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Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis

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Atopic dermatitis (AD) has long been associated with *Staphylococcus aureus* skin colonization or infection and is typically managed with regimens that include antimicrobial therapies. However, the role of microbial communities in the pathogenesis of AD is incompletely characterized. To assess the relationship between skin microbiota and disease progression, 16S ribosomal RNA bacterial gene sequencing was performed on DNA obtained directly from serial skin sampling of children with AD. The composition of bacterial communities was analyzed during AD disease states to identify characteristics associated with AD flares and improvement post-treatment. We found that microbial community structures at sites of disease predilection were dramatically different in AD patients compared with controls. Microbial diversity during AD flares was dependent on the presence or absence of recent AD treatments, with even intermittent treatment linked to greater bacterial diversity than no recent treatment. Treatment-associated changes in skin bacterial diversity suggest that AD treatments diversify skin bacteria preceding improvements in disease activity. In AD, the proportion of *Staphylococcus* sequences, particularly *S. aureus*, was greater during disease flares than at baseline or post-treatment, and correlated with worsened disease severity. Representation of the skin commensal *S. epidermidis* also significantly increased during flares. Increases in *Streptococcus, Propionibacterium,* and *Corynebacterium* species were observed following therapy. These findings reveal linkages between microbial communities and inflammatory diseases such as AD, and demonstrate that as compared with culture-based studies, higher resolution examination of microbiota associated with human disease provides novel insights into global shifts of bacteria relevant to disease progression and treatment.

[Supplemental material is available for this article.]

Atopic dermatitis (AD, "eczema," OMIM 603165) is a chronic, relapsing, intensely pruritic inflammatory skin disorder that can be successfully treated with varying combinations of topical or systemic antibiotics, corticosteroids, and dilute bleach baths (Huang et al. 2009). The prevalence of AD has more than doubled in industrialized countries with no clear cause (Asher et al. 2006; Shaw et al. 2011) and at a high cost (Bickers et al. 2006). More than half of children with moderate to severe AD develop allergic rhinitis and/or asthma, atopic disorders associated with significant morbidity and rare mortality. Genetic as well as environmental factors affect the expression of allergic diseases, including AD (von Mutius 2000; Cramer et al. 2010). Mutations in *FLG,* the gene encoding the skin barrier protein filaggrin, are associated with AD, particularly in patients who subsequently develop asthma and/or allergic rhinitis, suggesting that epicutaneous sensitization may contribute to atopic disease (Palmer et al. 2006; Sandilands et al. 2007).

AD patients experience frequent cutaneous infections, and *Staphylococcus aureus* is commonly cultured from lesional and nonlesional AD skin (Leyden et al. 1974). Reduced antimicrobial peptide expression in the skin of AD patients may contribute to this susceptibility (Ong et al. 2002). While individual microbes causative of common AD skin infections have been studied, it is increasingly clear that individual microbes function within larger bacterial communities (Cogen et al. 2010b).

High-throughput DNA sequencing of the bacterial 16S rRNA gene has revealed a vastly greater bacterial diversity on healthy human skin than demonstrated by culture-based methods (Costello et al. 2009; Grice et al. 2009). The skin's topography and microenvironments are powerful determinants of microbial community structure at particular skin sites (Gao et al. 2007; Costello et al. 2009; Grice et al. 2009). AD preferentially involves the antecubital and popliteal regions, sites that harbor similar groups of organisms and share distinct compositions of microbial communities (Grice et al. 2009). These findings suggest that microbial communities

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might underlie or contribute to observed predilections of some dermatologic disorders for stereotypical sites.

To examine the role of the skin microbiome in AD, we performed a skin microbiome study of AD disease states (baseline disease state, disease flare, and post-treatment for disease flare) in 12 pediatric patients with moderate-to-severe AD and 11 healthy controls, aged 2–15 yr (Table 1; Supplemental Table S1). In this study, we demonstrate how different disease states, severity, and treatments relate to microbial dynamics in this common skin disorder. We report dramatic reductions in the skin microbial diversity during AD flares, which is restored with common AD therapies.

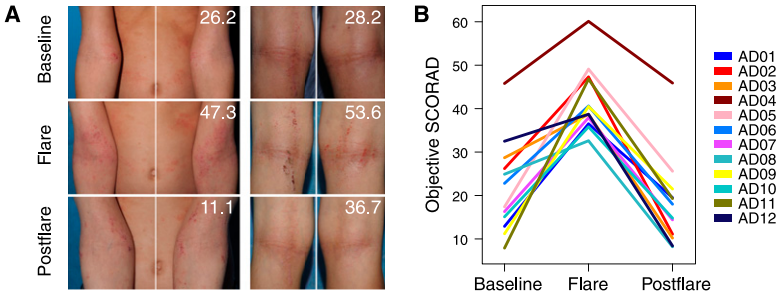

Figure 1. AD disease severity. (*A*) Representative clinical images of the antecubital (Ac, *left*) and popliteal creases (Pc, *right*) in two patients with overall disease severity scores (objective SCORAD). (*B*) Longitudinal objective SCORAD trend for each patient ($n = 12$) at baseline, flare, and postflare.

Results

Characteristics of bacterial communities associated with AD

To investigate the role of bacterial communities in AD, we obtained skin samples from 12 children with moderate-to-severe AD and from 11 controls recruited to the NIH Clinical Center between August 2008 and July 2010. Disease severity was assessed at predetermined sampling timepoints: baseline, stable disease state (B); acute disease flare (F); and 10–14-d post-flare treatment (PF). Disease severity was assessed quantitatively with SCORAD (SCORing AD), a well-validated clinical assessment tool (Williams et al. 1994b; Kunz et al. 1997; Oranje et al. 2007). We obtained skin samples from the left and right antecubital (Ac) and popliteal creases (Pc)—sites of predilection for AD in this age group (Fig. 1A). We also sampled volar forearms (Vf) as control skin sites that are adjacent to, but not a classical site of disease predilection itself, and the nares (N) as a reservoir for *S. aureus* colonization (Archer and Climo 2001; von Eiff et al. 2001). We sequenced skin samples from 36 AD patient visits and 24 control visits for a total of 151,924 near-full-length 16S ribosomal RNA (rRNA) genes (Supplemental Table S2). Study design is detailed in the Methods section.

Mean objective SCORAD scores designating AD disease severity were significantly elevated during flares (42 ± 2.2) as compared with baseline (22 ± 3.1, $P < 7.3 \times 10^{-4}$) and postflare (18 ± 3.0, $P < 0.0038$) (Fig. 1B). Categorization of baseline, flare, and postflare was based on patient clinical status and specified frequency of AD treatment administration (see Methods section for detailed classification criteria). Flare was defined by worsening disease irrespective of use of therapy >24 h prior to sampling. We examined the recent treatment history prior to flares because we predicted that the timing of treatments in relation to sampling might influence the skin microbiome. We found that while no significant differences were detected in objective SCORAD scores ($P < 0.22$), flare timepoints could be grouped into patients that used (1) "no-treatment" ($n = 7$), defined as patients who reported no use of topical medications (corticosteroids, calcineurin inhibitors, or antibiotics) for ≥7 d, and no ingestion of oral antibiotics ≥4 wk prior to flare skin sampling, or (2) "intermittent-treatment" ($n = 5$), defined as patients who reported use of topical medications in the previous 7 d and/or ingestion of oral antibiotics in the previous 4 wk before skin sampling (Table 2).

To investigate a possible relationship, we analyzed the association between AD disease severity and Shannon diversity, an ecological measure of microbial communities that considers: (1) richness, or the total number of bacterial types, and (2) evenness, or the relative proportion of these bacterial types. The antecubital and popliteal creases are considered sites of disease predilection and showed similar results. We averaged these sites per subject ("AcPc") for subsequent analyses, unless otherwise indicated, to avoid treating related samples from the same individual as biological replicates. The partial correlation between objective SCORAD and Shannon diversity of AcPc, adjusting for disease state, was significantly inversely correlated ($r = -0.57$, $P < 3.6 \times 10^{-4}$) (Fig. 2A), indicating that severe AD was associated with lower skin bacterial diversity at sites of disease predilection.

AD patients using no treatments during flares exhibited markedly reduced Shannon diversity in the antecubital creases as compared with intermittent-treatment use ($P < 0.025$) (Fig. 2B,C; Supplemental Table S3). Shannon diver-

Table 1. Clinical characteristics of the participants[a]

Characteristic	Atopic dermatitis (AD) patients	Age-matched healthy controls
Total subjects analyzed	12[a]	11
Age, mean (range)	7.8 yr (2–15)	8.0 yr (2–14)
Male:female	8:4	6:5
Baseline objective SCORAD[b,c]	21.8 ± 3.1	N/A
Flare objective SCORAD	42.1 ± 2.2	N/A
Post-flare objective SCORAD	18.1 ± 3.0	N/A
Asthma[d]	7 of 12	0
Allergic rhinitis[d]	8 of 12	0
Family history positive of AD	8 of 12	4 of 11
All nares cultures positive for *S. aureus* (%)	8 of 12	5 of 11
All nares cultures negative for *S. aureus* (%)	0	5 of 11
Eosinophils (%)[e]	8.43 ± 1.66	3.44 ± 0.92

[a]Evaluated during at least one full cycle of baseline, flare, postflare. Four additional patients were analyzed at flare.
[b]Baseline characteristics of participants at initial visit. Plus–minus values are means ± SE.
[c]SCORing atopic dermatitis.
[d]As defined by International Study of Asthma and Allergies in Childhood questionnaire.
[e]Normal range for eosinophils is 0.8%–7.0%.

