Skin Microbiota and Clinical Associations in Netherton Syndrome

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Netherton syndrome (NS) is a rare, life-threatening syndrome caused by serine protease inhibitor Kazal-type 5 gene (*SPINK5*) mutations, resulting in skin barrier defect, bacterial skin infections, and allergic sensitization in early childhood. Recent data on adult patients with NS suggest that the presence of *Staphylococcus aureus* further promotes barrier disruption and skin inflammation. We analyzed the skin microbiota by shotgun sequencing in 12 patients with NS from eight Finnish families with healthy family controls as the reference and correlated the findings with allergen-specific IgE prevalence, immune cell phenotype, and infection history of the patients. Compared with healthy family controls, skin microbiome diversity and normal skin site variability were measurably decreased in patients with NS. No correlation was found between allergic sensitization and skin microbiota as such, but low circulating CD57+ and/or CD8+ T cells significantly correlated with lower microbial diversity and less abundance of *S. aureus* ($P < 0.05$). *S. aureus* was the most prevalent species in patients with NS but also *Streptococcus agalactiae* was abundant in four patients. The genomic DNA relative abundance of *S. aureus* secreted virulence peptides and proteases PSMα, staphopain A, and staphopain B were increased in most of the samples of patients with NS, and their abundance was significantly ($P < 0.05$) associated with recurrent childhood skin infections, confirming the clinical relevance of *S. aureus* dominance in the NS skin microbiome.

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INTRODUCTION

Netherton syndrome (NS) (Online Mendelian Inheritance in Man #256500) is a severe ichthyosis characterized by desquamative skin inflammation, elevated IgE levels, broad allergic sensitizations, neonatal failure to thrive, and recurrent bacterial skin infections. It is known as an autosomal recessive disease with serine protease inhibitor Kazal-type 5 gene (*SPINK5*) mutations leading to uninhibited protease activity and thus to a barrier defect and constant inflammation in the skin (Chavanas et al., 2000; Hovnanian, 2013) with an increased level of proinflammatory and T helper type 17 pathway cytokines (Paller et al., 2017).

The composition of the skin microbiota is involved in the development and maintenance of the immune system (Belkaid and Hand, 2014). In NS, the immune system becomes dysfunctional (Eränkö et al., 2018; Hannula-Jouppi et al., 2016; Renner et al., 2009). The diversity of the normal skin microbiota increases during the first decade of life and resembles the microbiota of the mother (Zhu et al., 2019), but subsequently, the composition and temporal variation of an individual's microbiome remain highly personalized (Flores et al., 2014). NS skin inflammation resembles those of atopic dermatitis and IgE syndrome, in which the diversity of the microbiota is reduced, and *Staphylococcus aureus* is frequently isolated from skin lesions and during disease flares (Kong et al., 2012; Oh et al., 2013). The skin of adult patients with NS has recently been shown to have an increased relative abundance of *Sta. aureus* and *Sta. epidermidis*, where both species contributed to skin inflammation through bacterial proteases (Williams et al., 2020). Another study of three patients with NS reported that a higher proportion of *Firmicutes* and/or *Staphylococcus* correlated with transepidermal water loss, reflecting epidermal barrier impairment (Moosbrugger-Martinz et al., 2021). However, the factors contributing to the development of skin microbiome dysbiosis remain unknown.

In this study, we analyzed the skin microbiota of 12 patients with NS (11 children, 7 of whom came from three families) and compared them with those of their healthy family controls (HFCs). In addition, we investigated possible correlations with infection history, allergen-specific IgE prevalence, and immune cell phenotypes of patients with NS.

RESULTS

Common trends in skin microbiota

We compared the skin microbiome samples of 12 patients with NS with those of their corresponding HFCs to reduce the

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Abbreviations: HFC, healthy family control; NS, Netherton syndrome; ScpA, staphopain A; SspB, staphopain B

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effects of environmental factors on the skin microbiome. The samples were collected from each patient from different anatomical areas according to the skin sites with specific bacteria being associated with moist, dry, and sebaceous microenvironments (Dréno et al., 2016), that is, abdomen near the umbilicus, buttocks, and sternum (Table 1 and Figure 1).

The alpha diversity was clearly diminished in patients with NS compared with that in HFCs (Figure 2a and b). The analysis of beta diversity clearly separated patients with NS and HFCs, with two exceptions (Figures 2c and 3). The microbiome read coverage was found to be generally lower in NS samples than in HFCs samples. Patients with NS had a median of 3.5 ± 3.74% reads assigned to the microbiome, whereas HFCs had a median of 32.4 ± 21.1% (Table 2) owing to a higher proportion of human reads from lesional skin in patients with NS, which was likely caused by the nature of the damaged skin. The diversity calculations were, therefore, also performed using the same number of reads from NS and HFC samples. This result was found to not affect the diversity.

The use of systemic antibiotics or topical chlorhexidine during the 2 weeks before sampling did not have any significant effect on the NS skin microbiome (Figure 2c). The skin site variability of the microbiota was decreased in patients with NS and was more uniform among skin sites (Figure 2b) compared with that seen in HFCs. The differences between the skin microbiota of patients with NS and HFC were observed at every skin site but were less pronounced in the sternum ($P = 0.015$) than in the buttocks ($P = 0.000456$) or abdomen ($P = 0.0055$) (Figure 2b and d).

Sta. aureus was the most common bacterium in NS skin (Figure 4), occurring in 97.6% of the NS samples. The next most commonly occurring bacteria were *Micrococcus luteus* (76.2%), *Streptococcus agalactiae* (71.4%), *Sta. capitis* (66.7%), *Sta. epidermidis* (50.0%), and *Dermacoccus sp. PE3* (47.63%). Of the 30 most abundant species, *Cutibacterium acnes* and *Mi. luteus* dominated the HFC skin, except in two controls with *Sta. epidermidis* (Figure 4). *Sta. aureus*, *Corynebacterium bovis*, *Prevotella bivia*, *Sta. agalactiae*, *Str. dysgalactiae*, and *Co. striatum* were significantly more abundant in patients with NS (Figure 5), whereas *Cu. acnes*, *Dermacoccus sp. Ellin185*, *Gordonia paraffinivorans*, and *Lactococcus lactis* were significantly more abundant in the HFC skin (Figure 6). Anaerobic bacteria (such as *P. bivia*) were more abundant in HFCs than in patients with NS ($P < 0.001$), which is most likely attributed to more air exposure in the damaged skin of patients with NS.

While looking for the relation of *Sta. aureus* with the other microbes, we found a negative correlation between *Sta. aureus* and *Cu. acnes*, both in NS and HFC skin samples (rho = −0.57). No other significant associations with *Sta. aureus* were found. *Cu. acnes* was positively correlated with the prevalence of *Sta. epidermidis* (rho = 0.55).

