

Initial Evidence of Distinguishable Bacterial and Fungal Dysbiosis in the Skin of Patients with Atopic Dermatitis or Netherton Syndrome ^{JID}Open



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Atopic dermatitis (AD) is an inflammatory skin disease in which epidermal barrier impairment, often owing to *FLG* null mutations, precedes immune hyperresponsiveness. Ichthyosis vulgaris is characterized by *FLG* null mutations and noninflamed dry skin. Netherton syndrome (NS), caused by *SPINK5* null mutations, is characterized by generalized erythroderma with scaling and atopic manifestations. The goal of this work was to evaluate associations between specific skin disease features, such as ichthyotic and/or atopic manifestations, and the skin bacterial and fungal microbiota. Taxon diversity showed greater variation in the bacterial microbiota than in the fungal microbiota in the skin diseases. The relative abundances of Firmicutes (*Staphylococcus*) and Actinobacteria (*Corynebacterium*) were augmented in ichthyosis vulgaris, AD, and NS, whereas those of Proteobacteria/Enhydrobacter and Bacteroidetes were reduced, regardless of body site. Furthermore, proportions of *Staphylococcus* were correlated with transepidermal water loss and serum IgE levels. Nevertheless, the skin of patients with low to mild AD was overcolonized with *Staphylococcus epidermidis* and not with *Staphylococcus aureus*. Ascomycota were increased in both AD and NS, but from expansion of different fungal species. Finally, the expansion of pathogenic bacteria in AD and NS might be supported by surrounding fungi. Thus, distinguishable bacterial and fungal skin dysbiosis in AD, NS, and ichthyosis vulgaris emphasizes disease-specific pathomechanisms.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease characterized by eczematous lesions, pruritus, and skin dryness, with high prevalence worldwide (Odhambo et al., 2009; Weidinger et al., 2018). It is commonly associated with allergic conditions such as allergic rhinitis and asthma (Leung and Guttman-Yassky, 2014). Epidermal barrier impairment in AD—resulting from null mutations in the *FLG* gene in about 30% of cases that give rise to ichthyosis vulgaris (IV)—precedes immune hyperresponsiveness (Kelleher et al., 2015; Palmer et al., 2006; Weidinger et al., 2007). In nonlesional AD, a T helper type (Th) 2 immune response predominates, whereas in lesional AD a mixed Th1/Th2/Th17/Th22/Th9 immune response has been described (Leung and Guttman-Yassky,

2014). Clinical manifestations observed in AD partially overlap with those observed in ichthyotic patients with Netherton syndrome (NS) (e.g., erythema, high serum IgE, and eosinophilia) (Czarnowicki et al., 2018; Furio and Hovnanian, 2014; Paller et al., 2017). NS is caused by mutations in the *SPINK5* gene encoding a protein called LEKT1, a serine peptidase inhibitor. The phenotype of patients with NS typically is more severe because they have major cutaneous barrier defects (Furio and Hovnanian, 2014; Malik et al., 2019). In contrast, IV skin exhibits only subclinical signs of inflammation despite major skin dryness (Gruber et al., 2011; Oji et al., 2009).

AD skin lesions exhibit reduced alpha-diversity of the bacterial skin community owing to enrichment of *Staphylococcus*, which are suspected to contribute to disease aggravation and flares (Kennedy et al., 2018; Kong et al., 2012; Meylan et al., 2017; Paller et al., 2019). In *FLG*-deficient epidermis, regardless of the presence of AD, a low frequency of proteolytic Gram-positive anaerobic cocci, together with underrepresentation of bacterial taxa that utilize histidine as a substrate, has been observed (Zeeuwen et al., 2017a). Previous work has shown trends to increased alpha- and beta-diversities of fungi at the genus and species levels in AD (Han et al., 2018; Jagielski et al., 2014; Zhang et al., 2011). Although individuals with IV are prone to develop skin fungal infection, very little is known about the composition of their fungal microbiota (Agostini et al., 1992; Hoetzenecker et al., 2007). The bacterial skin microbiota of patients with NS has been recently studied (Williams et al., 2020). Moreover, most studies have been carried out in patients with severe AD;

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Abbreviations: AD, atopic dermatitis; CTRL, healthy control; FDR, false discovery rate; IV, ichthyosis vulgaris; NS, Netherton syndrome; PF, popliteal fossae; SR, lower scapular region; Th, T helper type; WT, wild-type

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thus, little is known in patients with milder disease. We analyzed the skin bacterial and fungal composition of patients with low to mild AD with or without *FLG* mutations (in both nonlesional and lesional skin), IV, or NS at two different body sites with the goal of evaluating the effects of specific skin disease features, such as ichthyotic and/or atopic manifestations, on the bacterial and fungal microbiota.

RESULTS

Proportions of Firmicutes/*Staphylococcus* and Actinobacteria/*Corynebacterium* are augmented in IV and NS