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Table 2. Atopic dermatitis treatments prior to flare sampling

	Topical medications[a]	Dilute bleach baths	Systemic antibiotics	Shannon diversity index (antecubital crease at flare)
Time prior to sampling	≤7 d	≤7 d	≤4 wk	
(A) No treatment[b]				
AD02	-	-	-	low (0.76)
AD03	-	-	-	low (0.11)
AD04	-	-	-	low (0.44)
AD05	-	-	-	low (0.59)
AD07	-	-	-	low (0.35)
AD10	-	-	-	high (1.37)
AD11	-	-	-	low (0.40)
(B) Intermittent treatment[c]				
AD01	+	+	+	high (3.27)
AD06	+	-	-	high (3.08)
AD08	+	-	-	high (3.26)
AD09	+	-	-	high (2.38)
AD12	+	-	-	high (2.27)
(C) Additional patient flares[d]				
AD13	-	Incomplete data		low (0.60)
AD14	-	-	-	low (0.27)
AD15-1st flare	-	-	-	low (0.63)
AD15-2nd flare	+	+	-	high (1.65)
AD16	-	+	-	high (3.75)

[a]Topical medications include corticosteroids, antibiotics, and calcineurin inhibitors.
[b]No treatment group (n = 7) represents AD patients who used topical medications >7 d and/or oral antibiotics >4 wk prior to disease flare sampling.
[c]Intermittent treatment group (n = 5) represents AD patients who used topical medications ≤7 d and/or oral antibiotics ≤4 wk prior to disease flare sampling timepoint.
[d]Additional AD patients (n = 4) sampled during flares. AD15 had two flare visits.

sity during intermittent-treatment flares was not significantly different from baseline, postflares, or controls. Similar trends were observed for popliteal creases, another site of disease predilection (Fig. 2C; Supplemental Table S3). These results suggest that cutaneous microbial diversity is associated with disease status in the context of recent treatment history, and that AD treatments can prevent or reverse the change in diversity associated with flares even before clinical improvement is seen. In contrast to the antecubital and popliteal creases, diversity in the nares as well as the volar forearm was relatively constant, demonstrating that changes in bacterial diversity were specific to sites of disease predilection (Fig. 2C; Supplemental Table S3).

To assess how distinct the skin microbiomes for each AD disease state and controls were from each other, we compared the level of similarity of bacterial community structures with the Yue-Clayton theta similarity coefficient (θ) (Yue and Clayton 2005). The θ index takes into consideration the number of bacterial species present and their relative abundances in the two communities being compared (Yue and Clayton 2005; Schloss and Handelsman 2006) with θ = 1 indicating identical community structure and θ = 0 indicating absolute dissimilarity. AD patients within the no-treatment flare group (θ = 0.91 ± 0.029) were statistically significantly more similar to each other than baseline, intermittent-treatment flares, and postflare (Fig. 2D; Supplemental Fig. S1; Supplemental Table S4), far exceeding the reference point of interpersonal variation observed in the within-group θ for controls (θ = 0.26 ± 0.025, $P < 4.3 \times 10^{-10}$). Our results underscored that no-treatment flares were distinguishable from intermittent-treatment flares and likely reflected the natural disease course unaltered by recent medications.

To investigate whether these bacterial community shifts across timepoints were unique to the AD cohort, we calculated both Shannon and Theta indices across visits for the healthy controls, who were sampled at similar time intervals as AD patients (Supplemental Table S5). Mean changes in Shannon diversity between visits were significantly higher for no-treatment AD flare patients (baseline/flare, and flare/postflare) as compared with healthy controls ($P < 0.002$ and $P < 0.02$, respectively, for Ac and Pc). Similarly, mean theta values between visits were significantly lower in the no-treatment AD flare group than healthy controls in the Ac ($P < 0.03$). We conclude from this analysis that the skin bacterial community shifts across timepoints are significantly different in AD patients as compared with similarly sampled healthy controls.

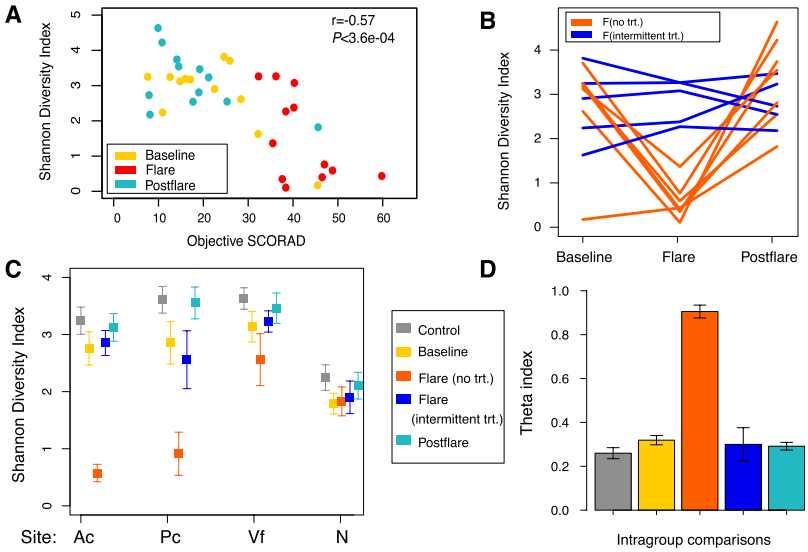

Figure 2. Microbial community-level statistics in the AD microbiome. (*A*) Relationship between objective SCORAD and Shannon diversity in the Ac of AD patients. Partial correlation (adjusting for disease state). (*B*) Longitudinal Shannon diversity trend in AD grouped by no-treatment (trt) and intermittent-trt flares (n = 12, Ac). (*C*) Mean Shannon diversity ± SEM in controls and all AD disease states (Ac, Pc, volar forearm [Vf], nares [*N*]). (*D*) Mean theta (θ) similarity coefficients ± SEM. Pairwise comparisons of community structure between individuals within a control or AD disease group. Bars represent average of all pairwise comparisons of community structure for control individuals to other controls, baselines to baselines, flares(no-trt)-flares(no-trt), flare(intermittent-trt)-flare(intermittent-trt), and postflares-postflares.

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Selective bacterial shifts and AD disease states

To identify bacteria contributing to the reduction in AD microbial diversity, we classified the phylum, order, and genus of the sequences (Supplemental Fig. S2; Supplemental Table S6) at each site. To determine the similarity of the bacterial communities between sites at a taxonomic level, we first performed cross-site correlations of the abundances of each taxa at the phylum-order level (Supplemental Fig. S3). In AD patients, the antecubital and popliteal creases were highly similar (partial correlation adjusting for disease state $r = 0.80$), exceeding the similarity of these sites in controls ($r = 0.68$, Fisher r-to-z transformation, $P < 0.0002$). The similarity between the antecubital and popliteal creases relates to the observed localization of AD in these sites and further justifies combining these sites for analysis. Antecubital creases were similar to the adjacent volar forearm in both AD and control individuals ($r = 0.79$ and 0.81, respectively), suggesting that the typically unaffected skin of the volar forearm significantly correlated with sites of disease predilection. In general, the nares taxa were poorly correlated with the skin sites for all individuals.

Examining the relative abundance of specific taxa at each timepoint, we observed that the antecubital and popliteal creases were strikingly dominated by the genus *Staphylococcus*. The no-treatment flares had the highest abundance of bacterial 16S genes classified as *Staphylococcus* at 90 ± 1.6% as compared with other AD patients and controls (35 ± 2.2%, $P < 0.013$ [baseline]; 31 ± 5.0%, $P < 0.050$ [intermittent-treatment flares]; 20 ± 1.3%, $P < 2.0 \times 10^{-4}$ [postflare]; 16 ± 1.8%, $P < 6.2 \times 10^{-4}$ [controls]) (Fig. 3A; Supplemental Table S7).

The genus *Staphylococcus* includes several bacterial species relevant to clinical disease, including *S. epidermidis* as a skin commensal (Wisplinghoff et al. 2004; Hidron et al. 2008) and *S. aureus* as a pathogen with a known association with AD. To distinguish among *Staphylococcal* species, we generated a custom 16S rRNA sequence database to uniquely classify the Staphylococcal sequences (Fig. 3B). The proportion of *S. aureus* in the no-treatment flares was 65 ± 3.5%, statistically significantly higher than baseline, postflare, and controls (Fig. 4A; Supplemental Table S7). The proportion of *S. aureus* in the intermittent-treatment flares (15 ± 2.5%) was significantly higher than controls ($P < 0.020$), but not significantly higher than baseline, when some AD patients carry large amounts of *S. aureus*.

With significant shifts in *S. aureus* abundance observed in AD, we then examined the relationship between the relative abundances of *S. aureus* and Shannon diversity. Adjusting for disease status, the partial correlation between *S. aureus* abundance and Shannon diversity was $r = -0.68$, $P < 6.3 \times 10^{-6}$, indicating that increases in *S. aureus* accounted for reductions in overall diversity observed in this AD cohort (Fig. 4B; Supplemental Table S8).

We then analyzed the relationship between the proportion of *S. aureus* and disease severity. Given the heterogeneity of the microbiome over the human skin surface (Grice et al. 2009), we evaluated the contribution of skin site. Partial correlation between *S. aureus* abundance and disease severity, adjusting for disease state and site, was significant: antecubital ($r = 0.59$, $P < 2.1 \times 10^{-4}$) and popliteal creases ($r = 0.57$, $P < 4.3 \times 10^{-4}$) (Fig. 4D; Supplemental Table S9). Volar forearm sites also showed a positive partial correlation ($r = 0.70$, $P < 1.4 \times 10^{-5}$), possibly related to a field effect, or extension of changes in the microbiome to adjacent skin. This positive partial correlation observed for the volar forearms appeared to be influenced by the increase in body surface area of affected skin extending to the volar forearms during AD disease flares. The nasal site did not demonstrate a positive correlation. These findings suggest that overabundance of cutaneous *S. aureus* and the associated loss of microbiome diversity are intimately tied to the pathogenesis of AD. Moreover, these data emphasize the regional variation of the skin microbiome at distinct sites and the importance of rational selection of sampling sites in microbiome studies.