In summary, the skin microbiome in NS showed clear differences from healthy skin microbiomes, including lower diversity and increased presence of potentially pathogenic

Table 1. Patients with NS and HFCs and Skin Sample Details

				Sample 1		Sample 2		Sample 3		
Family	Patient (Age)	HFC (Age)	Sex	Location	Antibiotic	Location	Antibiotic	Location	Antibiotic	Other
I	I.1 (10)		F	S, A, B	no	—	—	—	—	
		Mother (42)	F	S, A, B	no					
II	II.1 (9)		M	T	no	S, A, B	no	S, A, B	no	IVIG[1], TC
		Mother (38)	F	S	no	S, A, B	no	S		
		Brother 1 (7)	M	S, A, B	no					
		Brother 2 (6)	M	A, B	no					
III	III.1 (12)		M	A	no	S, A, B	no	—	—	—
	III.2 (9)		M	S	no	S, A, B	no	—	—	—
	III.3 (6)		F	S	no	S, A, B	no	—	—	—
		Half-brother (14)	M	S	no				—	—
IV	IV.1 (2)		M	S	A + C	S, A, B	no	—	—	—
		Sister (7)	F	S	no		—	—	—	—
V	V.1 (6)		M	S	A + C	S, A, B	A + C	—	—	—
	V.2 (2)		M	S	A + C	S, A, B	A + C	—	—	—
		Mother (29)	F	S	no		—	—	—	—
VI	VI.1 (4)		M	S	no	—	—	—	—	—
	VI.2 (2)		M	S	A + C	—	—	—	—	—
VII	VII.1 (53)		F	S, A, B	no	—	—	—	—	—
VIII	VIII.1 (16)		F	S, A, B	no	—	—	—	—	—
		Half-brother (9)	M	S, A, B	—	—	—	—	—	—

Abbreviations: A + C, amoxicillin + clavulanic acid; A, abdomen; B, buttock; F, female; HFC, healthy family control; IVIG, intravenous immunoglobulin; M, male; NS, Netherton syndrome; S, sternum; T, left posterior thigh; TC, topical chlorhexidine on abdomen.

Location of sampling: S, A, B, T. Antibiotics labeled if used within 2 weeks before sampling. Oral antibiotics: A + C. Second sampling 2−7 months and third sampling 8 months after first sampling.

[1]IVIG treatment started 3 months before the first sampling and continued during the study period at a dose of 400 mg/kg/month.

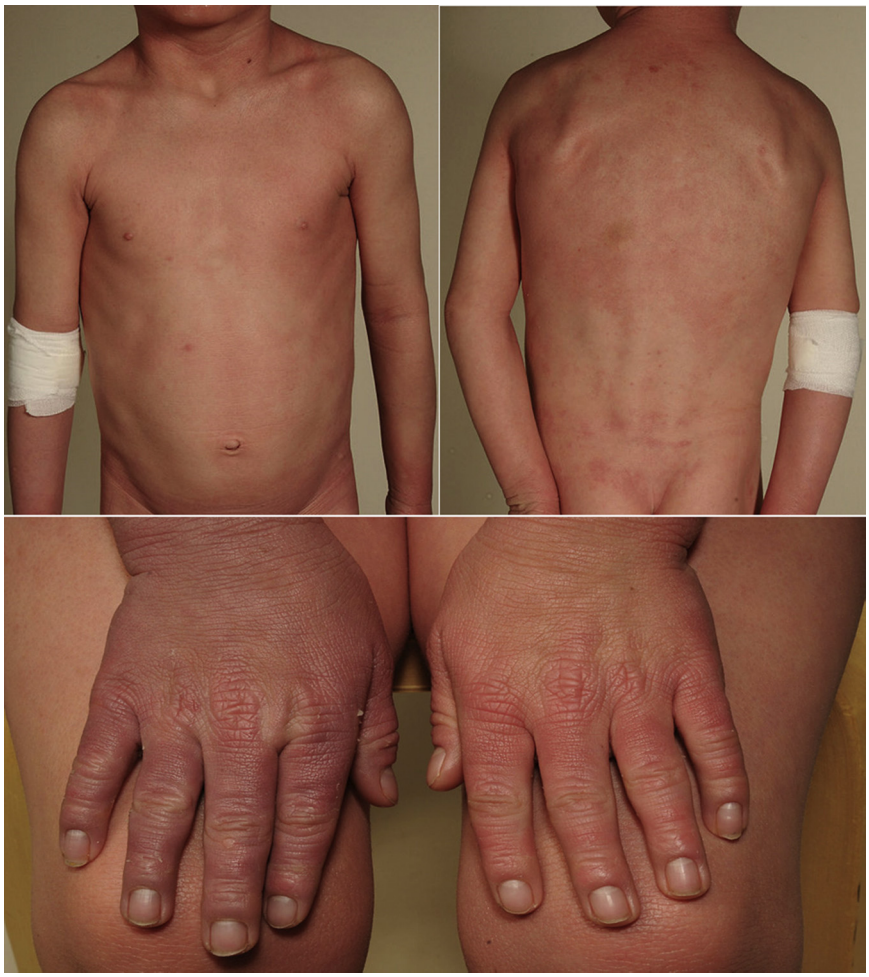

Figure 1. Clinical picture of patient with NS II.1. The patient has consented to the publication of the image. NS, Netherton syndrome.

bacteria, and these differences were independent of antibiotic use.

Fungal or viral colonization is sparse in NS skin

Although very little viral or fungal colonization was found in the skin of patients with NS, Cladosporium was found only in the HFC skin, whereas *Malassezia globosa* and *Ma. restricta* were more abundant in HFCs (Figure 6). Patient V.1 had *Verruconis gallopava* in his buttock sample. No human papillomavirus, molluscum contagiosum, or human polyomavirus DNA was found in the skin of patients with NS, in contrast to some HFC samples (Table 3).

Variation in skin microbiota among siblings with NS

Three of the studied families had more than one patient with NS (Table 1). The two boys from family V had serine protease inhibitor Kazal-type 5 gene (*SPINK5*) mutation, distinct from the other Finnish patients with NS (Hannula-Jouppi et al., 2016), and their skin microbiomes were the most similar to each other in the whole cohort (Figure 7a). Patient V.1 had suffered from neonatal sepsis caused by *Str. agalactiae* (Group B *Streptococcus*), and both patients from this family had their skin colonized by *Str. agalactiae* (Figure 7b).

The three affected siblings of family III had the most aberrant immunological phenotype (Eränkö et al., 2018), and the sibling (III.1) with most reduced skin microbiota diversity had suffered the most from recurrent skin and ear infections among all the siblings (Figure 8).

Patient VI.2 had antibiotic treatment while sampling; instead of *Sta. aureus*, the most dominant bacteria in his sample were *Cu. acnes, Propionibacterium* sp. 409-HC1, and *Sta. haemolyticus*, whereas *Sta. aureus* and *Sta. capitis* were the most prevalent in his brother's skin (Figure 9). VI.1 had frequent conjunctivitis, and both brothers have had recurrent skin and ear infections.

Association of lifetime skin infections with skin microbiota

Because patients with NS are prone to bacterial infections, we looked for associations between the skin microbiome and patients' lifetime infection history. On an average, there were more skin infections, otitis, and conjunctivitis in patients with NS than in peers without NS (Table 4; Eränkö et al., 2018). Prolonged antibiotic therapy was common among patients with NS (8 of 11, 73%). Four patients with NS used systemic antibiotics during the 2 weeks preceding skin microbiome sampling.

However, we could not find a statistically significant association between lifetime infections and skin microbiota alpha diversity ($P = 0.45$) (Figure 10).