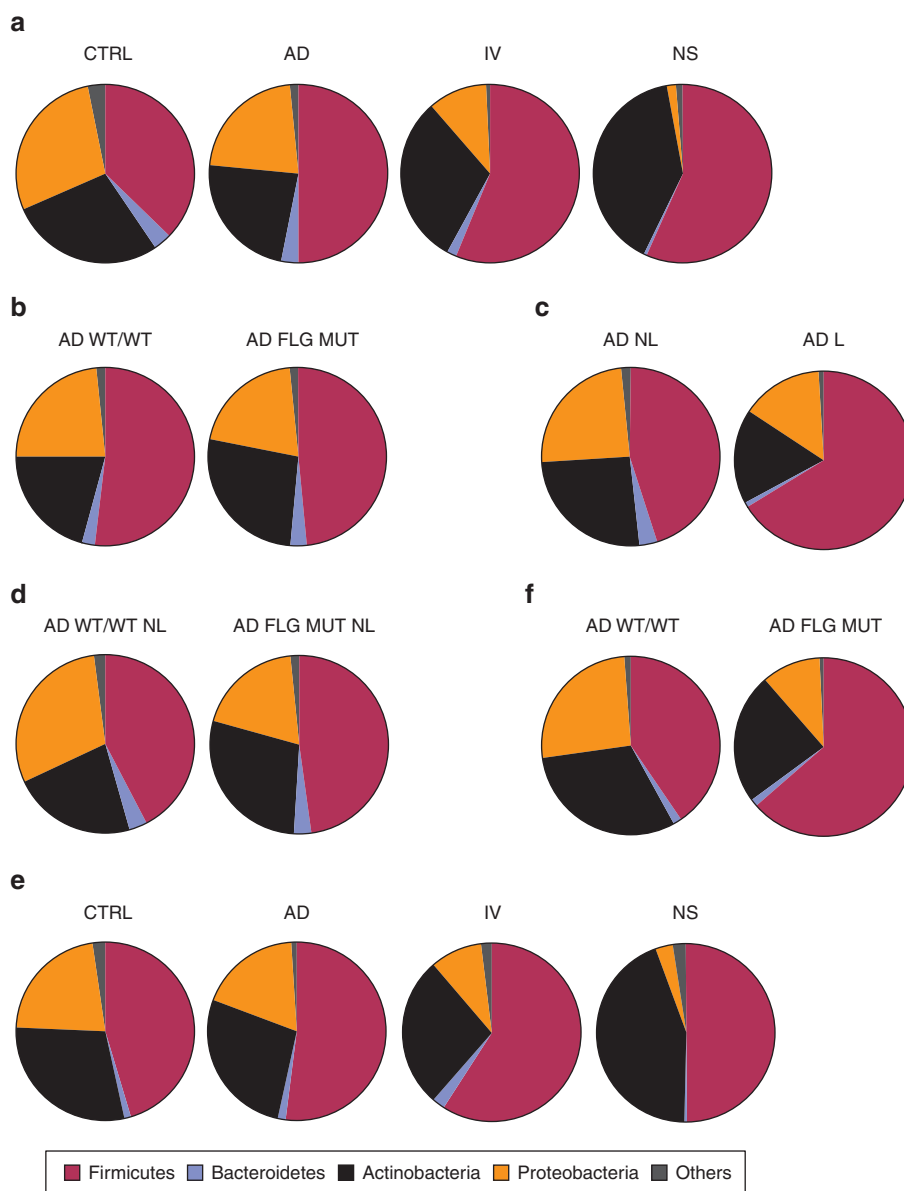
We compared the composition of the skin microbiota in healthy controls (CTRL, $n = 9$) and patients with AD ($n = 17$), IV ($n = 4$), and NS ($n = 3$) in two different body locations, the popliteal fossae (PF), which is a privileged site of AD skin lesions, and the lower scapular region (SR) (Supplementary Figure S1). Each disease exhibited a specific bacterial signature, irrespective of body site. Firmicutes (Bacilli, Bacillales, Staphylococcaceae, and *Staphylococcus*) were augmented in AD and IV when compared with CTRLs, and even more so in NS, at the expense of Proteobacteria/Enhydrobacter (Figure 1a and e, Figure 6a, Supplementary Figures S2a–S5a and S2e–S5e). Moreover, in NS, Clostridia were markedly reduced (Supplementary Figure S2a and e), suggesting potential competition within the Firmicutes. Lactobacillus were not significantly altered in AD and IV or in AD skin lesions when compared with CTRLs but nearly disappeared in NS (Supplementary Figure S5a and e). Actinobacteria were increased in IV and to an even greater extent in NS as a result of expansion of *Corynebacterium* when compared with other groups (Figure 1a and e, Figure 6a, Supplementary Figure S5a and e). In PF of patients with AD, the relative abundance of Firmicutes/*Staphylococcus* was further increased in association with skin lesions (Baurecht et al., 2018) but not by *FLG* null mutations (Figure 1b–d, Figure 6a, Supplementary Figure S5b–d), whereas in SR, *FLG* null status was associated with increased Firmicutes/*Staphylococcus* (Figures 1f and 6a, Supplementary Figure S5f). We found low proportions of *Propionibacterium* (*Cutibacterium*) in SR (Supplementary Figure S5e), a nonsebaceous area of the back (Smith and Thiboutot, 2008), in agreement with previous work (Bouslimani et al., 2015; Ross et al., 2017); however, a technical pitfall owing to a bias of primer pairs (modified 515f–806r) cannot totally be excluded (Meisel et al., 2016; Zeeuwen et al., 2017b). Thus, these results show that the bacterial dysbiosis, observed in all patients, similarly affects two distinct body sites, at least at the phylum level and, to some extent, at other levels. Moreover, the expansion of Firmicutes/*Staphylococcus* is promoted in AD skin lesions and in patients with loss-of-function mutations in genes encoding important structural and functional proteins of the stratum corneum such as *FLG* and *LEKTI*. This suggests that the expansion of *Staphylococcus* might correlate with the severity of the epidermal barrier impairment, irrespective of its genetic or mechanical (scratching) origin, rather than with skin inflammation; IV displays only subclinical signs of inflammation but still has a high relative abundance of *Staphylococcus*. In contrast, *Corynebacterium* might expand preferentially on ichthyotic skin. The effects of *FLG* mutations

on *Staphylococcus* in AD were different in PF and SR, suggesting that the expansion of *Staphylococcus* in patients with AD with *FLG* mutations might be modulated by skin properties or the microenvironment (e.g., local fungal populations).