Given the dominance of *S. aureus* during AD flares, we sought to determine whether this reduction in diversity was attributed solely to a relative overexpansion of *S. aureus* in the local microbiome. Interestingly, trends in decreased microbial diversity and the similarities of the microbiomes of flaring patients persisted even when the abundance of *S. aureus* sequences was excluded from the analysis and sequence numbers were normalized post-removal (Supplemental Fig. S4; Supplemental Table S10). After subtracting *S. aureus* sequences, we recalculated Shannon and θ diversity metrics. The decrease in Shannon diversity during the no-treatment flares remained significantly different in Ac and Pc ($P < 0.05$ flare vs. baseline, postflare, and controls), and was significantly correlated with increasing disease severity (Ac: $r = -0.48$, $P = 0.004$; Pc: $r = -0.44$, $P = 0.009$) as compared with the other timepoints and with controls. After removal of *S. aureus* sequences, the no-treatment flares in Ac and Pc remained similar to each other based on Theta index as well. These results suggested that similarities in microbiomes extended beyond *S. aureus* during no-treatment AD flares.

Therefore, in addition to shifts in *S. aureus*, we sought to determine whether the presence of novel bacteria or absence of protective bacteria could play a role in AD flares. While the abundance of *S. epidermidis* in AD is conflicting in the literature (Gloor et al. 1982; Williams et al. 1990; Higaki et al. 1999), the proportion of *S. epidermidis* during flares was significantly higher than during postflares and

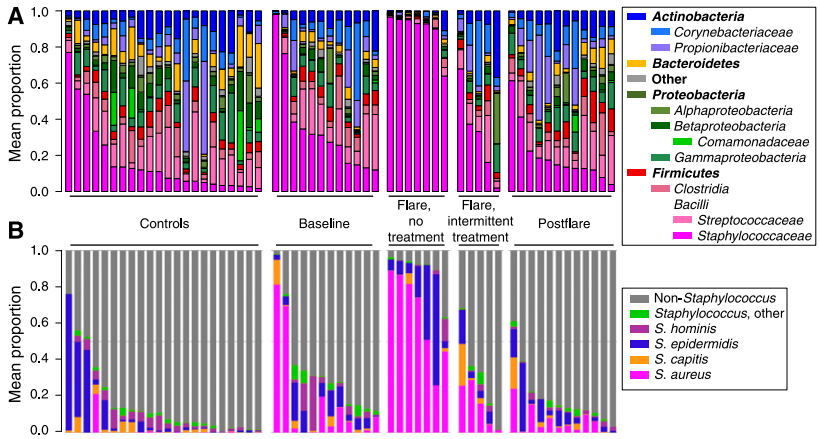

Figure 3. Bacterial taxonomic classifications in the AD skin microbiome. (*A*) Mean relative abundance of the 14 major phyla-order in the antecubital (Ac) and popliteal creases (Pc) for controls and AD disease states: baseline, flare (no-treatment [trt] and intermittent-trt), and postflare (Supplemental Table S13 for order of subjects). (*B*) Mean relative abundances for Ac and Pc of species-level classifications of staphylococcal species. Order of subjects follows *A*.

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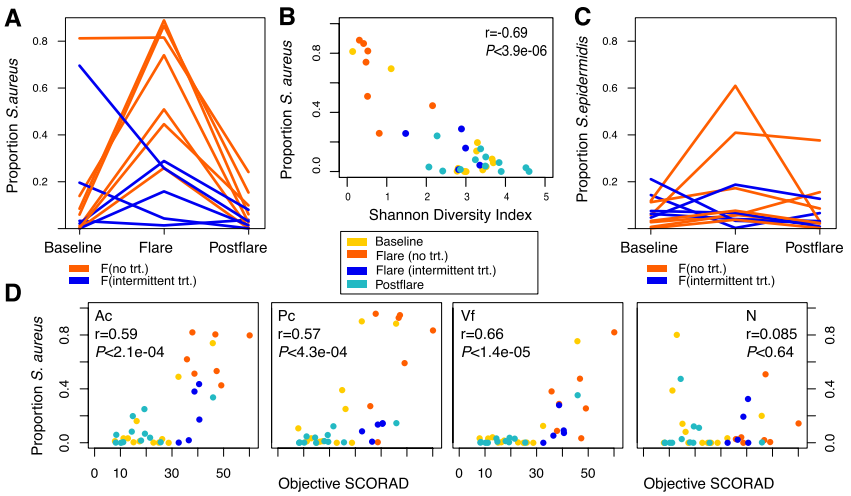

Figure 4. Relationship between staphylococcal species and AD. (*A*) Longitudinal trend of mean proportion of *S. aureus* in AD in antecubital and popliteal creases (AcPc, $n = 12$) grouped by no-treatment (trt) and intermittent-trt flares. (*B*) Proportion of *S. aureus* and Shannon diversity index in AcPc. Partial correlation (adjusting for disease state, AcPc). (*C*) Longitudinal trend of mean proportion of *S. epidermidis* in AcPc. (*D*) Correlation of proportion of *S. aureus* versus objective SCORAD for each site (Ac, Pc, Volar forearm/Vf, Nares/N). Partial correlation (adjusting for disease state and site).

in controls ($P < 0.034$ and $P < 0.006$, respectively) (Fig 4C). *S. epidermidis* was also the dominant staphylococcal species in the AcPc of controls (Spearman correlation, r = 0.98, $P < 8.9 \times 10^{-8}$; Supplemental Table S7). These findings demonstrate distinct selective shifts among at least two staphylococcal species relative to disease status.

We also identified additional genera over-represented at baseline and postflare. We used the Wilcoxon rank-sum test for comparisons of no-treatment flares with baseline and postflare. We observed a significant relative increase in *Streptococcus, Corynebacterium,* and *Propionibacterium* species post-treatment (Supplemental Table S11). With the sequencing depth in this study (Supplemental Fig. S5; Supplemental Table S12) we also identified several lower-abundance bacteria such as the *Rothia* species, which were also statistically significantly increased between disease timepoints (Supplemental Table S11). To compare the taxa present during baseline and postflare, we used matched-pairs analyses to examine whether the same taxa were abundant, or whether new taxa appeared during postflare. In total, 58% of taxa (272 of 469) observed at either baseline or postflare were present at both timepoints. Of the taxa present in >9/12 patients (Supplemental Table S11), all taxa were present at both baseline and postflare, none at statistically significantly different quantities. We also determined that the abundance of genera between baseline and postflare was significantly correlated (partial correlation adjusting for patient, $r = 0.61$, $P < 2.2 \times 10^{-16}$), indicating that there is a relative expansion of taxa already present in the microbiome with a relatively low number of newly introduced bacteria detected at our level of sampling.

Genomic analyses and culture data comparison

Since cultivation studies have demonstrated a higher likelihood of *S. aureus* colonization of AD skin in positive nasal carriers (Williams et al. 1998; Breuer et al. 2002), we examined the results from *S. aureus* cultures concurrently obtained with genomic sampling. Assessment of *S. aureus* nares bioburden (measured as colonies per sample: 0, 1–10, >10) was significantly associated with relative abundance of *S. aureus* nares genomic sequences $P < 0.0072$ (0 vs. 1–10 colonies/sample); $P < 2.1 \times 10^{-6}$ (0 vs. >10 colonies/sample) (Supplemental Fig. S6; Supplemental Table S13), suggesting that genomic identification tracked with traditional cultivation methods for this easily cultured microbe. All AD patients had ≥1 nares culture positive for *S. aureus* as compared with 55% of controls (Table 1). Genomic analysis of nares samples did not detect an association between mean objective SCORAD and the relative abundance of *S. aureus* genomic sequences (Fig. 4D; Supplemental Table S9).

In an effort to determine whether a particular *S. aureus* subtype predominated during flares, we examined the microbial population dynamics of unique *S. aureus* 16S rRNA sequences. The heterogeneity of the 16S rRNA sequences supports earlier studies demonstrating absence of predominant *S. aureus* clones or biotypes (Hoeger et al. 1992; Lomholt et al. 2005; Kim et al. 2009; Yeung et al. 2011).

Associations with treatment

Prior studies have shown conflicting results regarding the effect of topical steroids and antimicrobials on the abundance of *S. aureus* (Lever et al. 1988; Stalder et al. 1994; Williams et al. 1998; Ravenscroft et al. 2003; Gong et al. 2006; Hung et al. 2007; Bath-Hextall et al. 2010). One study demonstrated that use of dilute sodium hypochlorite baths did not completely eliminate *S. aureus,* yet resulted in improved clinical outcomes for AD patients (Huang et al. 2009). Since bacterial diversity in AD skin differed between intermittent-treatment and no-treatment flares, we sought to confirm whether treatment within a defined period of time prior to flares influenced Shannon diversity. We examined samples obtained from four additional AD patients during disease flares. Other sampling timepoints for these four patients did not fulfill strict predetermined criteria for classification as baseline and flare, and were not included in original analyses. For the patients who used no treatment prior to flare, relative Shannon diversity was low, similar to findings from the no-treatment flare group (Table 2; Supplemental Table S14). In contrast, AD patients who used any treatment prior to flare sampling exhibited the higher diversity characteristic of the intermittent-treatment flare group. One particular AD patient was sampled during two different flares, but with two different treatment regimens prior to flare sampling. During the first flare, the patient used no topical treatments in the week prior to sampling and demonstrated reduced Shannon diversity (0.626). Before the second flare sampling, the patient used intermittent dilute bleach baths and had a higher Shannon diversity (1.650). Using the Wilcoxon rank-sum test, the mean Shannon diversity for all flares was statistically significantly different between all no-treatment and intermittent-treatment flares (Fig. 5) ($P < 0.0025$, $n = 16$). These findings suggest that antimicrobial or anti-inflammatory medications decreased *S. aureus* predominance (Supplemental Table S14), affecting bacterial diversity during flares.