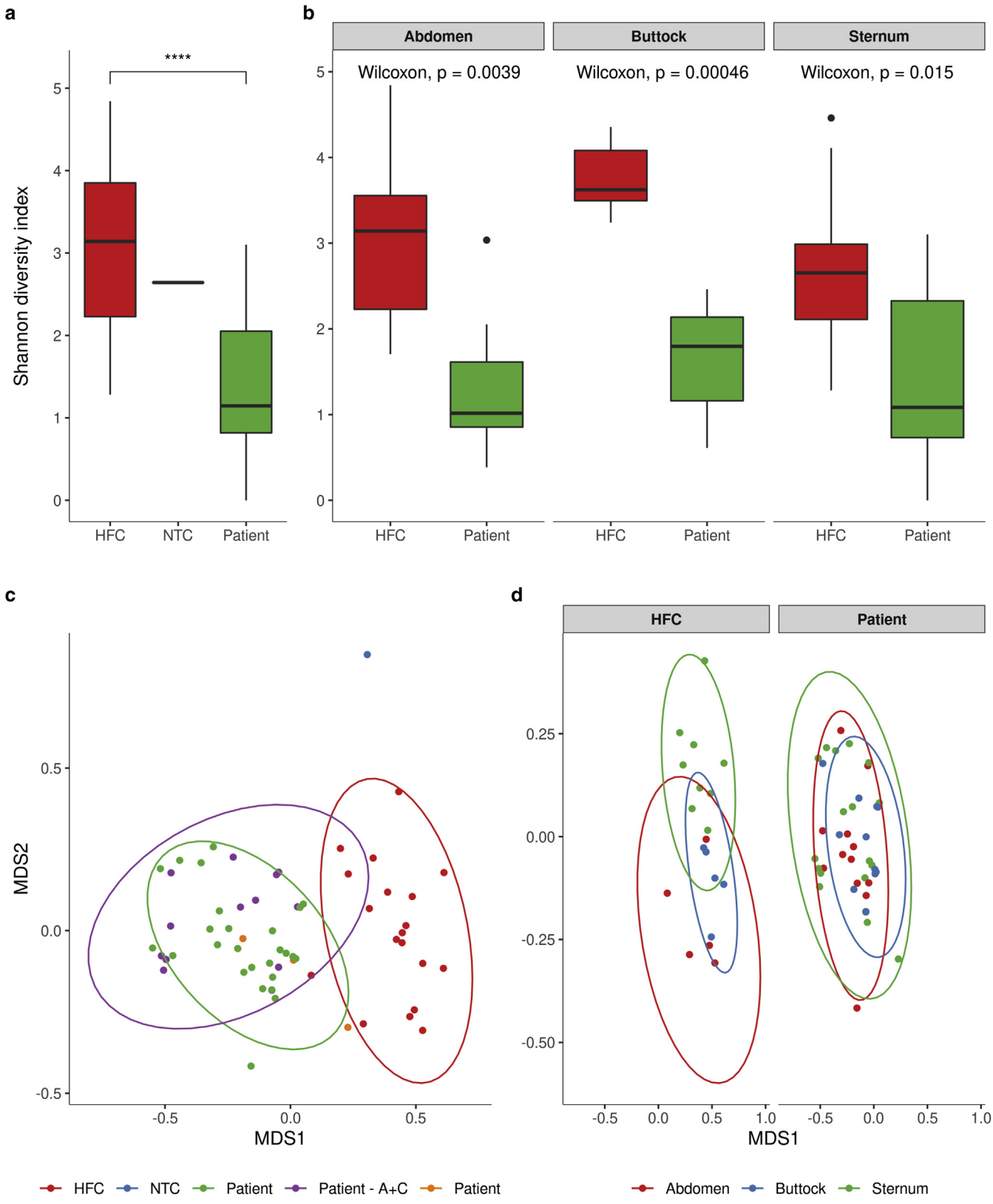

Figure 2. Skin microbiome alpha and beta diversities of patients with NS versus HFCs. (**a**) Shannon diversity index of patients with NS, HFCs, and NTC. Significant differences between groups were found (Kruskal–Wallis, $P < 0.001$). (**b**) Shannon diversity index divided by the skin site. Significant differences between groups were found ($P < 0.05$). (**c**) MDS-based ordination of the samples using a reference-free Mash distance clustering. Samples clustered by clinical status. A, C, TC on abdomen within 2 weeks before sampling. (**d**) MDS-based ordination, control, and patient samples were ordinated together; the visualization was split into two identical axes to highlight the site-specific dynamics. ****Wilcoxon, $P < 0.0001$. A, amoxicillin; C, clavulanic acid; HFC, healthy family control; MDS, multidimensional scaling; NS, Netherton syndrome; NTC, negative control; TC, topical chlorhexidine.

