcriptome defines the ecological niche (sebaceous gland-rich versus—poor, lipid-production), and the niche defines the existence and coexistence of microbes.

Reduced microbial diversity is usually associated with AD, but we observed such a decrease only in posterior thigh, and similar

discrepancies exist in the literature.[14,42,43] Our results suggest the existence of an "*AD-like microbiome*," consistent with Baurecht et al[29]: The disease exhibits an environment that is enriched in a particular *Staphylococcus-rich* microbial community in the different skin sites independent of the influence of local physiology. The AD-like microbiome model helps to explain why alpha diversity is not always decreased; the less diverse healthy sites like "upper back" can transition to an "AD-like" state without a visible change in their alpha diversity metrics. Upper back lesions showed somewhat higher diversity than thigh lesions in these patients. A possible explanation for this could be that the thigh lesions were more severe on average. We also observed that the effect of the disease for selecting microbes is particularly strong in lesions, but it can also be observed in nonlesional skin.

The availability of transcriptome data from the very same skin sites that had been sampled for the microbiota, allowed us to examine host processes that could explain differences in the skin physiology of the different sites and the disease conditions. Lipid metabolism constituted the main difference between thigh and back in the transcriptome analysis, and perturbations to lipid metabolism genes were observed in both skin sites between healthy controls and nonlesional skin. We speculate that this may have major implications on microbial colonization in AD skin, and consequently on the disease manifestations. Two studies have suggested a connection between epidermal lipid composition and bacterial community composition.[44] Here, linoleic, alpha-linolenic, and arachidonic acid metabolism were shown to be stimulated in the back and these molecules have been shown to have antimicrobial activity, and may selectively inhibit certain groups of bacteria such as *Staphylococcus*.[45] It has also been shown that certain strains of *S aureus* have evolved intrinsic defense mechanisms to cope with these fatty acids.[46-48]

Keratinocyte differentiation, which we found to be dysregulated in AD, contributes to skin barrier function.[49] In our data, keratinocyte differentiation and related biological processes showed differences between nonlesional skin and healthy control skin in thigh samples, and between thigh and back in AD. Furthermore, inflammatory responses were activated in nonlesional thigh skin compared with healthy control skin, which was not seen in the upper back samples. These results indicate that while there are differences between nonlesional and healthy control skin in both of the skin sites, nonlesional thigh skin shows signs of subclinical inflammation and an increased susceptibility to lesions, as reflected in the clinical predilection. Previous transcriptome studies have identified alterations in the expression of epidermal cornification genes on AD lesional and nonlesional skin but did not report which skin sites were sampled.[20,21]

In lesional skin, genes associated with inflammation were differentially expressed in skin of both body sites when compared to nonlesional skin of AD patients. Well-described type 2 inflammation pathway genes such as *IL4R*, C-C motif chemokine receptor type 4 (*CCR4*), and C-C motif chemokine 22 (*CCL22*) were upregulated in both lesional thigh and back in comparison with nonlesional skin. These genes were not differentially expressed in nonlesional AD samples compared with healthy control samples. Activation of the Th2 pathway has been previously reported in acute AD lesions,[23] and more recently in a subgroup of AD patients characterized as type 2-high endotype, exhibiting a more severe form of the disease.[26] While AD is currently recognized as a multifactorial, heterogeneous disease with different immune subsets, Th2-responses do play an important role in both acute lesions and chronic disease states.[50]

Despite the reduction in inter-skin site variability driven by the disease, the detected differences in transcriptomes across the sites in both nonlesional and lesional sites could help explain the observed skin site-specific microbial dynamics in AD, which have been reported also for other skin diseases like psoriasis.[51] In particular, we see an increase in *S aureus* abundance from nonlesional skin to lesional skin in thigh, which is not observed in upper back. Additionally, we show that the previously described positive association between *S aureus* and disease severity (measured by SCORAD)[52] is observed only in posterior thigh, but not in the upper back region. We obtained comparable results when considering a lesion-specific measure of severity. Similar results were obtained by Kong et al in antecubital and popliteal creases (moist), as well as volar forearm (dry), but not in nasal sites (moist and *S aureus* rich).[14] This raises questions regarding the presence and role of different *S aureus* strains in AD, such as whether specific *S aureus* strains actively contribute to an increase in severity, and whether certain strains preferentially colonize specific locations. These results stress the need to further explore the role of skin site variability as a crucial part of the experimental design in studies of AD and other skin diseases.