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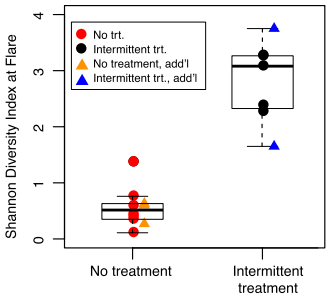

Figure 5. Shannon diversity index during flares. Circles indicate AD patients with a full longitudinal cycle (baseline, flare, and postflare). Triangles indicate flares from four additional (add'l) patients. Means between no-treatment (trt) and intermittent-trt tested with Wilcoxon rank-sum test ($P < 0.0025$, $n = 16$).

Discussion

AD represents a classic inflammatory skin disorder associated with a specific bacterial species. It is often managed with antimicrobial approaches and as such, AD serves as an example of a disorder in which the contribution of microorganisms to disease course can be examined via metagenomic and microbial community analyses. Using direct 16S rRNA gene sequencing, we defined and characterized the fluctuation of skin bacterial communities during the representative basal (baseline), exacerbation (flare), and resolution (postflare) phases of AD. Our microbiome analyses demonstrated a topographic and temporal shift in the predominant bacterial species and the bacterial diversity in the skin of pediatric AD patients.

A hallmark of AD is the chronic, recurrent flaring of intensely itchy skin. By serially analyzing AD patients, we demonstrated a strong association between worsening disease severity and lower skin bacterial diversity. We also determined that this microbiome shift is primarily localized to sites of disease predilection. Because many dermatologic disorders present with a stereotypical body distribution, the site-specificity of skin bacterial communities suggests that not only do particular ecological niches of the skin favor the growth of certain bacteria, but that communities of microbes are also important in the initiation and perpetuation of certain skin diseases.

Direct 16S rRNA gene sequencing of samples obtained during different disease states of AD provided a less-biased microbiological survey of the selective shifts of *Staphylococcus*, particularly of *S. aureus*. While *S. aureus* skin colonization and infection is a common feature of AD (Leyden et al. 1974), we have expanded these observations by demonstrating concurrent fluctuations of other skin bacteria. For example, *S. epidermidis* is the predominant *Staphylococcus* species in the skin of controls and has been considered a commensal in healthy skin (Iwase et al. 2010; Lai et al. 2010), with the ability to inhibit *S. aureus* (Iwase et al. 2010). Although results from previous culture-based studies are conflicting (Gloor et al. 1982; Williams et al. 1990; Higaki et al. 1999), our genomic analysis showed that the proportion of *S. epidermidis* consistently increased during no-treatment flares. The simultaneous prevalence of both *S. aureus* and *S. epidermidis* that we observed during disease flares provides new insights into the relationship between staphylococci. These staphylococcal species may share a mutualistic or commensal relationship to enhance common resistance to antimicrobial peptides (Peschel et al. 2001; Sieprawska-Lupa et al. 2004; Lai et al. 2007; Li et al. 2007) or enhance binding to exposed extracellular matrix proteins in inflamed AD skin (Nilsson et al. 1998; McCrea et al. 2000; Cho et al. 2001; Williams et al. 2002). Alternatively, the observed concordance may represent a compensatory or antagonistic mechanism of *S. epidermidis,* increasing in an attempt to control *S. aureus*.

In addition to the selective shifts in *Staphylococcus* species, the relative abundance of *Streptococcus, Corynebacterium,* and *Propionibacterium* species varied across the AD disease states, highlighting the complex relationships among bacteria and the importance of comprehensive investigation of microbial communities. *S. aureus* and *S. epidermidis* production of antibacterial compounds, including bacteriocins and antimicrobial peptides (Cogen et al. 2010a,b; Iwase et al. 2010; Lai et al. 2010; Joo et al. 2011), may contribute to the relative decrease in *Streptococcus, Corynebacterium,* and *Propionibacterium* species observed during AD flares. In addition to identifying changes in the skin commensal population, we determined that low-abundance genera including *Rothia* species were over-represented during different disease states in all patients. Furthermore, our data suggested that following treatment, increasing diversity of the microbiome, similar to the diversity observed in healthy skin, arises from a relative expansion of taxa already present in the skin microbial community with colonization of few additional new genera not present at baseline. Greater sampling depth in future studies may demonstrate that the majority of taxa present at baseline persist in low numbers during flares, then re-expand following treatment. Overall, the ability to globally examine bacterial community dynamics highlights a major advantage of microbial genomics, defining parameters needed for the development of new methods to maintain or enhance beneficial microbes and microbial diversity to promote health.

While bacterial culture methods are readily implemented and are relatively inexpensive in comparison to 16S rRNA gene sequencing, genomic methods are highly complementary, are rapidly dropping in cost and turnaround time, and can identify bacteria that are not easily cultivatable, yet may be important contributors to disease pathophysiology or progression. For easily cultured bacteria such as *S. aureus*, we have shown that the relative abundance of genomic sequences from nasal samples corresponds to the bacterial bioburden assessed using traditional cultivation techniques. However, these results strengthen assertions that genomic analyses of more difficult-to-cultivate bacteria represent the true relative abundance of those bacteria in a sample. No association was identified between the relative abundance of *S. aureus* genomic sequences in nasal samples and disease severity, suggesting that although the nares may serve as a reservoir for *S. aureus*, there may not be a dose-response between the abundance of nasal *S. aureus* and AD severity. Culture-based methods remain important and provide vital information on bioburden. However, the higher resolution and reduced bias of sequence-based and culture-independent microbiome studies in human diseases is of great benefit.

We determined that AD flares are characterized by low bacterial diversity in the absence of recent treatment. In contrast, intermittent or active treatment is associated with higher bacterial diversity. Although the impact of common AD therapies on *S. aureus* levels has been debated (Stalder et al. 1994; Williams et al. 1998; Ravenscroft et al. 2003), our results and prior studies (Huang et al. 2009) indicate that the clinical effectiveness of AD treatments does not rely on elimination of *S. aureus*. However, AD therapeutic modalities may act to recalibrate the diversity of the skin microbiome. For intermittent-treatment flare patients who used sporadic AD treatments in the week preceding flare sampling, the early shift toward a diverse microbiome in the presence of active clinical disease suggests that known effective AD treatments diversify the AD skin bacterial community. Intermittent-treatment flares likely

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represent a point in the continuum of the disease during transition from flare to postflare. However, the presence of active clinical disease during intermittent-treatment flares suggests that lesional skin requires continued intensive treatment to sufficiently reduce the inflammatory response toward that observed in the postflare state. Increases in diversity associated with AD treatments may be due to therapies that preferentially kill *S. aureus,* allowing relative expansion of other bacteria, promotion of microbes that control *S. aureus* predominance, or total reduction of skin microbes, followed by rapid repopulation with a diverse community.

In summary, specific AD disease states are characterized by concurrent and anticorrelated shifts in microbial diversity and proportion of *Staphylococcus*. Since untreated flares have reduced diversity and high *Staphylococcus* proportions as compared with baseline, postflares, and, notably, intermittent-treatment flares, we propose that increases in the proportion of *Staphylococcus* and reductions in microbial diversity precede worsening of AD disease severity as observed in no-treatment flares (Fig. 6). Relatively high *Staphylococcus* proportions observed in a few of the baseline timepoints could represent the pre-flare stage, in which staphylococcal levels increase, yet clinical disease has not begun to dramatically worsen. In contrast, reduction in *S. aureus* proportions and restoration of microbial diversity was observed in the intermittent-treatment flares, which are characterized as having clinically worsened disease. Based on this, we propose that use of AD treatments modify microbial diversity and proportions of *Staphylococcus*, but that consistent and continued treatment over a period of time is required to induce the "resolving flare" stage, which transitions into a restoration of full microbial diversity and low population levels of *Staphyloccocus*, typical of a true postflare.

With public health concerns regarding the development of antibiotic resistance, understanding how current treatments affect bacteria and subsequent disease activity will allow us to identify and develop directed therapies for AD that modify the skin microbiome, potentially reducing use of systemic antibiotics. Our findings highlight the importance of performing longitudinal microbiome studies to examine the influence of microbial communities on health and disease. Advances in technologies including whole-genome shotgun sequencing will continue to enhance our ability to probe microbial communities via metagenomics and functional metabolomics. The ability to interrogate microbial communities en toto and in situ has begun to provide insights regarding the important and diverse roles of microbes in human disorders and how shifts in microbial communities may improve human health.

Methods

Study design

A prospective natural history study was approved by the institutional review board of NHGRI, NIH. The study was designed to evaluate pediatric AD patients at three timepoints (baseline, flare, and postflare) to capture clinically distinct timepoints that characterize this chronic relapsing, remitting skin disease. Age-matched children were sampled at similar time intervals to serve as healthy pediatric controls.

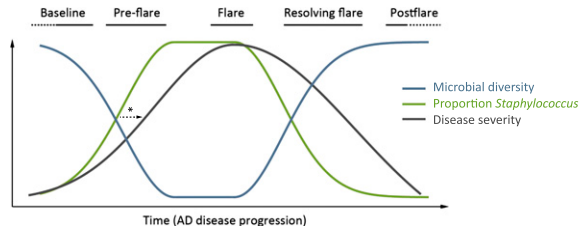

Figure 6. AD microbiome progression hypothesis. (*) Proposed relationship among shifts in skin microbial diversity, the proportion of *Staphylococcus*, and disease severity.