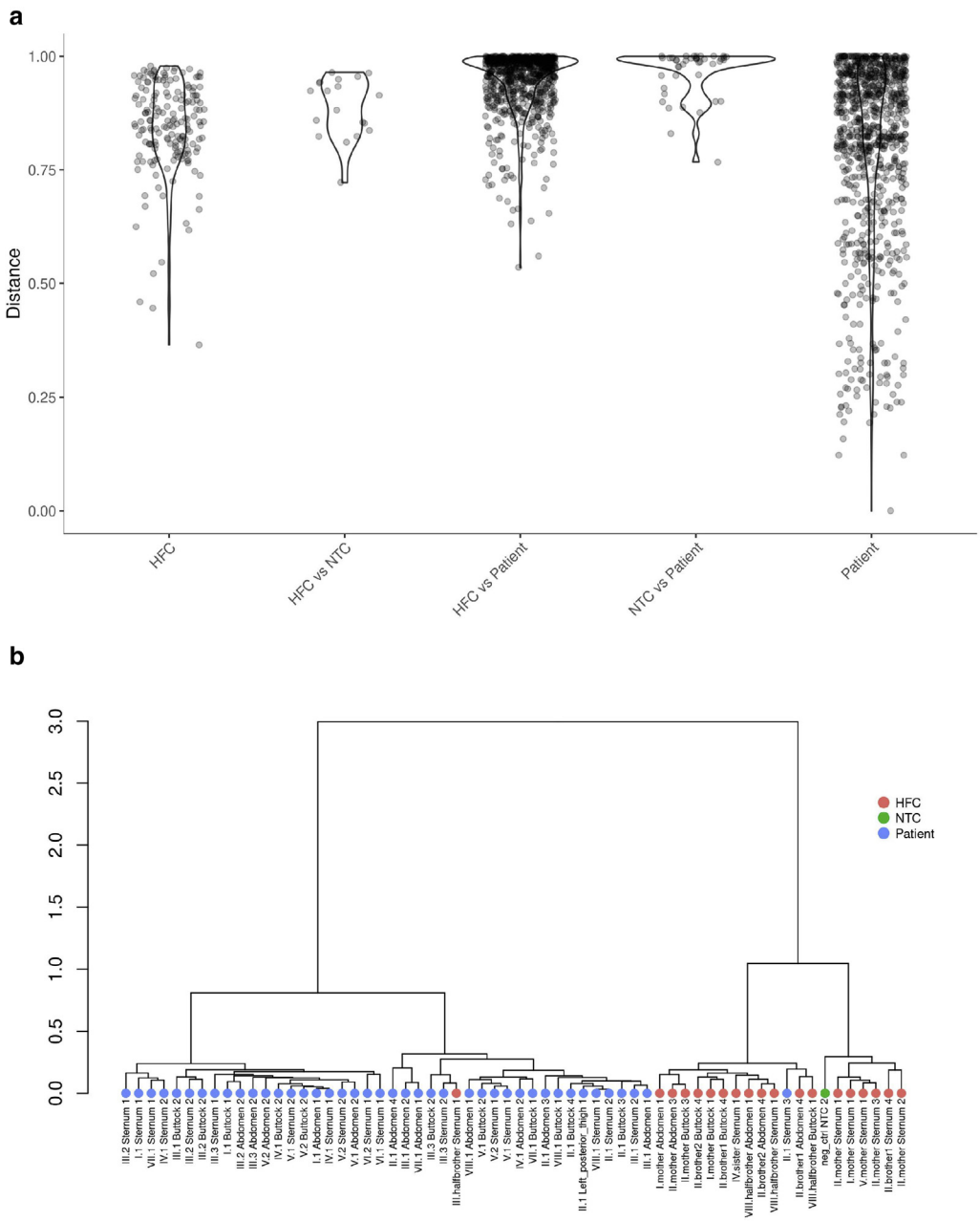

Figure 3. Beta diversity of patients with NS versus HFCs. (**a**) Beta diversity distance between and within sample by clinical group based on the Jaccard similarity coefficient. (**b**) Cluster dendrogram generated using a reference-free Mash distance clustering. Samples clustered by clinical status with the exception of two samples. HFC, healthy family control; NS, Netherton syndrome; NTC, negative control.

No correlation between allergic sensitization and skin microbiota

Patients with NS become sensitized early in life and develop multiple allergic symptoms ranging from asthma to anaphylactic reactions (Hannula-Jouppi et al., 2014; Hovnanian, 2013; Sun and Linden, 2006). To investigate the association between allergic sensitization and skin microbiota, we used total IgE and allergen component–specific IgE levels in patient sera. For statistical comparison, the allergen components were first grouped as aeroallergens, food allergens, and others and then grouped as food allergens, aeroallergens, pollen allergens, pan-allergens, lipid transfer protein allergens, and others. We analyzed all groups separately and also combined them but could not find any significant correlations between allergic sensitization and microbiota diversity or *Sta. aureus* colonization (data not shown). Neither did serum total IgE associate with microbiome diversity ($P = 0.13$) nor with *Sta. aureus* relative abundance ($P = 0.21$).

Skin microbiota diversity and *Sta. aureus* abundance associate with NK cells

We analyzed whether the skin microbiota diversity was associated with any specific lymphocyte or NK cell subclass, which we have previously shown to be altered in these same patients with NS (Eränkö et al., 2018). Statistically significant associations were found for the Shannon

Table 2. Metagenomic Sequence Read Count Data

Subject ID[1]	Skin Site[2]	Sampling Date	Clinical Group	Total Reads	Trimmed Reads	% after Trimmed	Human Reads	Nonhuman Reads	% Nonhuman Reads
VIII.halfbrother	Buttock 1	6 October 2015	Control	4.22E + 07	4.14E + 07	97.97	5.91E + 06	3.55E + 07	85.71
II.brother2	Buttock 4	8 September 2015	Control	2.15E + 07	2.12E + 07	98.71	4.14E + 06	1.71E + 07	80.48
II.brother1	Abdomen 4	8 September 2015	Control	2.37E + 07	2.34E + 07	98.70	8.14E + 06	1.53E + 07	65.23
II.mother	Abdomen 3	10 August 2015	Control	4.26E + 07	4.12E + 07	96.93	1.49E + 07	2.63E + 07	63.80
II.mother	Buttock 3	10 August 2015	Control	2.64E + 07	2.55E + 07	96.71	1.35E + 07	1.20E + 07	47.12
III.halfbrother	Sternum 1	9 July 2015	Control	8.46E + 06	8.09E + 06	95.65	4.45E + 06	3.64E + 06	45.02
I.mother	Abdomen 1	12 August 2015	Control	3.57E + 07	3.48E + 07	97.57	2.09E + 07	1.39E + 07	39.98
II.mother	Sternum 2	16 June 2015	Control	4.68E + 05	4.38E+05	93.59	2.66E + 05	1.72E + 05	39.26
II.brother1	Buttock 4	8 September 2015	Control	1.66E + 07	1.64E + 07	98.45	1.09E + 07	5.51E + 06	33.66
II.mother	Sternum 3	10 August 2015	Control	3.01E + 07	2.89E + 07	95.85	1.95E + 07	9.37E + 06	32.44
I.mother	Buttock 1	12 August 2015	Control	3.05E + 07	2.94E + 07	96.35	2.00E + 07	9.37E + 06	31.86
VIII.halfbrother	Sternum 1	6 October 2015	Control	1.78E + 07	1.74E + 07	97.65	1.23E + 07	5.14E + 06	29.49
I.mother	Sternum 1	12 August 2015	Control	3.69E + 07	3.55E + 07	96.14	2.59E + 07	9.65E + 06	27.16
VIII.halfbrother	Abdomen 1	6 October 2015	Control	6.52E + 07	6.42E + 07	98.38	4.80E + 07	1.62E + 07	25.22
II.brother1	Sternum 4	8 September 2015	Control	5.97E + 06	5.73E + 06	95.98	4.37E + 06	1.36E + 06	23.77
IV.sister	Sternum 1	9 July 2015	Control	3.61E + 07	3.49E + 07	96.59	2.74E + 07	7.49E + 06	21.46
V.mother	Sternum 1	9 July 2015	Control	2.60E + 07	2.56E + 07	98.47	2.07E+07	4.89E + 06	19.09
II.mother	Sternum 1	28 January 2015	Control	2.26E + 07	2.22E + 07	98.35	1.99E + 07	2.31E+06	10.41
II.brother2	Abdomen 4	9 September 2015	Control	6.16E + 07	6.05E + 07	98.12	5.48E + 07	5.71E+06	9.45
II.1	Sternum 3	12 August 2015	Patient	7.30E + 07	7.11E + 07	97.29	5.34E + 07	1.77E + 07	24.87
III.1	Buttock 2	20 November 2015	Patient	2.47E + 07	2.36E + 07	95.33	2.14E+07	2.14E + 06	9.08
I.1	Sternum 1	10 August 2015	Patient	1.05E + 07	9.87E + 06	94.43	9.07E + 06	8.02E + 05	8.13
VIII.1	Abdomen 1	6 October 2015	Patient	2.54E + 07	2.37E + 07	93.08	2.21E + 07	1.62E + 06	6.84
III.1	Abdomen 2	20 November 2015	Patient	2.93E + 07	2.85E + 07	97.08	2.66E + 07	1.84E+06	6.47
II.1	Abdomen 3	12 August 2015	Patient	1.07E + 07	1.03E + 07	96.20	9.62E + 06	6.35E+05	6.19
VII.1	Abdomen 1	19 November 2015	Patient	2.79E + 07	2.69E + 07	96.33	2.52E + 07	1.65E + 06	6.14
III.1	Sternum 2	20 November 2015	Patient	2.30E + 07	2.22E+07	96.69	2.09E + 07	1.33E + 06	5.98
III.2	Sternum 1	9 July 2015	Patient	9.21E + 06	8.62E + 06	93.57	8.12E + 06	5.04E + 05	5.84
III.2	Abdomen 2	20 November 2015	Patient	2.61E + 07	2.40E + 07	92.14	2.27E + 07	1.30E + 06	5.40
III.2	Buttock 2	20 November 2015	Patient	1.92E + 07	1.75E + 07	91.31	1.66E + 07	9.14E+05	5.22
VIII.1	Sternum 1	6 October 2015	Patient	5.72E + 07	5.41E+07	94.54	5.13E + 07	2.77E + 06	5.12
II.1	Sternum 2	16 June 2015	Patient	1.14E + 07	1.10E+07	95.72	1.04E + 07	5.56E + 05	5.08
I.1	Abdomen 1	10 August 2015	Patient	4.07E + 07	3.95E + 07	96.95	3.76E+07	1.91E + 06	4.84
VII.1	Sternum 1	19 November 2015	Patient	1.06E + 07	1.02E + 07	96.25	9.69E + 06	4.87E + 05	4.79
III.1	Abdomen 1	9 July 2015	Patient	2.91E + 07	2.81E+07	96.76	2.68E + 07	1.32E + 06	4.69
II.1	Abdomen 4	8 September 2015	Patient	6.93E + 07	6.64E + 07	95.81	6.35E + 07	2.89E + 06	4.36
V.1	Buttock 2	20 November 2015	Patient	2.27E+07	2.15E + 07	94.52	2.05E + 07	9.08E + 05	4.23
IV.1	Sternum 2	20 November 2015	Patient	1.11E + 07	1.07E + 07	96.13	1.03E + 07	4.28E + 05	4.00
III.3	Abdomen 2	20 November 2015	Patient	1.66E+07	1.58E + 07	95.08	1.52E + 07	6.29E + 05	3.98
III.2	Sternum 2	20 November 2015	Patient	2.10E + 07	1.85E + 07	88.28	1.79E + 07	6.43E + 05	3.48
V.2	Buttock 2	20 November 2015	Patient	2.70E + 07	2.55E + 07	94.39	2.46E+07	8.79E + 05	3.44
IV.1	Sternum 1	9 July 2015	Patient	4.95E + 07	4.80E + 07	96.91	4.64E + 07	1.64E + 06	3.41