Ascomycota and especially Cladosporium is associated with atopic manifestations

The ITS region has been recommended and widely accepted as a suitable barcode to analyze fungal microbiota (Findley et al., 2013; Walters et al., 2015), and the ITS1F and ITS2 primer pair has recently been modified to provide greater specificity (Smith and Peay, 2014; Walters et al., 2015). Our results are in line with previous work showing that skin fungal composition is site-dependent and displays frequent outliers (Figures 2 and 6b, Supplementary Figures S6–S9) (White et al., 2014). In PF, Ascomycota predominated over Basidiomycota, but the reverse was true in SR (Figure 2). Moreover, in AD and NS, Ascomycota relative abundance was enhanced when compared with CTRL in both PF and SR (Figure 2a and e). In IV, the relative abundances of Ascomycota, Basidiomycota, and Chytridiomycota were very similar between body sites (Figure 2a and e), suggesting that the lack of *FLG*, in a noninflammatory context, could be a strong determinant of fungal communities. In AD, skin lesions and *FLG* mutations had no significant effects on fungal relative abundance (Figure 2b–d, f). Moreover, the fungal microbiota in AD and NS exhibited similar variations at the phylum level when compared with CTRL, suggesting that the expansion of Ascomycota was associated with pathologic commonalities, such as atopy, observed in both AD and NS. In line with this, we found a positive correlation between serum IgE levels and proportions of Ascomycota (Supplementary Table S2). Furthermore, strong effects on fungal communities, owing to *FLG* mutations (IV in Figure 2a and e), were masked by physiopathological changes occurring in AD skin (Figure 2b–d). All results on fungal classes, orders, families, genera, and species were compiled in Supplementary Figures S6–S10. For the PF site, AD and NS displayed similar profiles at the class level (e.g., a similar marked increase in Dothideomycetes) (Supplementary Figure S6a) but not at other levels (Supplementary Figures S7a–S9a, S10b), with the exception of Cladosporium, which was increased in AD and NS when compared with CTRL and IV (Supplementary Figure S9). Of note, Cladosporium (Ascomycota) was positively correlated with transepidermal water loss in PF (Supplementary Table S2). Moreover, IV exhibited a fungal profile different from that of CTRL, AD, and NS at all levels (Figure 6b, Supplementary Figures S6a–S9a, S10b). *FLG* mutations in AD had little or no effect in contrast to AD lesions (Supplementary Figures S6–S9b–d), which particularly favored *Strobilurus*, *Saccharomyces*, and *Kabatiella* (Supplementary Figure S9b–d). In SR, *Malassezia* was reduced in all disease groups when compared with CTRL (Figure 6b, Supplementary Figure S9e), owing to the expansion of *Cladosporium*, *Gymnopilus*, and *Penicillium* in NS and of *Cladosporium* and to the apparition of *Leptosphaeria* and *Debaryomyces* in AD (Supplementary Figure S9e). However, this was only observed in AD wild-type (WT/WT)

Figure 1. Proportions of Firmicutes/ *Staphylococcus* and Actinobacteria/ *Corynebacterium* are augmented in IV and NS. Mean relative abundance of bacterial phyla in (a–d) the popliteal fossae and (e, f) the lower scapular region. CTRL, n = 9; AD, n = 17; IV, n = 4; NS, n = 3; AD WT/WT, n = 9; AD *FLG* MUT, n = 8; AD NL, n = 13; AD L, n = 4; AD WT/WT NL, n = 6; AD *FLG* MUT NL, n = 7. AD, atopic dermatitis; CTRL, healthy control; IV, ichthyosis vulgaris; L, lesional; MUT, mutation; NL, nonlesional; NS, Netherton syndrome; WT, wild-type.



(Supplementary Figure S9f). Relative abundance of each *Malassezia* species in both PF and SR was reported in Supplementary Figure S11 and showed disease-specific interspecies competition. Altogether, these results show a greater fungal diversity in AD than in CTRL (Han et al., 2018; Jagielski et al., 2014; Zhang et al., 2011), IV, and NS. Moreover, they highlight the clearest alterations in skin fungal composition in NS. In addition, they unravel a correlation between serum IgE levels and Ascomycota, a fungal phylum increased in AD and NS, largely because of the expansion of *Cladosporium*.

Genus diversity shows greater variation in bacteria than in fungi in AD, IV and NS

We then studied in more detail bacterial and fungal genera in PF and SR. The results show diminished diversity of bacterial genera in patients with IV and a more drastic reduction of those parameters in patients with NS (0.34-fold,

false discovery rate [FDR] < 0.05) when compared with CTRL and patients with AD in PF and, to a lesser extent, in SR (Figure 3a). Moreover, *FLG* mutations apparently exert no significant effects (Clausen et al., 2018) (Figure 3b). AD skin lesions were associated with reduction of alpha-diversity (0.73-fold, FDR > 0.1) (Figure 3b), as reported previously (Kennedy et al., 2018). Of note, the bacterial diversity was reduced in NS, IV, and AD skin lesions (Figure 3), suggesting a tight relationship between epidermal barrier abnormalities and the overrepresentation of specific bacteria (e.g., *Staphylococcus*) on the skin. The diversity of fungal genera was only significantly altered in AD SR (12.20-fold, FDR < 0.05) and showed a trend in IV (1.48-fold, FDR > 0.1) and NS (1.63-fold, FDR > 0.1) when compared with CTRL (Figure 4a). In PF, AD lesions were associated with reduced diversity of fungal genera (0.73-fold, FDR < 0.05) (Figure 4b). Thus, our results show that bacterial diversity has greater variation than does fungal diversity in AD, IV, or