Inclusion criteria for AD patients included ages 2–18 yr, moderate-to-severe disease, presence of more than or equal to one affected antecubital or popliteal crease at enrollment, and ability to tolerate >7 d without topical AD treatments and >4 wk off both systemic antibiotics and corticosteroids. The diagnosis of AD was based on the UK Working Party definition (Williams et al. 1994a). Objective SCORAD as assessed by one individual (HHK) was used to determine study eligibility and disease status at each clinic visit (Williams et al. 1994b; Kunz et al. 1997; Oranje et al. 2007). Moderate-to-severe disease was defined by objective SCORAD ≥15 (range 0–83) (Oranje et al. 2007).

Exclusion criteria for all subjects included receiving investigational new treatments, ultraviolet light therapy, monoclonal antibodies, systemic immunosuppressants within 7 d or five half-lives (taking the longer time period) of skin sampling; a history of bone-marrow transplant or gene therapy; and clinically apparent underlying immunodeficiency. Exclusion criteria specific for AD patients included systemic antibiotics during the preceding 2 wk (except for post-flare timepoint). Exclusion criteria for healthy controls also included current or prior chronic skin disorders such as AD or psoriasis; asthma and allergic rhinitis, via International Study of Asthma and Allergies in Childhood questionnaire (Asher et al. 1995); other chronic medical conditions; and use of systemic antibiotics in the preceding 6 mo.

For AD patients, baseline was defined as usual and stable disease state and ability to tolerate >7 d without topical AD treatments to intended sample sites and >4 wk off both oral antibiotics and corticosteroids. Flare was defined as acute exacerbation of disease on any skin region prior to initiation of intensified AD treatment and without restriction of usual treatments >24 h prior to sampling. Subjects were instructed to promptly contact the research team when skin disease worsening was apparent. Flares were evaluated and sampled within 24 h of notification to avoid delay in intensified treatment. *Postflare* was defined as 10–14 d after the initiation of intensified AD treatment. Intensified AD treatment recommendations could include the following based on their typical regimen: dilute bleach baths (0.25 cup of 6% bleach into bath half filled with water for a final concentration of 0.0005%) three to four times per week, higher potency topical steroids twice daily, bland emollients twice daily, and, in certain cases of suspected clinical infection, systemic antibiotics prescribed by primary care physicians (Supplemental Table S1).

Subjects

AD patients aged 2–15 yr of age, and similarly aged healthy controls were recruited from the larger Washington, DC metropolitan region. Written informed consent was obtained from parents or guardians of all participating children. Complete medical and medication history and skin examination was performed at all clinic visits.

Sample collection

Skin preparation instructions included avoiding bathing and application of topical medications and emollients for 24 h at sampling sites prior to all sampling timepoints (baseline, flare, and postflare). Samples were obtained as described (Grice et al. 2009). Nasal cultures were obtained at each timepoint. Blood was drawn during the first visit.

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Cultures

Nasal cultures were performed by the Department of Laboratory Medicine (DLM), Clinical Center, NIH. Cultures were obtained with swabs (BBLtm CultureSwabTM, made by Copan for Becton, Dickinson and Company) and plated on three different growth mediums (Remel Products). After incubation, the colonies were counted and identified on the basis of gross and microscopic assessment after treatment with Gram's stain. Culture results were grouped into 0, 1–10, and >10 colonies of *Staphylococcus aureus* per sample.

DNA extraction and sequencing

Skin swabs were incubated in enzymatic lysis buffer and lysozyme (20 mg/mL) for 30 min at 37°C. Two 5-mm stainless steel beads (Qiagen) were added to the solution, placed in a Tissuelyser (Qiagen), and processed for 2 min at 30Hz.The standard protocol for the PureLink Genomic DNA kit (Invitrogen) was followed for all subsequent steps. rRNA genes (16S) were amplified from purified genomic DNA using primers 8F (5′-AGAGTTTGATCCTGGCT CAG-3′) and 1391R (5′-GACGGGCGGTGWGTRCA-3′). PCR amplification, cloning, and sequencing were performed as described (Grice et al. 2009). Sequence assembly, alignment, and chimera elimination were performed as described, with specified modifications (Grice et al. 2009; Schloss et al. 2009; Huse et al. 2010).

Analysis pipeline

Community analysis

Operational taxonomic units (OTUs) were identified and a distance matrix calculated using mothur software v14.0 (Schloss et al. 2009). No lane masking was applied. The distance matrix was then analyzed to calculate OTUs using the average neighbor algorithm. Community evenness and richness (Shannon diversity index "shannon"), shared community membership, and shared community structure (theta index "thetayc") at a 98% similarity cutoff were calculated using summary.shared() and summary. single() in mothur. Shannon diversity was calculated as follows: $H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} \ln \frac{n_i}{N}$, where S_{obs} = number of observed OTUs, n_i = number of individuals in OTU i, and N = total number of individuals in the community. The theta index, or Yue and Clayton measure of similarity between two community structures (Yue and Clayton 2005), was calculated as follows:

$$C_{\theta_{YC}} = \frac{\sum_{i=1}^{S_T} a_i b_i}{\sum_{i=1}^{S_T} (a_i - b_i)^2 + \sum_{i=1}^{S_T} a_i b_i},$$

where S_T = total number of OTUs in communities A and B, a_i = relative abundance of OTU i in community A, and b_i = relative abundance of OTU i in community B. To calculate alpha and beta diversity metrics minus *S. aureus*, we removed sequences classified as *S. aureus* from the analysis. Resulting samples were then subsampled 100× to the lowest number of sequences per group (Ac = 129, Pc = 31, N = 68, Vf = 133) and subsampled results were averaged. Results without subsampling yielded similar results (data not shown).

Because left and right symmetric sites generally exhibit low variability (Grice et al. 2009), the sites were grouped prior to analysis. For timepoint categories baseline, no-treatment flare, intermittent-treatment flare, and control calculations of Shannon diversity and theta indices, values were averaged for the antecubital and popliteal crease unless otherwise indicated. To estimate depth of sequence sampling, rarefaction curve data for each individual at each site was calculated using rarefaction.single in mothur, and individuals for each group were averaged to obtain group-wide rarefaction curves.

Genus–level analysis

Following sequence trimming, alignment, and other pre-processing steps as previously described, 16S rRNA sequences were classified to the genus level using the Ribosomal Database Project (RDP) classifications v1.0, training set 4 (Cole et al. 2009). For 14 major order-genera that represented >1% of total 16S rRNA sequences, relative abundances were computed by dividing sequence counts by the total sequences obtained for that site, with proportions for antecubital and popliteal crease averaged. Finally, custom scripts were generated for taxonomic classifications to the species level for staphylococcal species. Staphylococcal sequences were speciated by alignment to a curated collection of staphylococcal reference sequences from complete genome sequences and type strains. Each sequence was assigned a label based on the consensus call of sequence alignments with the lowest edit distance between a query and reference.

Statistics

All data are represented as mean ± SEM unless otherwise indicated. For controls with >1 timepoint, the relevant data was averaged for each control. As disease severity differed minimally from left to right symmetric sites, left and right values were averaged prior to statistical comparisons. AcPc indicates the mean of values obtained from the antecubital and popliteal crease for each individual (post-averaging of left and right symmetric sites, and for controls, also post-averaging of timepoints).

Analyses involved calculating partial correlations by using the relevant variables for each subject over all timepoints. Calculations were performed on Fisher-transformed r values. For statistical testing of comparisons of differences between groups, the nonparametric Wilcoxon rank sum test was used for all comparisons (wilcox.test in the statistical software program R). Where indicated, within-subjects analysis was performed by using the option "paired = T" in wilcox.test. All P-values were adjusted using p.adjust in R using Bonferroni (# comparisons ≤ 10) or false discovery rate (# comparisons > 10) corrections. For testing of significantly over-represented genera between the baseline, flare, and post-flare groups, we reduced the number of multiple comparisons by prefiltering the search to the seven genera that were present in 100% of baseline or no-treatment flares (for baseline-flare comparisons), or the 12 genera that were present in 100% of no-treatment flares or postflare (for flare-postflare comparisons) prior to performing a paired Wilcoxon rank sum test. For comparisons between baseline and postflare, we limited the search to genera that were present in >75% of individuals and performed a paired Wilcoxon rank sum test.

Data access

The sequence data from this study have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank) and can be accessed through BioProject ID 46333. Patient and sample metadata have been deposited in the controlled access database dbGaP under study accession phs000266.v1.p1.

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Supplemental Figures and Tables.

Supplemental Tables.

Table S1. Treatments used by AD patients prior to sampling

Table S2. Summary of full-length 16S rRNA sequence data for 16 AD patients and 11 controls

Table S3. Shannon diversity indices for 11 HCs and 12 AD patients

Table S4. Yue & Clayton theta and Jaccard similarity coefficients for 11 HC and 12 AD patients for the Ac and Pc

Table S5. Community shifts across timepoints, AD vs. HC

Table S6. Summary of Ribosomal Database Project (RDP) classifications for 11 HC and 12 AD

Table S7. Proportion of *Staphylococcus spp.* averaged for Ac and Pc

Table S8. Shannon diversity index, SCORAD, and proportion of *S. aureus* for the AcPc

Table S9. Comparison of *S. aureus* proportion with SCORAD for all sites

Table S10. Alpha and beta diversity metrics for communities with *S. aureus* removed

Table S11. Genera significantly overrepresented in AcPc present in all timepoints

Table S12. Summary of rarefaction analysis

Table S13. Culture data, SCORAD, and proportion of *S. aureus* proportion for nares

Table S14. Data for additional AD flare patients (n=4)

Supplemental Figures.

Figure S1. Comparisons of within-group and between-group bacterial community structure. Mean Theta (θ) similarity coefficients $\pm$ SEM of all pairwise comparisons of community structure between individuals within a control or AD disease group. θ=1 indicates identical community structure; θ=0 indicates dissimilar community structure. Pairwise comparisons are listed on the x-axis. See Fig. 2D for additional description. Values for Ac and Pc are averaged.