(*continued*)

Table 2. Continued

Subject ID[1]	Skin Site[2]	Sampling Date	Clinical Group	Total Reads	Trimmed Reads	% after Trimmed	Human Reads	Nonhuman Reads	% Nonhuman Reads
III.3	Sternum 1	9 July 2015	Patient	7.84E+06	7.49E + 06	95.52	7.23E + 06	2.54E + 05	3.40
IV.1	Abdomen 2	20 November 2015	Patient	4.94E + 07	4.82E + 07	97.57	4.66E+07	1.53E + 06	3.19
VII.1	Buttock 1	19 November 2015	Patient	1.58E + 07	1.51E + 07	95.80	1.46E + 07	4.69E + 05	3.11
V.1	Sternum 1	9 July 2015	Patient	3.68E + 07	3.47E+07	94.18	3.37E + 07	1.05E + 06	3.02
III.3	Buttock 2	20 November 2015	Patient	1.08E + 07	1.05E + 07	97.07	1.02E + 07	3.09E+05	2.94
V.2	Abdomen 2	20 November 2015	Patient	1.88E + 07	1.80E + 07	95.74	1.75E + 07	5.29E + 05	2.93
V.1	Abdomen 2	20 November 2015	Patient	3.13E + 07	2.98E + 07	95.20	2.90E + 07	7.93E + 05	2.66
II.1	Buttock 3	12 August 2015	Patient	1.80E + 07	1.73E + 07	96.06	1.68E + 07	4.38E + 05	2.54
VIII.1	Buttock 1	6 October 2015	Patient	2.94E+07	2.82E + 07	96.00	2.76E + 07	6.60E + 05	2.34
V.1	Sternum 2	20 November 2015	Patient	2.65E+07	2.51E + 07	94.73	2.45E + 07	5.78E + 05	2.30
VI.1	Sternum 1	9 July 2015	Patient	1.33E + 07	1.25E + 07	94.56	1.23E+07	2.45E + 05	1.95
II.1	Buttock 4	8 September 2015	Patient	2.16E + 07	2.09E + 07	96.94	2.06E + 07	3.83E + 05	1.83
IV.1	Buttock 2	20 November 2015	Patient	5.20E + 07	5.02E + 07	96.53	4.94E + 07	7.95E + 05	1.58
III.3	Sternum 2	20 November 2015	Patient	4.65E + 07	4.54E + 07	97.68	4.47E + 07	7.17E + 05	1.58
II.1	Left posterior thigh 1	28 January 2015	Patient	1.22E + 07	1.16E + 07	95.27	1.15E + 07	1.62E + 05	1.39
VI.2	Sternum 1	9 July 2015	Patient	2.42E + 07	2.34E + 07	96.60	2.31E + 07	2.83E + 05	1.21
V.2	Sternum 2	20 November 2015	Patient	2.76E + 07	2.65E + 07	95.71	2.61E + 07	3.14E + 05	1.19
I.1	Buttock 1	10 August 2015	Patient	2.57E + 07	2.28E+07	88.47	2.25E + 07	2.54E + 05	1.12
V.2	Sternum 1	9 July 2015	Patient	4.28E + 07	3.94E+07	92.14	3.90E + 07	3.86E + 05	0.98
NTC	—	20 November 2015	NTC	1.76E + 05	1.67E + 05	95.38	4.57E + 04	1.22E + 05	72.71

Abbreviations: ID, identification; NTC, negative control.
Samples in the order of nonhuman reads.
[1]Roman number refers to a family.
[2]Number refers to the sampling round.

diversity index and CD57+ and/or $CD8^{+}$+ T cells (Figure 11). Patients with NS with a lowered effector memory CD57+ and/or CD8+ T cells had lower microbial diversity than patients with elevated corresponding cell numbers ($P < 0.05$). Furthermore, *Sta. aureus* relative abundance correlated with the proportion of circulating CD27+ and/or CD56 bright (Figure 12a) and CD57+ and/or NK cells (Figure 12b). In both cases, patients with the indicated cell population above reference values had a lower *Sta. aureus* abundance ($P < 0.05$). We previously showed the proportion of CD27+ NK cells to be significantly decreased in these same patients with NS (Eränkö et al., 2018).

Staphylococcal virulence toxins and proteases associate with recurrent skin infections in NS

Sta. aureus cysteine proteases staphopain A (ScpA) and staphopain B (SspB) as well as *Sta. epidermidis* extracellular cysteine protease have been related to skin damage in adult patients with NS (Williams et al., 2020). In the pediatric patients with NS, we found, as expected, an increased ScpA and SspB genomic DNA abundance in subjects with elevated *Sta. aureus* abundance (Figure 13). ScpA and SspB were most increased in patient II.1, in whom *Sta. aureus* was the most dominant species. Notably, his healthy mother had the second highest level of ScpA and the highest level of SspB among the HFC.

The *Sta. epidermidis* extracellular cysteine protease genomic DNA was only found in the control group, which was expected as *Sta. epidermidis* is more abundant in this group (Figure 14a). The metagenomic analysis showed that DNA for the secreted membrane–injuring toxin PSMα operon was elevated in samples with *Sta. aureus* (Figure 14b).