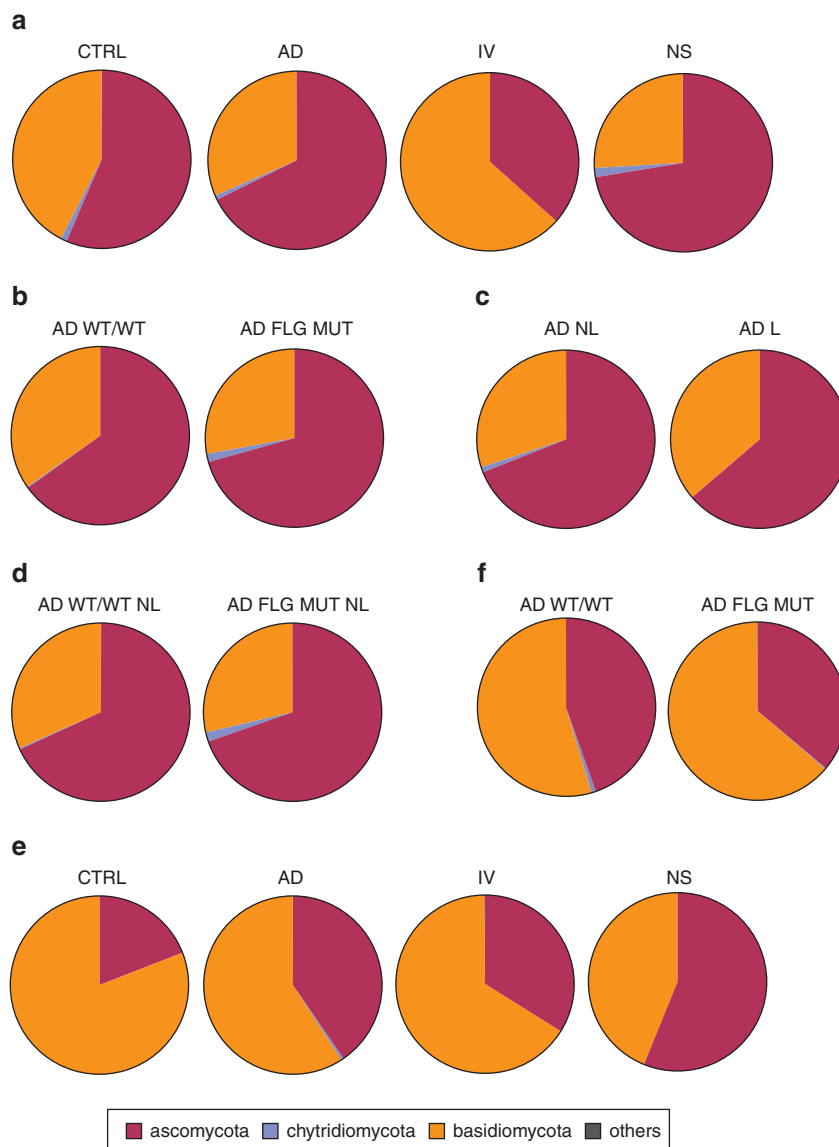


Figure 2. Ascomycota and especially *Cladosporium* is associated with atopic manifestations. Mean relative abundance of fungal phyla in (a–d) the popliteal fossae and (e, f) the lower scapular region. CTRL, n = 9; AD, n = 17; IV, n = 4; NS, n = 3; AD WT/WT, n = 9; AD *FLG* MUT, n = 8; AD NL, n = 13; AD L, n = 4; AD WT/WT NL, n = 6; AD *FLG* MUT NL, n = 7. AD, atopic dermatitis; CTRL, healthy control; IV, ichthyosis vulgaris; L, lesional; MUT, mutation; NL, nonlesional; NS, Netherton syndrome; WT, wild-type.

NS. Moreover, AD skin lesions reduce both bacterial and fungal diversity. We then studied the beta-diversity of the bacterial and fungal genera in all our samples by using principal coordinate analysis based on Bray-Curtis dissimilarities. Results show that NS clustered separately from CTRL, AD, and IV in PF and SR for bacteria and mainly in SR for fungi (Figure 5). Moreover, AD and IV diverged similarly from CTRL in PF for bacteria and in PF and SR for fungi (Figure 5). Additional analysis showed that AD with *FLG* mutation clustered differently from AD WT/WT for bacteria in SR, but not in PF, and not for fungi (Supplementary Figure S12a). Lesional AD and NS clustered separately from each other and from other groups, whereas nonlesional AD clustered with CTRL and, to a certain extent, with IV in PF for bacteria but not for fungi (Supplementary Figure S12b). These results suggest that the severity of epidermal barrier impairment, regardless of its origin, might have a greater role in shaping the composition of the skin bacterial microbiota than in controlling the composition of the fungal microbiota.

No superinfection with *Staphylococcus aureus* in low to mild AD

Shotgun sequencing, species-specific PCR, or single-locus sequence typing are privileged technical approaches to study bacterial species. Modified 515f–806r primers, as well as other primer sets, might be used to perform analysis down to the species level. However, results might lack stringency to detect specific bacterial species (Castelino et al., 2017; Conlan et al., 2012; Grice et al., 2009; Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012; Kong, 2016; Meisel et al., 2016; Palmer et al., 2007; Zeeuwen et al., 2017a, 2017b) and require complementary analyses with other techniques. Of note, *Streptococcus pneumoniae* and *Propionibacterium acnes* might be underrepresented with modified 515f–806r primers (Meisel et al., 2016). Results on bacterial species present in PF and SR were summarized in Supplementary Figure S10a. We found relative proportions of *S. aureus* under the cutoff of 0.5% in all samples. To validate these data, we carried out additional PCR (data not shown) and quantitative PCR (Supplementary Table S3). Lesional AD skin

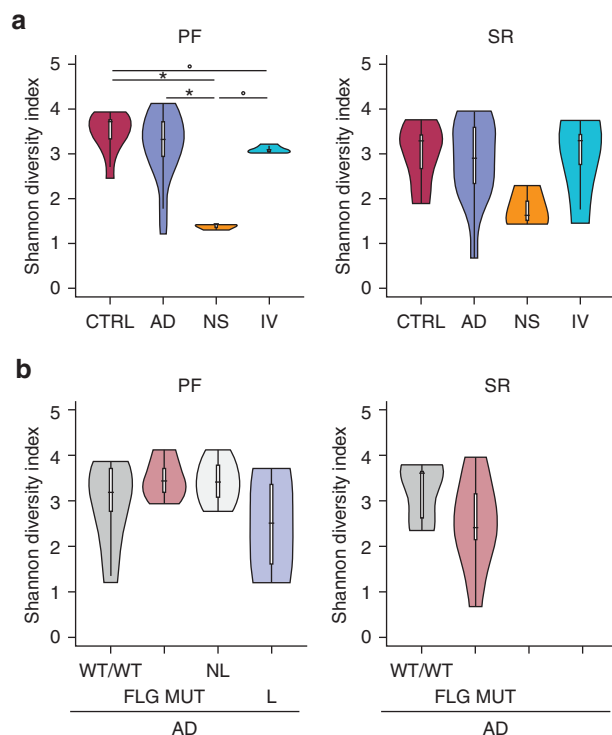


Figure 3. Bacteria diversity in AD, IV, or NS. The diversity of bacterial genera in PF and SR were evaluated with the Shannon diversity index, and differences were assessed with the Wilcoxon rank-sum test (P -values were adjusted for multiple testing). (a) CTRL, $n = 9$; AD, $n = 17$; IV, $n = 4$; NS, $n = 3$. (b) AD WT/WT, $n = 9$; AD FLG MUT, $n = 8$; AD NL, $n = 13$; AD L, $n = 4$. o, $0.05 \leq \text{adj. } P < 0.1$; *, adj. $P < 0.05$. AD, atopic dermatitis; adj., adjusted; CTRL, healthy control; IV, ichthyosis vulgaris; L, lesional; MUT, mutation; NL, nonlesional; NS, Netherton syndrome; PF, popliteal fossae; SR, lower scapular region; WT, wild-type.

was not enriched in *S. aureus* in our cohort of patients with low to mild AD, and *S. aureus* was detected on the skin of two patients with NS at low levels (Supplementary Table S3). In contrast, we observed a significant increase in *Staphylococcus epidermidis* and *Staphylococcus hominis* in NS when compared with all other groups in PF but not in SR; similar results, although less stringent, pertain to AD and IV (Supplementary Figure S13a and b). Quantitative PCR additionally showed increased amounts of *S. epidermidis* in NS and lesional AD when compared with nonlesional AD, CTRL, and IV, as well as in AD with FLG mutations when compared with AD WT/WT (Supplementary Table S3). Moreover, amounts of *P. acnes* were augmented in AD and IV and even more so in NS when compared with CTRL and higher in AD with FLG mutations when compared with AD WT/WT in PF (Supplementary Table S3). Of note, *Corynebacterium tuberculoostearicum*, a lipophilic bacterium involved in nosocomial infections, was markedly increased in PF of two patients with NS (Supplementary Figure S13c). These results show an expansion of *S. epidermidis* and *S. hominis* in low to mild AD, including in skin lesions.