Figure S2. Bacterial taxonomic classifications of the skin microbiome over AD disease progression versus controls, by site. Each panel shows the mean relative abundance for 14 major bacterial phyla-order across the skin microbiomes for controls, AD B, F(no-treatment and intermittent-treatment), and PF, sorted from highest to lowest for relative *Staphylococcus* abundance, by site. For the ordering of subjects for each site, see Table S13.

Figure S3. Cross-site comparisons of abundant taxonomic classifications. In this correlation matrix, each point represents a taxonomic classification as in Figure S2. Site comparisons are indicated in the upper left with the partial correlations adjusting for disease state, which are colored as in the legend. Upper left quadrant of comparisons are between-site in AD patients; the bottom right quadrant of comparisons (in gray) are between-site comparisons of controls.

Figure S4. Alpha and beta diversity metrics for communities with *S. aureus* removed. *S. aureus* sequences were removed from the analysis and samples were normalized to have an equivalent number of sequences prior to calculating diversity statistics. To obtain an accurate representation of the community, sequences were subsampled 100X and diversity indices calculated were averaged. (A) Mean Shannon diversity ± SEM in controls and all AD disease states (Ac, Pc, volar forearm (Vf), nares (N)). (B) Heatmap of mean Theta similarity coefficients of pairwise comparisons of communities within or between individuals at each comparison indicated. Darker color indicates higher similarity: $\theta=0$ indicates that two samples have no species in common and $\theta=1$ indicates that two samples are identical. Site abbreviations are as in (A). (C) Relationship between objective SCORAD and Shannon diversity in the Ac (left) and Pc (right) of AD patients.

Figure S5. Rarefaction curves for the skin microbiota sampling at each site (Ac, Pc, Vf, and N) calculated as operational taxonomic units (OTUs) at a cutoff of 98% similarity. Each point represents mean ± SEM of all individuals at specified site and disease category.

Figure S6. *S. aureus* culture and genomic sequenced-based analyses in AD patients. Cultures were scored based on the following criteria: 0 colonies of *S. aureus* (negative for *S. aureus*), 1-10 colonies (light *S. aureus* bioburden), and >10 colonies per plate (moderate to heavy *S. aureus* bioburden). (*A*) Nose culture results for *S. aureus* compared to the sequenced-based relative abundance values of *S. aureus*. (*B*) Nose culture results for *S. aureus* compared to objective SCORAD disease severity scores. (*C*) Mean relative abundance for 14 major bacterial phyla-order across the skin microbiomes for controls, AD B, F(no-treatment (trt) and intermittent-trt), and PF, sorted from highest to lowest for relative *Staphylococcus* abundance. (*D*) Relative abundance of *S. aureus* genomic sequences for controls as compared to AD disease baseline, flare (no-trt and intermittent-trt), and post-flare.

Figure S1

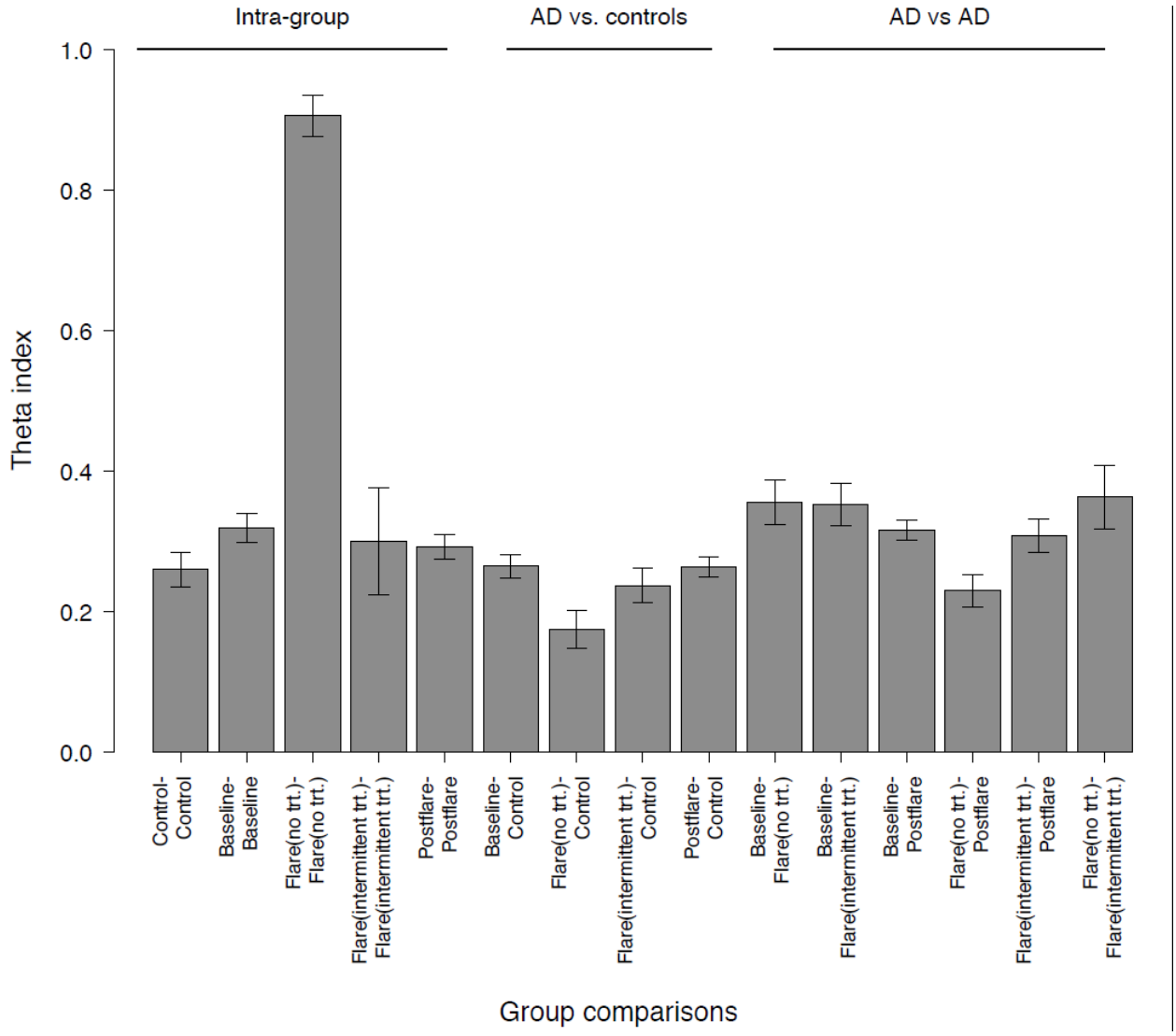

Intra-group
AD vs. controls
AD vs AD
Theta index
1.0
0.8
0.6
0.4
0.2
0.0
Control-Control
Baseline-Baseline
Flare(no trt.)-Flare(no trt.)
Flare(intermittent trt.)-Flare(intermittent trt.)
Postflare-Postflare
Baseline-Control
Flare(no trt.)-Control
Flare(intermittent trt.)-Control
Postflare-Control
Baseline-Flare(no trt.)
Baseline-Flare(intermittent trt.)
Baseline-Postflare
Flare(no trt.)-Postflare
Flare(intermittent trt.)-Postflare
Flare(no trt.)-Flare(intermittent trt.)
Group comparisons