We found a correlation between ScpA abundance and bacterial skin infections because ScpA was found to be increased in patients aged over 1.5 years with recurrent bacterial skin infections ($P < 0.05$) (Figure 15). The SspB abundance correlated with frequent occurrence of conjunctivitis ($P < 0.01$). The SspB was also increased in patients with a history of sepsis after the neonatal

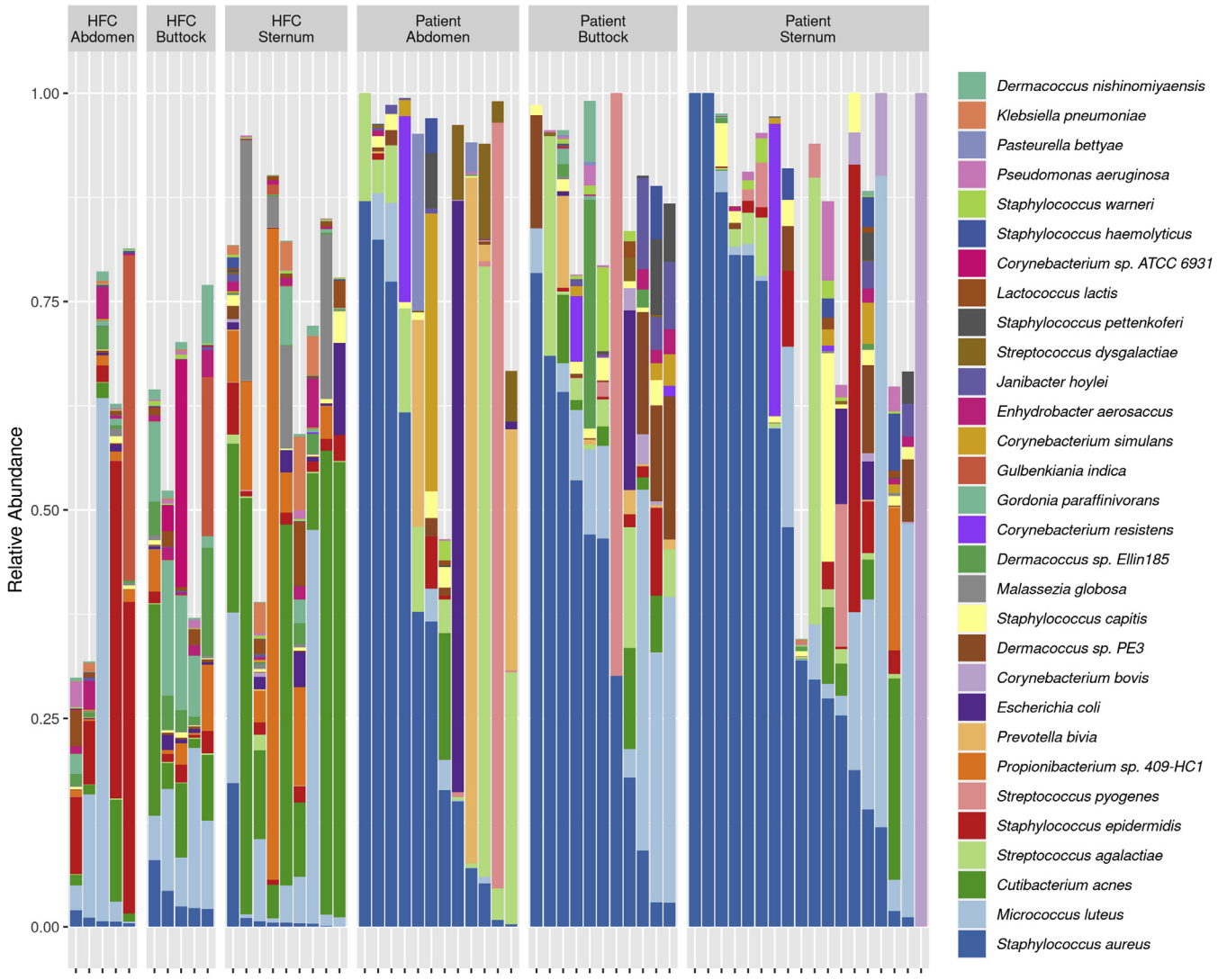

Figure 4. Microbial composition of NS skin microbiome versus HFCs. Sp. level bar plot showing the top 30 most abundant species across all samples. HFC, healthy family control; NS, Netherton syndrome.

period ($P < 0.05$), caused by *Sta. aureus* or *Sta. epidermidis*.

DISCUSSION

We report a remarkably diminished skin microbiota alpha diversity in a cohort of 12 predominantly pediatric patients with NS as compared with healthy, cohabitating age-matched siblings, and parents. In addition, the skin site variability was decreased in patients with NS compared with that in HFCs. The main difference regarding bacterial species was the dominance of *Sta. aureus* in multiple skin sites of these pediatric patients with NS. These findings are concordant with two other recent studies on teenage and adult patients with NS (Moosbrugger-Martinz et al., 2021; Williams et al., 2020). However, we did not detect *Sta. epidermidis* dominance in NS skin, as reported by Williams et al. (2020),but detected it in the HFC samples. We found anaerobic bacteria to be more abundant in HFC samples than in patients with NS, as has been previously found for atopic dermatitis (Fyhrquist et al., 2019). In HFCs, we found the same microbes that are commonly found on healthy skin (Dréno et al., 2016).

In contrast with adult patients with NS, the skin of our patients with NS was practically erythrodermic at all times, and no truly healthy skin sites could be identified. In addition, there was a wider skin site sampling in this study, and the reference skin samples were environmentally closer than those reported by Williams et al. (2020). The lack of viral and fungal colonization fits well with the infrequency of clinical viral or fungal infections despite the immunological dysfunction observed in patients with NS (Eränkö et al., 2018) and the recent observations in three other patients with NS