The expansion of pathologic bacteria in patients with AD or NS might be supported by fungi

Although the production of antimicrobial peptides and lipids by skin microbes has been demonstrated to reciprocally

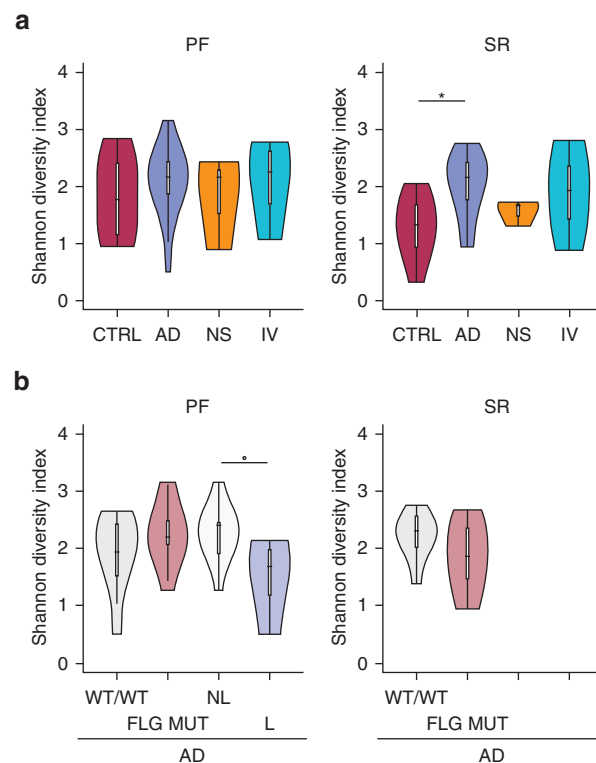


Figure 4. Fungal diversity in AD, IV, or NS. The diversity of fungal genera in PF and SR were evaluated with Shannon diversity index and differences with a Wilcoxon rank-sum test (P -values were adjusted for multiple testing). (a) CTRL, $n = 9$; AD, $n = 17$; IV, $n = 4$; NS, $n = 3$. (b) AD WT/WT, $n = 9$; AD FLG MUT, $n = 8$; AD NL, $n = 13$; AD L, $n = 4$. o, $0.05 \leq \text{adj. } P < 0.1$; *, adj. $P < 0.05$. AD, atopic dermatitis; adj., adjusted; CTRL, healthy control; IV, ichthyosis vulgaris; L, lesional; MUT, mutation; NL, nonlesional; NS, Netherton syndrome; PF, popliteal fossae; SR, lower scapular region; WT, wild-type.

control their growth (Nakatsuji et al., 2017; Nakatsuji and Gallo, 2012), details on coexisting pathogenic skin bacteria and fungi might shed new light on the establishment of skin dysbiosis in AD, IV, and NS. Thus, we next performed correlation analyses between bacterial and fungal genera (Supplementary Table S4, Supplementary Figure S14). *Staphylococcus* was positively correlated with *Cladosporium* in PF, both increased in patients with atopy. Moreover, potentially pathogenic bacteria (*Peptoniphilus*, *Prevotella*, *Fingoldia*, and *Streptococcus*) were correlated with *Penicillium* or *Eupenicillium*. These results might emphasize an insensitivity of these bacteria to antimicrobial factors (e.g., penicillin) produced by *Penicillium* and/or an opportunistic expansion because of a growth inhibition of other bacteria. For the PF site, Supplementary Figure S15 shows, inter alia, positive correlations between *C. tuberculoostearicum*, a lipophilic bacterium, and *Malassezia globosa*, exhibiting high lipase activity (Dawson, 2007); both were increased in NS. Moreover, *S. epidermidis* and *S. hominis* were positively correlated with *Cladosporium* spp. and with *Debaryomyces hansenii* (all being overrepresented in PF from patients with AD and NS), both of which might provide *Staphylococcus* with substrates (e.g., palmitic and oleic or pyruvic acid, respectively) (Krebs, 1937; Nguyen et al., 2015). Moreover, *Staphylococcus* was positively, whereas *Rothia* was negatively, correlated with