In our study, thigh lesional samples with high *S aureus* levels had a distinct skin transcriptome profile compared to samples with low *S aureus*. In particular, genes related to keratinization and circadian rhythm were differentially expressed between the sample groups. Keratinization relates to disruptions in the skin barrier, as noted above, whereas circadian rhythm influences cutaneous blood flow as well as epidermal barrier function. While it has been proposed that circadian rhythm plays a role in AD pathogenesis, the mechanistic evidence for this is still scarce,[53] especially with regard to the relevance of circadian gene expression in the skin. However, there are studies linking circadian genes to psoriasis-like skin inflammation.[44,54] Furthermore, pruritus and scratching, which are integral parts of the AD, could lead to sleep disruption and in turn may also influence genes which are regulated by circadian rhythm.[55] Further research is required to determine the significance of circadian rhythm genes in the pathogenesis of AD.

The relative abundance of *S aureus* and *S epidermidis* displayed an inverse correlation in lesional skin of the posterior thigh. Relative abundance of *S aureus*, but not that of *S epidermidis*, was also positively correlated with disease severity. In line with this, patients with severe AD have been reported to be colonized more dominantly by *S aureus* strains whereas patients with milder disease demonstrate more *S epidermidis* in skin flares.[16] To explore possible interaction between *S aureus* and *S epidermidis*, skin cell types and gene expression profiles on lesional thigh skin, we employed leukocyte deconvolution as well as WGCNA co-expression analysis. We identified two co-expression modules correlating positively

with the *S aureus* and *S epidermidis* abundances. The *S aureus* associated module displayed enrichment of *extracellular matrix organization* and *leukocyte migration*, but this module could not be assigned to any specific immune cell type. On the contrary, the *S epidermidis*-associated expression module exhibited enrichment of *epidermis development* and was highly associated with estimated mast cell proportions in the skin. Considering the inverse relationship between *S aureus* and *S epidermidis* abundances in lesional skin sites, these results suggest that *S epidermidis* might play a role in mast cell function which in turn may have also an impact on milder disease severity compared to *S aureus*-dominated skin flares.

We have utilized in this study a large patient cohort that includes samples from body sites representing two different ecological microenvironments: dry and sebaceous. This dataset allowed us to examine the influence of epidermal surface lipids on microbial colonization and gene expression on the skin, and identify features that are common for the disease regardless of site. The present study also included a larger number of samples than most previous studies focusing on the effect of skin microbiota composition and host transcriptome on AD pathophysiology. Further studies with shotgun metagenomics are warranted to further elucidate the functional role of microbes in the disease.

In conclusion, these findings suggest that in AD, the skin microbiota interacts through local, host-driven mechanisms, forming different ecological niches and thereby distinct microbe-host interactions, which should be taken into account when considering treatment options. Moreover, we propose that increased lipid metabolism in sebaceous areas may be a contributing factor to decreased *S aureus* colonization through the production of antimicrobial lipids.

CONFLICT OF INTEREST

Dr Ottman reports grants from BIOMAP IMI2 821511, during the conduct of the study; Dr Barrientos-Somarribas has nothing to disclose; Dr Fyhrquist has nothing to disclose; Dr Alexander has nothing to disclose; Dr Wisgrill has nothing to disclose; Dr Olah has nothing to disclose; Dr Tsoka has nothing to disclose; Dr Greco has nothing to disclose; Dr Levi-Schaffer has nothing to disclose; Dr Soumelis has nothing to disclose; Dr Schröder has nothing to disclose; Dr Kere has nothing to disclose; Dr Nestle reports other from Sanofi, outside the submitted work; Dr Barker has nothing to disclose; Dr Ranki reports grants from EU FP7/2007-2013, during the conduct of the study; Dr Lauerma reports grants from Orion Corporation, outside the submitted work; Dr Homey reports grants from EU-MAARS, grants from EU-BIOMAP, grants from DFG-FOR2690-HO 2092/7-1, during the conduct of the study; grants and personal fees from Galderma, personal fees from AbbVie, personal fees from Janssen, personal fees from Sanofi/Regeneron, personal fees from Leo Pharmaceuticals, outside the submitted work; Dr Andersson has nothing to disclose; and Dr Alenius reports grants from BIOMAP IMI2 821511, during the conduct of the study.

AUTHOR CONTRIBUTIONS

HA, BA, NO, and MB-S conceptualized the study; JB, BH, AL, and AR contributed to resources; JK, LW, BA, VS, MB-S, NO, and NF carried out methodology; HA, NO, MB-S, LW, and BA involved in formal analysis; HA, NO, MB-S, and LW visualized the study; HA, BA, NO, and MB-S involved in writing—original draft; NO, MB-S, NF, HeA, LW, PO, ST, DG, FL-S, VS, JMS, JK, FON, JB, AR, AL, BH, BA, and HA involved in writing—review and editing; NO and MB-S contributed equally; HA carried out project administration.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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