Figure S2

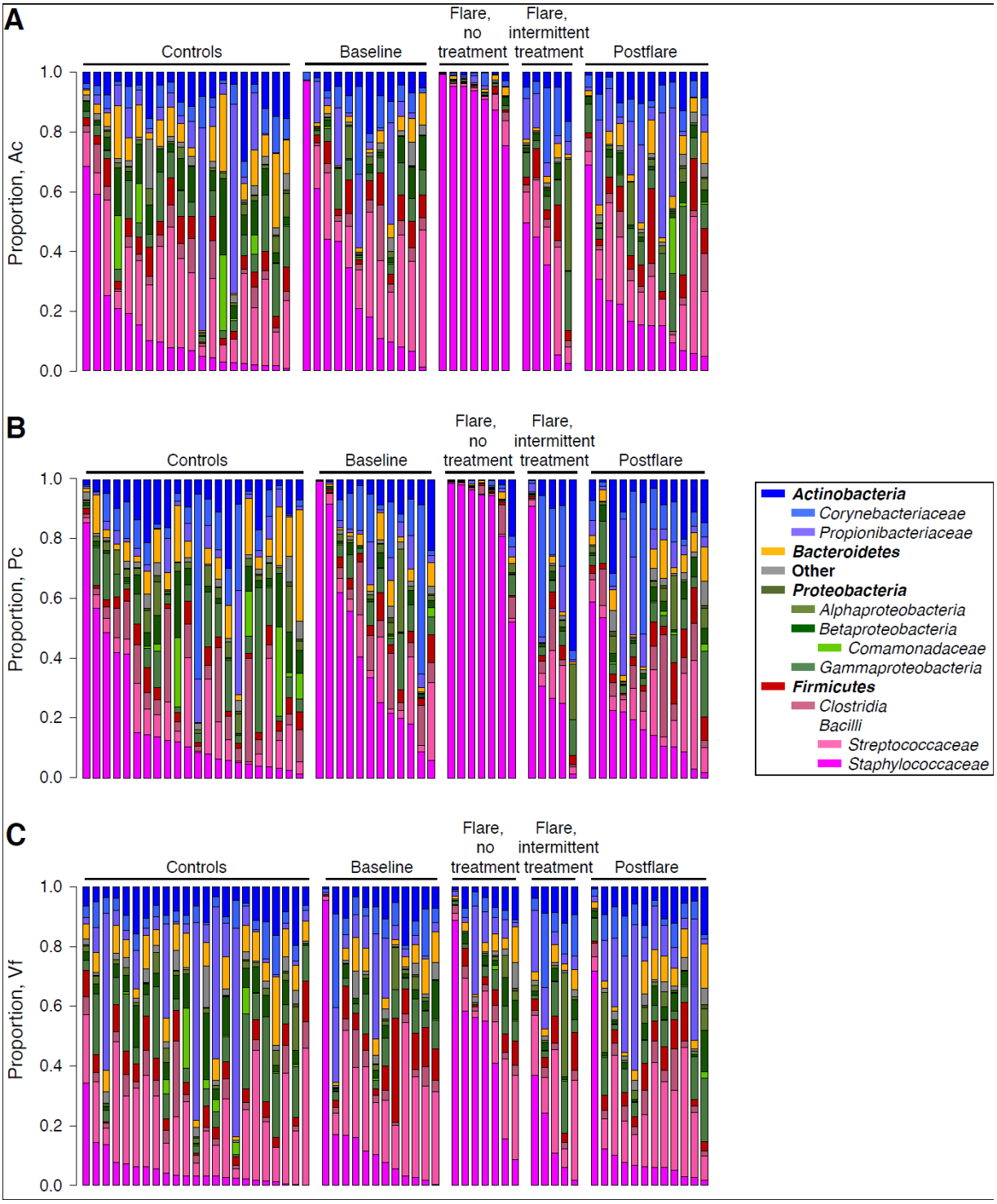

A
Controls
Baseline
Flare, no treatment
Flare, intermittent treatment
Postflare
Proportion, Ac
1.0
0.8
0.6
0.4
0.2
0.0
B
Controls
Baseline
Flare, no treatment
Flare, intermittent treatment
Postflare
Proportion, Pc
1.0
0.8
0.6
0.4
0.2
0.0
C
Controls
Baseline
Flare, no treatment
Flare, intermittent treatment
Postflare
Proportion, Vf
1.0
0.8
0.6
0.4
0.2
0.0
Actinobacteria
Corynebacteriaceae
Propionibacteriaceae
Bacteroidetes
Other
Proteobacteria
Alphaproteobacteria
Betaproteobacteria
Comamonadaceae
Gammaproteobacteria
Firmicutes
Clostridia
Bacilli
Streptococcaceae
Staphylococcaceae

Figure S3

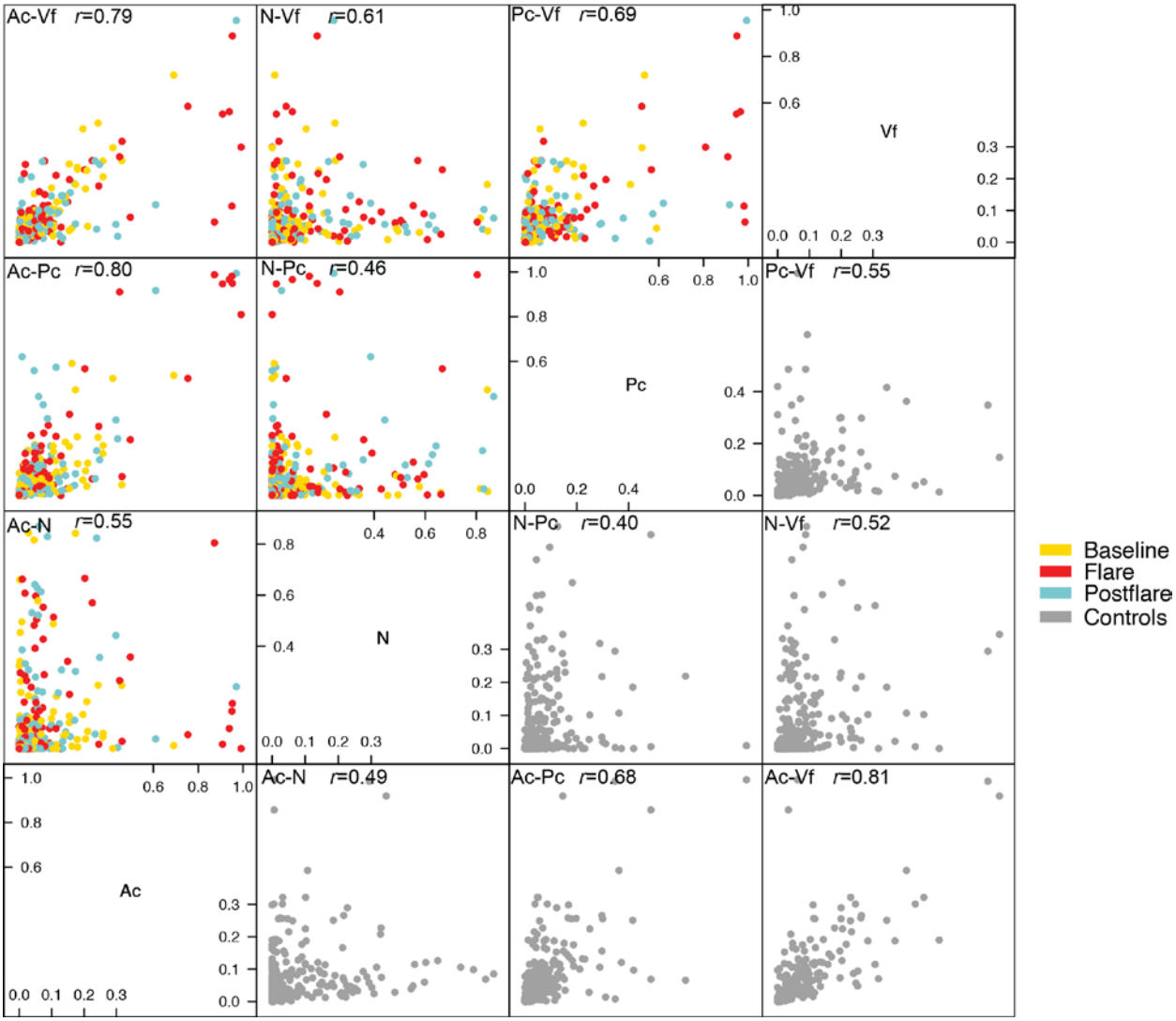

Ac-Vf r=0.79
N-Vf r=0.61
Pc-Vf r=0.69
Vf
Ac-Pc r=0.80
N-Pc r=0.46
Pc
Pc-Vf r=0.55
Ac-N r=0.55
N
N-Pc r=0.40
N-Vf r=0.52
Ac
Ac-N r=0.49
Ac-Pc r=0.68
Ac-Vf r=0.81
Baseline
Flare
Postflare
Controls

Figure S4

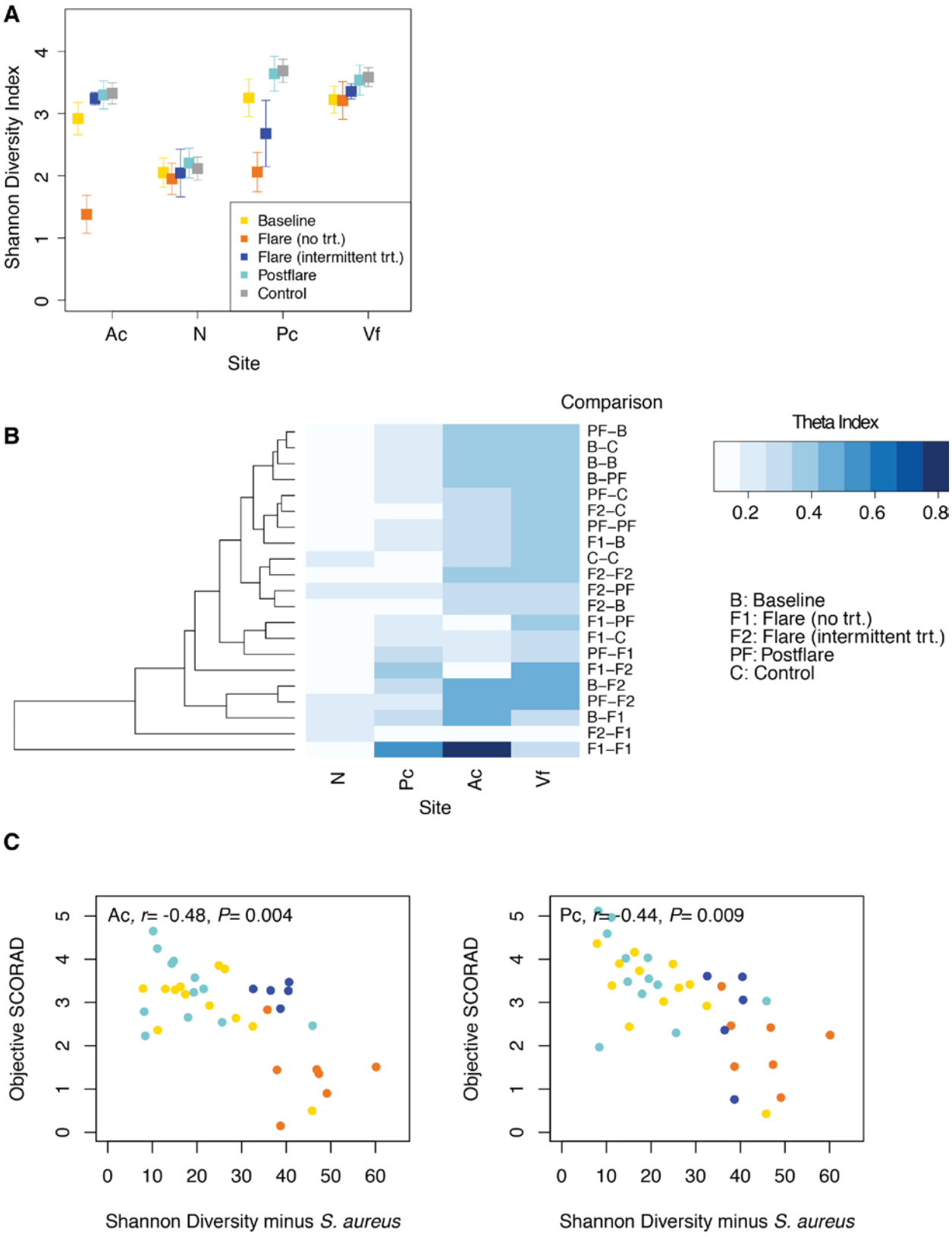

A
Shannon Diversity Index
0
1
2
3
4
Ac
N
Pc
Vf
Site
Baseline
Flare (no trt.)
Flare (intermittent trt.)
Postflare
Control
B
Comparison
PF–B
B–C
B–B
B–PF
PF–C
F2–C
PF–PF
F1–B
C–C
F2–F2
F2–PF
F2–B
F1–PF
F1–C
PF–F1
F1–F2
B–F2
PF–F2
B–F1
F2–F1
F1–F1
N
Pc
Ac
Vf
Site
Theta Index
0.2
0.4
0.6
0.8
B: Baseline
F1: Flare (no trt.)
F2: Flare (intermittent trt.)
PF: Postflare
C: Control
C
Ac, r= -0.48, P= 0.004
Objective SCORAD
0
1
2
3
4
5
0
10
20
30
40
50
60
Shannon Diversity minus S. aureus
Pc, r= -0.44, P= 0.009
Objective SCORAD
0
1
2
3
4
5
0
10
20
30
40
50
60
Shannon Diversity minus S. aureus