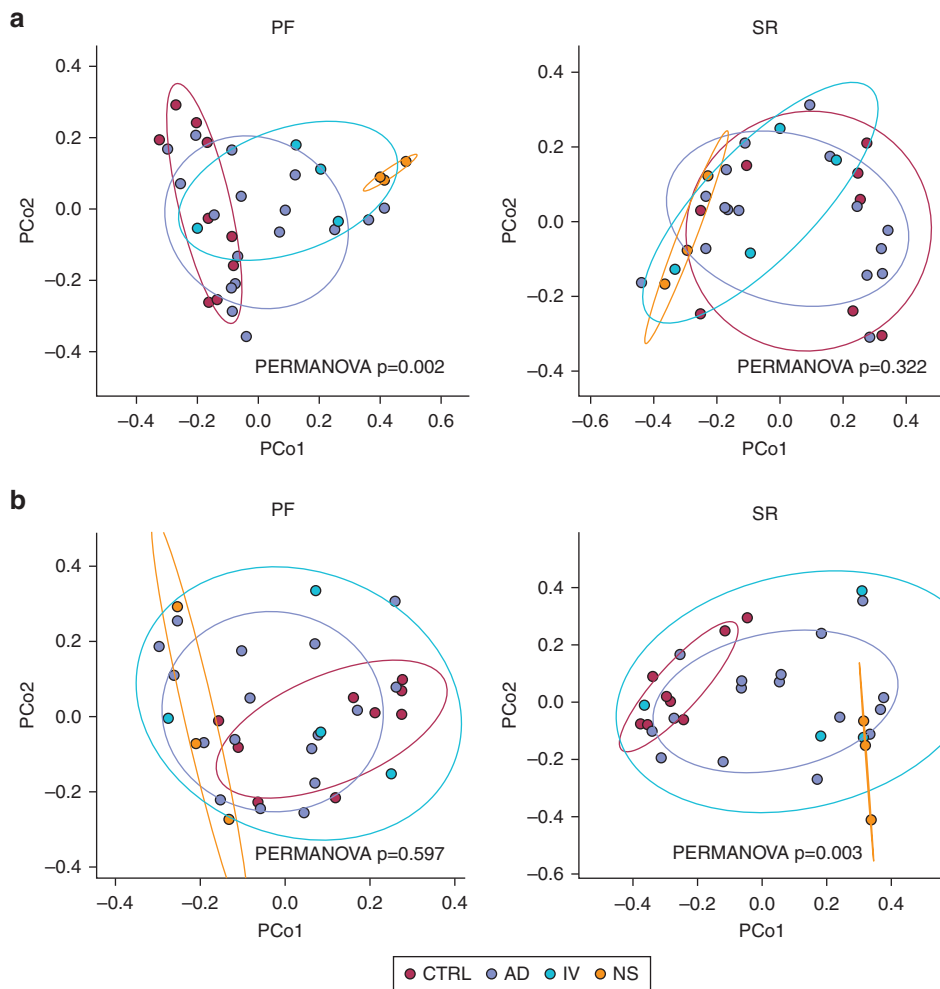


Figure 5. Severity of epidermal barrier impairment is associated with bacterial β -diversity. PCoA based on unweighted Unifrac distances and significances were evaluated with PERMANOVA as described in Methods in PF and SR for (a) bacterial and (b) fungal clustering. CTRL, $n = 9$, orange; AD, $n = 17$, red; IV, $n = 4$, blue; NS, $n = 3$, green. AD, atopic dermatitis; CTRL, healthy control; IV, ichthyosis vulgaris; NS, Netherton syndrome; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariable analysis of variance; PF, popliteal fossae; SR, lower scapular region.

transepidermal water loss and serum IgE levels (Supplementary Table S2), suggesting that severe epidermal barrier impairment might favor the expansion of specific potentially pathogenic bacteria at the detriment of others. These data also suggest a superior capacity of *Staphylococcus* to proliferate on the skin of patients with AD or NS, potentially by using fungal-derived lipid substrates; *Rothia* utilize sugars. Thus, the expansion of *Staphylococcus* in patients with AD or NS might be supported by surrounding fungi.

DISCUSSION

In this work, we discovered a more pronounced increase in the proportions of Firmicutes (*Staphylococcus*) in NS, IV, and lesional AD than in nonlesional AD and CTRL, regardless of body site, opposite to the relative abundances we observed for Proteobacteria (Figure 1, Supplementary Figure S5). Moreover, proportions of Firmicutes and *Staphylococcus* were correlated with transepidermal water loss, suggesting that they might be a marker for increasing severity of epidermal barrier impairment, in contradistinction to Proteobacteria. In line with this, a crescendo in histologic and ultrastructural abnormalities is observed in nonlesional AD, IV (Blunder et al., 2017; Gruber et al., 2011), lesional AD (Byrd et al., 2018; Kennedy et al., 2018; Kong et al., 2012; Meylan et al., 2017; Paller et al., 2019) and NS (Fartasch

et al., 1999; Hachem et al., 2006). Actinobacteria (*Corynebacterium*) were increased in IV (Baurecht et al., 2018) and NS when compared with AD and CTRL, irrespective of body site (Figure 1, Supplementary Figure S5). Recent work has demonstrated that colonization of the skin by *Staphylococcus* precedes AD onset, suggesting a major pathophysiological role of skin superinfection in AD (Meylan et al., 2017). However, our data, showing similar increases in the proportions of *Staphylococci* in AD and IV, might challenge this hypothesis, although further studies are required.

Patients with AD and NS display increased *S. epidermidis* and *S. hominis* in PF (Supplementary Figures S10a, S13a and b; Supplementary Table S3). Both bacteria can secrete antimicrobial peptides able to kill *S. aureus*, emphasizing a contextual pathogenicity (Chen et al., 2018). In line with this, the relative abundances of *S. aureus* were under the cutoff of 0.5% in all healthy controls and patients, in line with results from quantitative PCR (Supplementary Table S3). Previous studies reported high levels of *S. aureus* in skin lesions of adult patients with disease flare or severe AD, in contrast to nonlesional skin, which exhibits low to absent *S. aureus*, similar to healthy skin (Baurecht et al., 2018; Clausen et al., 2018; Francuzik et al., 2018; Smits et al., 2020; van Mierlo et al., 2019; Zeeuwen et al., 2017a). A predominance of *S. epidermidis* was already observed in patients with low to