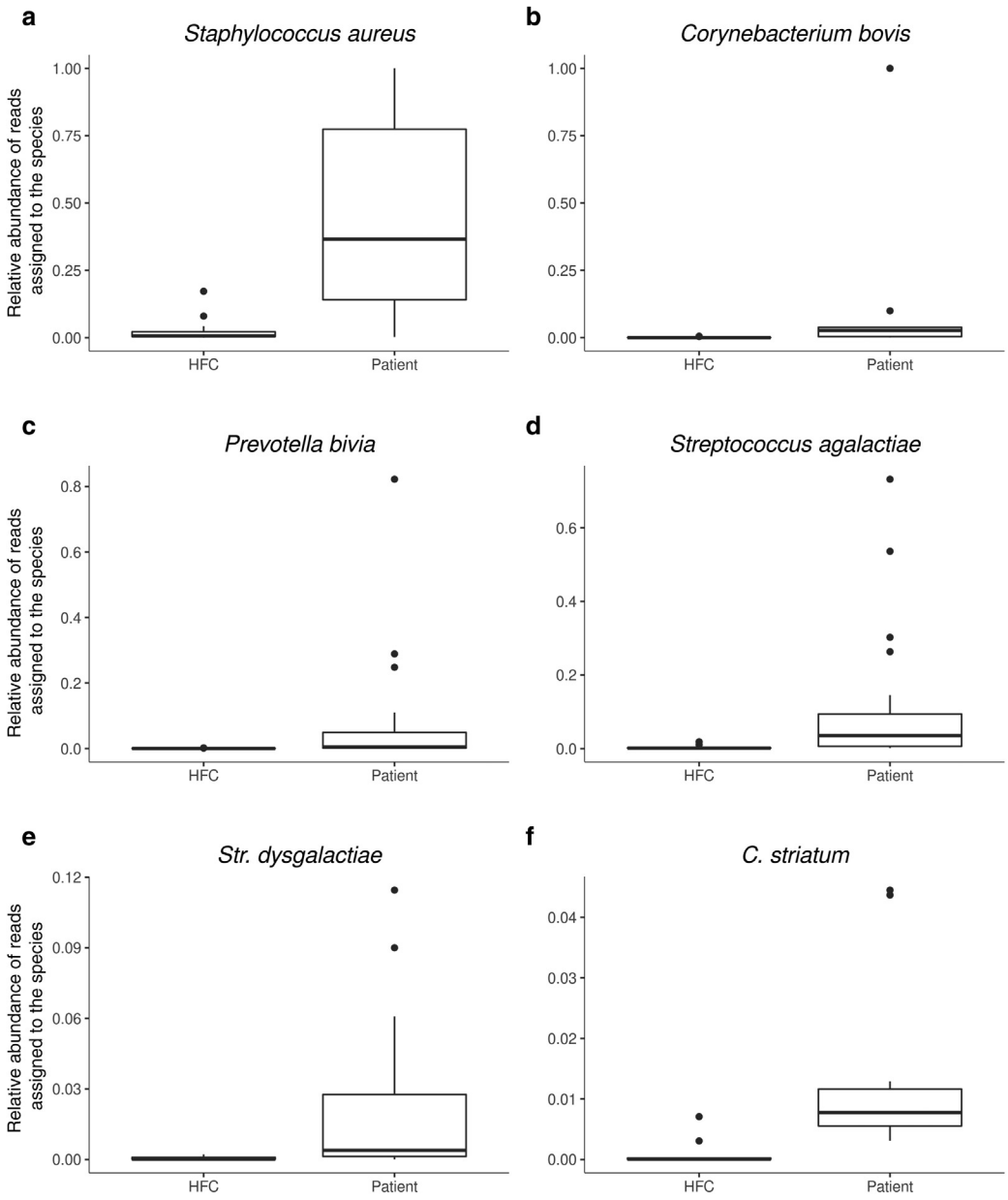

Figure 5. ANCOM abundance bar plots of differentially abundant species between HFCs and patients with NS. (**a**) *Staphylococcus aureus*, (**b**) *Corynebacterium bovis*, (**c**) *Prevotella bivia*, (**d**) *Streptococcus agalactiae*, (**e**) *Str. dysgalactiae*, and (**f**) *C. striatum* show an increasing trend from HFCs to patients with NS. ANCOM, analysis of composition of microbiomes; HFC, healthy family control; NS, Netherton syndrome.

(Moosbrugger-Martinz et al., 2021). One of our patients had *V. gallopava*, which is found in hot springs and is a neurotropic opportunist in immunocompromised humans (Samerpitak et al., 2014).

We had the unique opportunity to analyze the skin microbiome of three families with two or three patients with NS each. Some of the siblings with NS showed extremely similar microbiomes. In one of the families, the child with NS with the most reduced skin microbiome diversity had suffered from recurrent skin and external ear infections. However, in the whole NS cohort, we could not find any significant association between lifetime infections and microbiome diversity.

Instead, we found an association of skin microbiota diversity with specific lymphocyte and NK cell subclasses, which we have previously shown to be altered in the same patients with NS and to correlate with skin infection frequency and a need for antibiotic usage (Eränkö et al., 2018). NK cells are known to be instrumental in innate immune responses, in particular by rapidly producing cytokines necessary to control certain bacterial, parasitic, and viral infections. We found a significant positive association between skin microbiome Shannon diversity and the CD57+ and/or $CD8^{+}$+ T cell proportion, a T-cell subclass known to control several viral infections and also known to be involved in staphylococcal

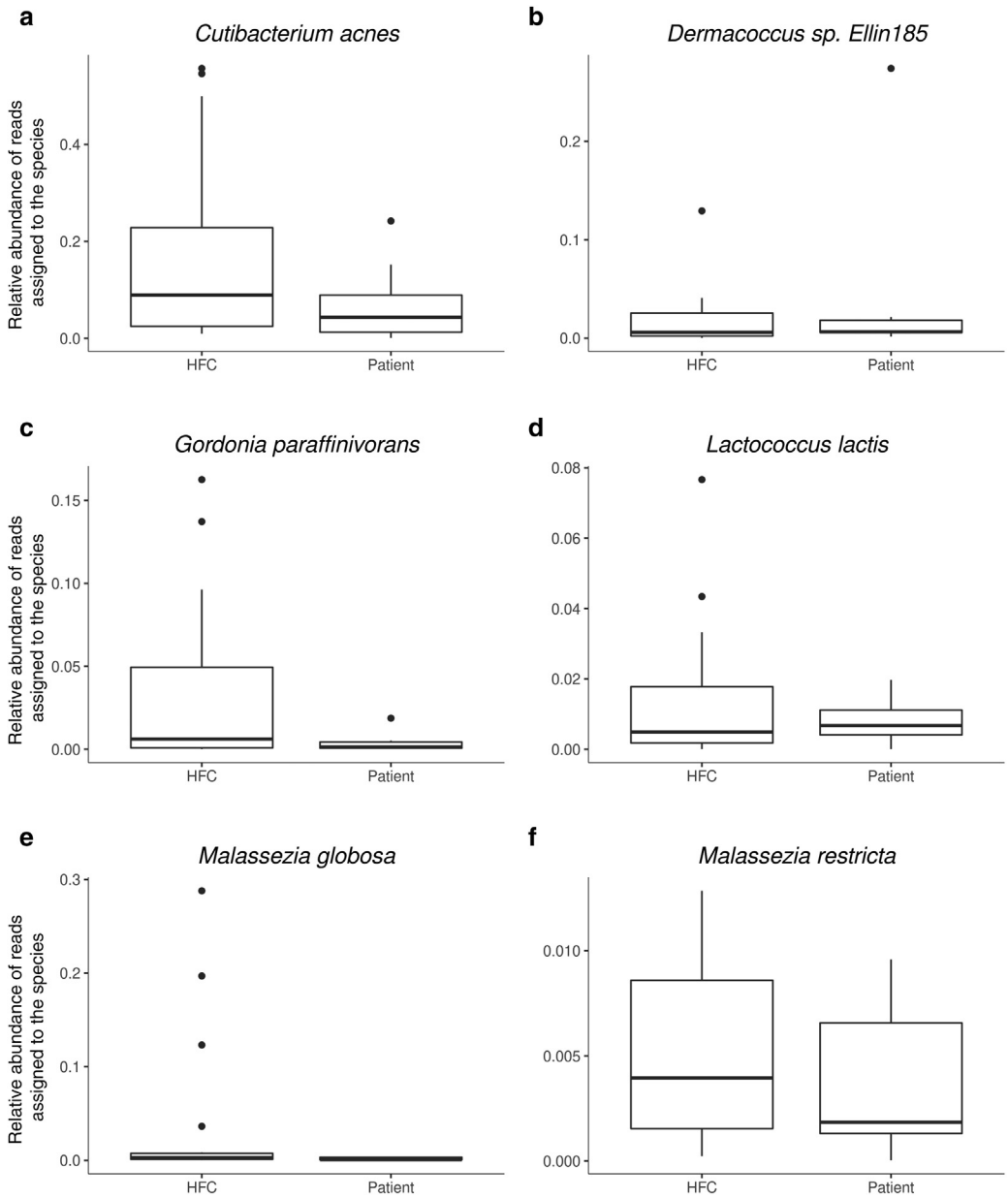

Figure 6. ANCOM abundance bar plots of differentially abundant species between clinical groups. (**a**) *Cutibacterium acnes*, (**b**) *Dermacoccus sp. Ellin185*, (**c**) *Gordonia paraffinivorans*, (**d**) *Lactococcus lactis*, (**e**) *Malassezia globosa*, and (**f**) *Malassezia restricta* show the decreasing trend from HFCs to patients with NS. ANCOM, analysis of composition of microbiomes; HFC, healthy family control; NS, Netherton syndrome.