Figure S5

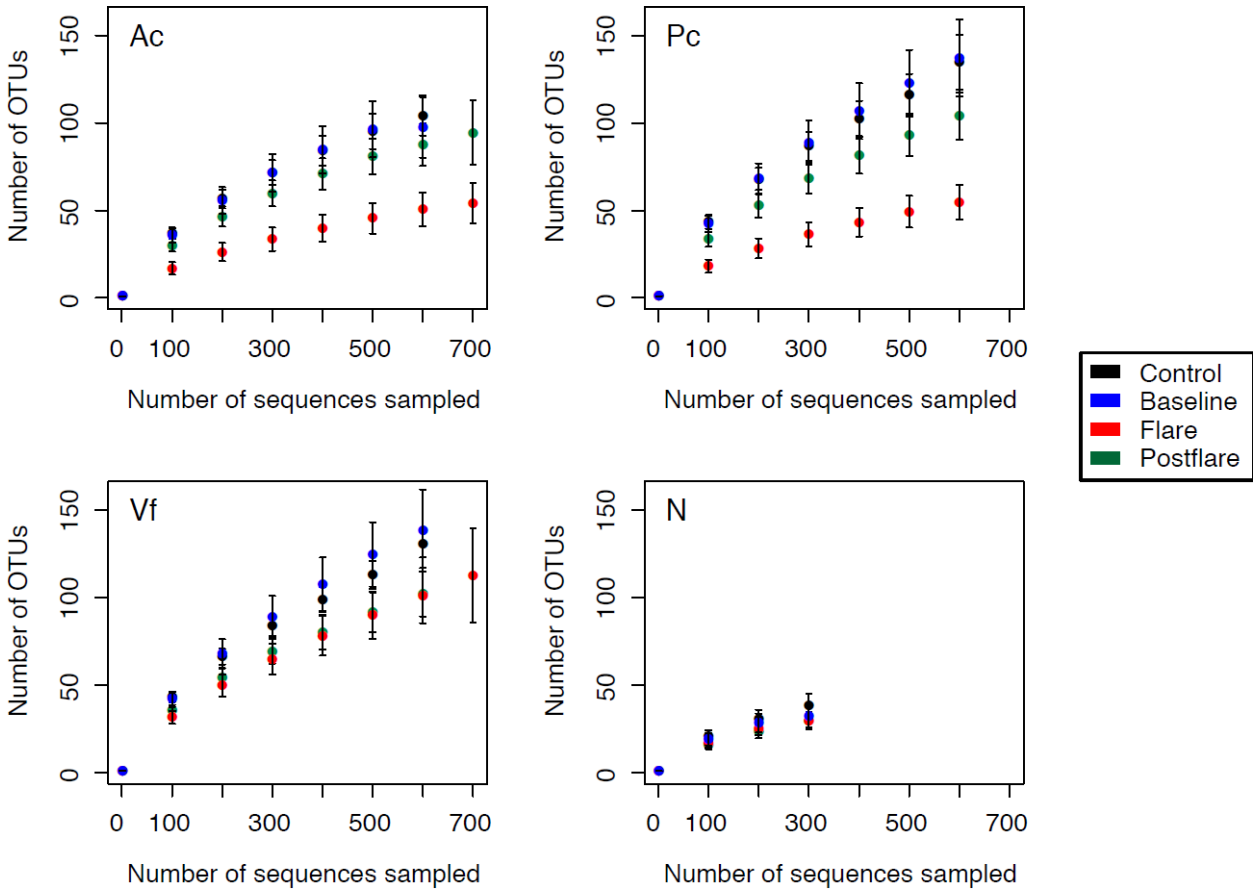
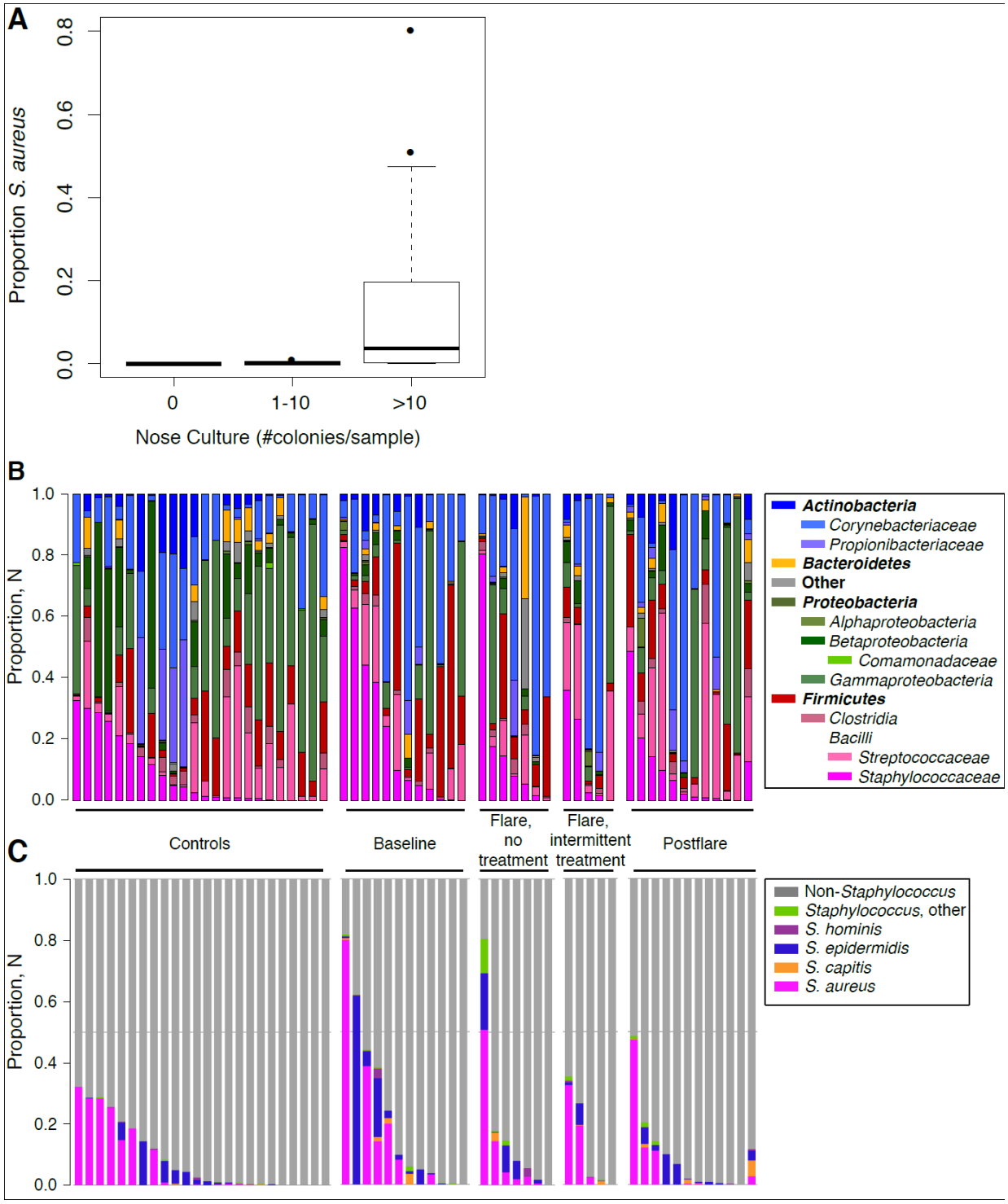

Ac
Pc
Vf
N
Number of OTUs
Number of sequences sampled
0
50
100
150
0
100
300
500
700
Control
Baseline
Flare
Postflare

Figure S6


A
Proportion S. aureus
0.0
0.2
0.4
0.6
0.8
0
1-10
>10
Nose Culture (#colonies/sample)
B
Proportion, N
1.0
0.8
0.6
0.4
0.2
0.0
Controls
Baseline
Flare, no treatment
Flare, intermittent treatment
Postflare
Actinobacteria
Corynebacteriaceae
Propionibacteriaceae
Bacteroidetes
Other
Proteobacteria
Alphaproteobacteria
Betaproteobacteria
Comamonadaceae
Gammaproteobacteria
Firmicutes
Clostridia
Bacilli
Streptococcaceae
Staphylococcaceae
C
Proportion, N
1.0
0.8
0.6
0.4
0.2
0.0
Non-Staphylococcus
Staphylococcus, other
S. hominis
S. epidermidis
S. capitis
S. aureus