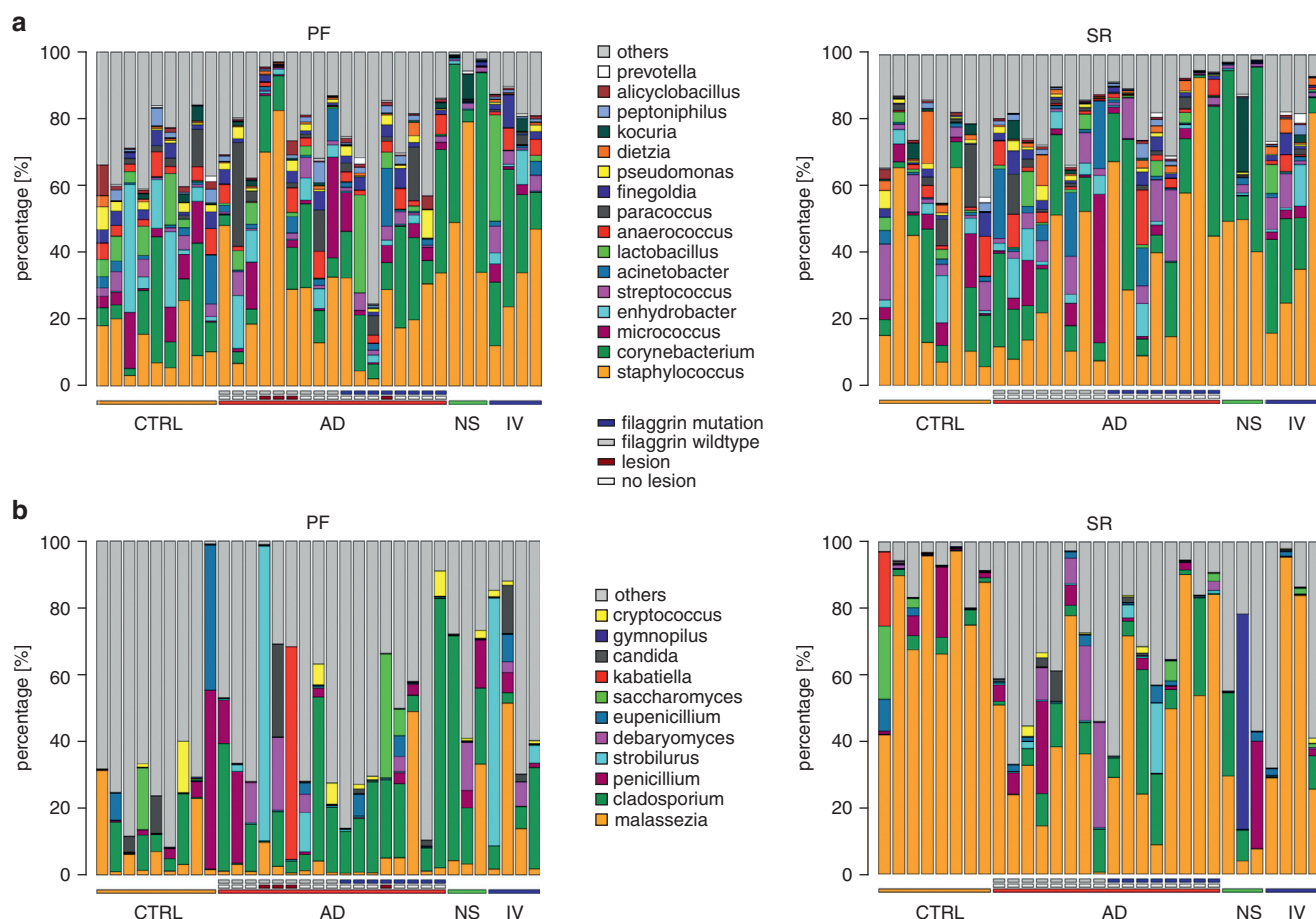


Figure 6. Bacterial and fungal genera in AD, IV, and NS. Mean relative abundance of (a) bacterial and (b) fungal genera within the total bacterial and fungal population in PF and SR. CTRL, n = 9; AD, n = 17; IV, n = 4; NS, n = 3; AD WT/WT, n = 9; AD FLG MUT, n = 8; AD NL, n = 13; AD L, n = 4. AD, atopic dermatitis; CTRL, healthy control; IV, ichthyosis vulgaris; L, lesional; MUT, mutation; NL, nonlesional; NS, Netherton syndrome; PF, popliteal fossae; SR, lower scapular region; WT, wild-type.

mild disease (Byrd et al., 2017), identical to patients with AD from our cohort (mean Eczema Area and Severity Index [EASI] < 5). Our results show that the nonlesional skin of patients with NS, although exhibiting a severe skin phenotype, including strong erythema, and several major pathophysiological commonalities with patients with AD such as atopic manifestations (Malik et al., 2019; Paller et al., 2017), is not overcolonized with *S. aureus*, albeit the detection of the bacterium by quantitative PCR in two patients with NS (Supplementary Table S3). Williams et al. (2020) recently showed, in six patients with NS with ages ranging between 12–47 years old, increasing amounts of *S. aureus* according to disease severity but regardless of skin lesions when compared with healthy controls. In patients with NS with absent *S. aureus*, *S. epidermidis* predominated (Williams et al., 2020), hereby confirming our data. Thus, these results emphasize that atopic manifestations are probably not linked to the expansion of *S. aureus* and vice versa. This hypothesis is further proven by recent work showing increased *S. aureus* in psoriasis when compared with healthy controls (Chang et al., 2018; Ng et al., 2017), although to a lesser extent than in severe AD (Fyhrquist et al., 2019). Moreover, antistaphylococcal treatments efficiently eliminate *S. aureus* on the skin of patients with AD but do not

significantly improve disease when compared with non-antimicrobial agents (Bath-Hextall et al., 2010), suggesting that *S. aureus* might be a marker of disease severity rather than a major pathogenic factor.

Studies on skin fungal microbiota have been carried out by using superficial skin scrapes (Findley et al., 2013), swabbing (Paulino et al., 2006), or tape stripping (Zhang et al., 2011) for subsequent fungal cultures or direct genetic analysis. Malassezia-rich skin microbiota have been evidenced with predominant *M. restricta* or *M. globosa* according to body sites (Chng et al., 2016; Findley et al., 2013; Paulino et al., 2006; Zhang et al., 2011), similar to our results (Supplementary Figure S11). Previous work on skin mycobacteria and especially on Malassezia in AD has shown inconsistent results, potentially because of the use of different analytic methods and the inclusion of patients with topical as well as systemic therapies in several studies (Chng et al., 2016; Han et al., 2018; Jagielski et al., 2014; Nowicka and Nawrot, 2019; Prohic et al., 2016; Zhang et al., 2011). We showed that, although all disease groups exhibited changes in the fungal microbiota, the biggest alterations were found in patients with NS. Moreover, although NS and AD shared similar increases in Ascomycota, fungi expanding on AD and NS skin were different. Thus, these results demonstrate

specific fungal dysbiosis in AD and NS, suggesting that the skin microenvironment in NS diverges more from AD than initially believed (Czarnowicki et al., 2018; Furio and Hovnanian, 2014; Paller et al., 2017). Several studies have emphasized clinical commonalities between NS and AD, especially atopic manifestations, *SPINK5* polymorphisms (mutated in patients with NS) (Malik et al., 2019), and impaired epidermal barrier function, although differing in severity. However, AD and NS exhibit distinct histologic and immunologic features associated with differential expression of genes involved in epidermal barrier function and lipid metabolism (Leclerc-Mercier et al., 2016; Malik et al., 2019). This might explain disease-specific bacterial and fungal skin profiles when observed in detail; NS exhibits the highest bacterial and fungal dysbiosis when compared with CTRL, AD, and IV. *Cladosporium* has been associated with asthma and a Th2 immune deviation (Sharma et al., 2019; Sharpe et al., 2015). In line with this, we found increased *Cladosporium* in AD and NS when compared with CTRL and IV, regardless of body site (Supplementary Figure S9), and a positive correlation between *Cladosporium* and trans-epidermal water loss (Supplementary Table S2). These data suggest that the ingress of *Cladosporium* into the epidermis might be favored by major epidermal barrier impairment, hence exacerbating Th2 immunity and atopic manifestations. Further investigations showed a 2-fold increase in *Cladosporium colombiae* and *Cladosporium cladosporioides* in NS when compared with AD (Supplementary Figure S10b). *C. cladosporioides* induces robust airway hyper-responsiveness, eosinophilia, and a predominately Th2/Th17 immune response (Mintz-Cole et al., 2013). Confirmation of this finding and deeper research on those specific fungi might provide a better understanding of processes involved in the atopic march. Moreover, *FLG* mutations might cause greater alterations to skin fungi than to bacteria, as evidenced in IV skin. Furthermore, interspecific competition within *Malassezia* (lipophilic) in AD, NS, and IV (Supplementary Figure S11) might stress differential alterations in lipid metabolism in the skin of these patients.

To conclude, this work shows, to our knowledge, first evidences of specific bacterial and fungal skin signature in AD, IV, and NS, suggesting distinguishable disease features. Further work including higher numbers of ichthyotic patients will be of interest to confirm these results.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Medical University of Innsbruck and conducted in accordance with the Declaration of Helsinki principles. All study subjects gave written informed consent and participated voluntarily. Detailed information on techniques used in this study is provided in Supplementary Materials and Methods.

DNA isolation and sequencing

DNA was extracted with an Ultraclean swab DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA was subjected to a 30-cycle PCR using the Hot-StarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) under the following conditions: 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds, and 72 °C for 1 minute,

after which a final elongation step at 72 °C for 5 minutes was performed. After amplification, PCR products were visualized in a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentration. Pooled samples were purified using calibrated Ampure XP beads. Then, the pooled and purified PCR product was used to prepare an Illumina DNA library. Two assays were run, one for bacteria and the other for fungi, using the following primers: illicu515F GTGYCAGCMGCCGCGGTAA and new806RB GGACTACNVGGGTWTCTAAT (variable region 4) for bacteria and ITS1F-Bt1 CTTGGTCATTTAGAGGAAGTAA and ITS2R GCTCGCTTCTTCATCGATGC for fungi, reported to give overall improved performance compared with the original primers (Walters et al., 2015). Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX) on a MiSeq instrument following the manufacturer's guidelines. Sequence data were processed using the MR DNA microbiota analysis pipeline (MR DNA, Shallowater, TX). In summary, paired sequences were joined and depleted of barcodes; sequences < 150 base pairs or with ambiguous base calls were removed. Sequences were denoised, operational taxonomic units generated, and chimeras removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity). Final operational taxonomic units were taxonomically classified using BLASTn against a curated database derived from RDP II and NCBI (www.ncbi.nlm.nih.gov, <http://rdp.cme.msu.edu>). To classify *Malassezia* ITS1 sequences from the genus to species level, we used the `get.lineage` command in `mothur`.

Data and statistical analysis

Statistical analysis and visualizations were performed using R statistical software (version 3.4.1) (R core Team. R Foundation for Statistical Computing. R: A language and environment for Statistical Computing. 2018. Available at: <http://www.R-project.org/>). Bacterial (fungal) species/genera were filtered for a detected presence of at least 0.5% (0.1%) in $\geq 25\%$ of all samples in each of the two studied sites, PF and SR. Stacked barplots were used to compare distributions of selected bacterial and fungal species between patients and sites. For comparison of individual species between groups, boxplots were used (individual percentages were indicated using the package `beeswarm`). Differences between groups were assessed by the Kruskal-Wallis test. A Wilcoxon rank-sum test was used as a post-hoc test. *P*-values were adjusted for multiple testing at the respective level based on FDR according to the Benjamini-Hochberg method. Alpha-diversity was measured based on the Shannon diversity index on raw unfiltered data using the package `VEGAN` (Dixon, 2003). The Shannon diversity index takes into account both the richness (number of different species) and evenness (how evenly the species are distributed) of the bacterial or fungal community. Violinplots were used to compare distributions of Shannon diversity indices between groups and tested by (pairwise) Wilcoxon rank-sum tests with adjusted *P*-values for multiple testing based on FDR according to the Benjamini-Hochberg method. To compare overall differences between groups, principal coordinate analysis was performed and visualized using the first two principal coordinates. `DataEllipse` from the package `car` was used to indicate related sample groups at the 65% confidence level, and *P*-values from permutational multivariable analysis of variance based on Bray-Curtis dissimilarity using the `VEGAN` package and 1,000 permutations were calculated ($P < 0.05$ indicates that at least one group was

significantly different from the other groups). Correlations were calculated by Spearman rank correlation (test). Selected species were grouped into their respective phyla and percentages were visualized as heatmaps using Genesis (Sturn et al., 2002). Statistical analyses for histograms were performed using Graphpad Prism software. Results were expressed as mean $\pm$ SD and analyzed for statistical significance by using Student's *t*-test or one-way ANOVA followed by a Tukey post-hoc test.

Data availability statement

Datasets related to this article can be found at <https://data.mendeley.com/datasets/vsx5xcktwx/draft?a=60fd840f-7c71-4a72-bdb9-0efaa57e28a8>, hosted at (Mendeley Data) (10.17632/vsx5xcktwx.2).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: HH, VMM, SD; Data Curation: HH, SD; Formal Analysis: HH, SD; Funding Acquisition: SD; Investigation: VMM, RG, MP, LK; Methodology: HH, SD; Project Administration: SD; Resources: VMM, RG, DOH, HH; Software: HH; Supervision: SD; Validation: VMM, HH, DOH, SD; Visualization: HH, SD; Writing - Original Draft Preparation: SD; Writing - Review and Editing: VMM, HH, RG, LK, DOH, SD

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2020.05.102>.

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