superantigen–induced immune responses (Ami et al., 2002). The CD57+ and/or CD8+ T cells are absent in newborns, but this T-cell subtype percentage is increased in chronic immune activation and clinical conditions with functional immune deficiency (Focosi et al., 2010), including NS (Eränkö et al., 2018). Accumulating evidence also points to the utility of peripheral blood CD57+ T cells to indicate a threshold beyond which the risk of opportunistic infections becomes significant (Focosi et al., 2010).

The circulating CD27+ and/or CD56bright and CD57+ and/or NK cells, whose proportion correlated with lower *Sta. aureus* abundance in the skin, have previously been shown to become activated on stimulation with *Staphylococcus* and *Corynebacterium* (D'Orazio et al., 1995). The CD27+ and/or CD56bright NK cells denote regulatory NK cells, which are abundant cytokine producers, albeit a numerical minority (6%) of human peripheral blood NK cells (Cooper et al., 2001). The CD57 expression on peripheral NK cells identifies the final stages of maturation and is associated with chronic infections (Focosi et al., 2010). The $CD57^{+}$ NK cells are highly cytotoxic, and their presence seems to be beneficial in several noncommunicable diseases.

As known previously, patients with NS suffer from multiple allergies (Hannula-Jouppi et al., 2014; Hovnanian, 2013). *Sta. aureus* colonization has been associated with food sensitization and allergy in children

Table 3. Viruses Found in HFC Skin Samples

Subject	Skin Site	Virus	Abundance
I. mother	Buttock	*Molluscum contagiosum* virus subtype 1	50,226.0
II. brother1	Buttock	*Human polyomavirus* 6	145.0
II. brother1	Buttock	*Human papillomavirus* -1	77.0
II. brother2	Buttock	*Human papillomavirus* -1	649.0

Abbreviation: HFC, healthy family control.
Nonphage viruses' abundance calculated by FastViromeExplorer.

with eczema (Jones et al., 2016); thus, we anticipated that the same association could be found in patients with NS with a predominance of *Sta. aureus* in their skin. We have previously speculated whether the loss of LEKTI inhibition of allergen proteases combined with an increased allergen penetration due to the barrier defect

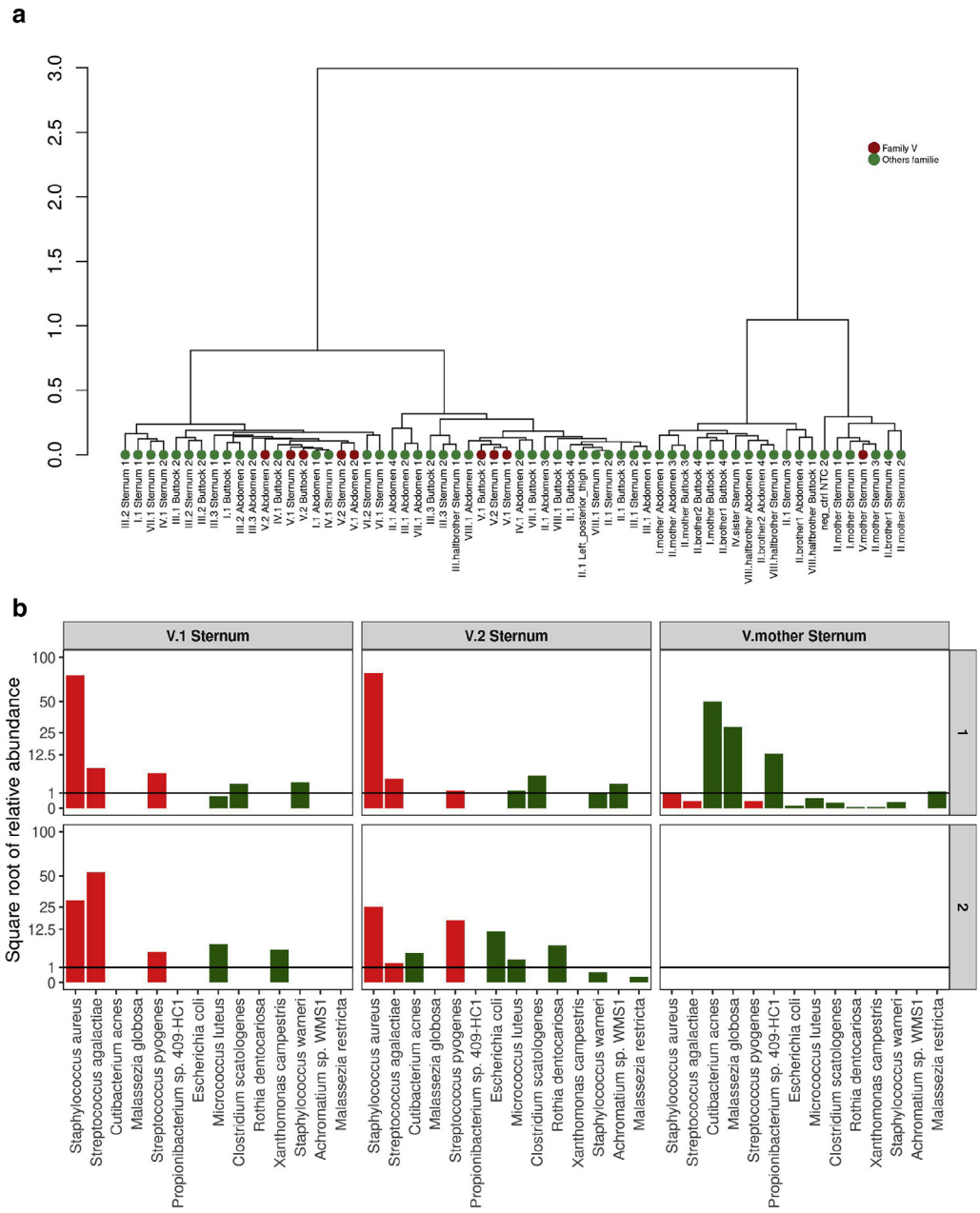

Figure 7. Skin microbiome of family V with two brothers with NS with antibiotic treatment at time of sampling. (**a**) Cluster dendrogram generated using a reference-free Mash distance clustering. Family V NS brothers cluster together. (**b**) The most abundant species in the family. Infectious species indicated in red. NS, Netherton syndrome.

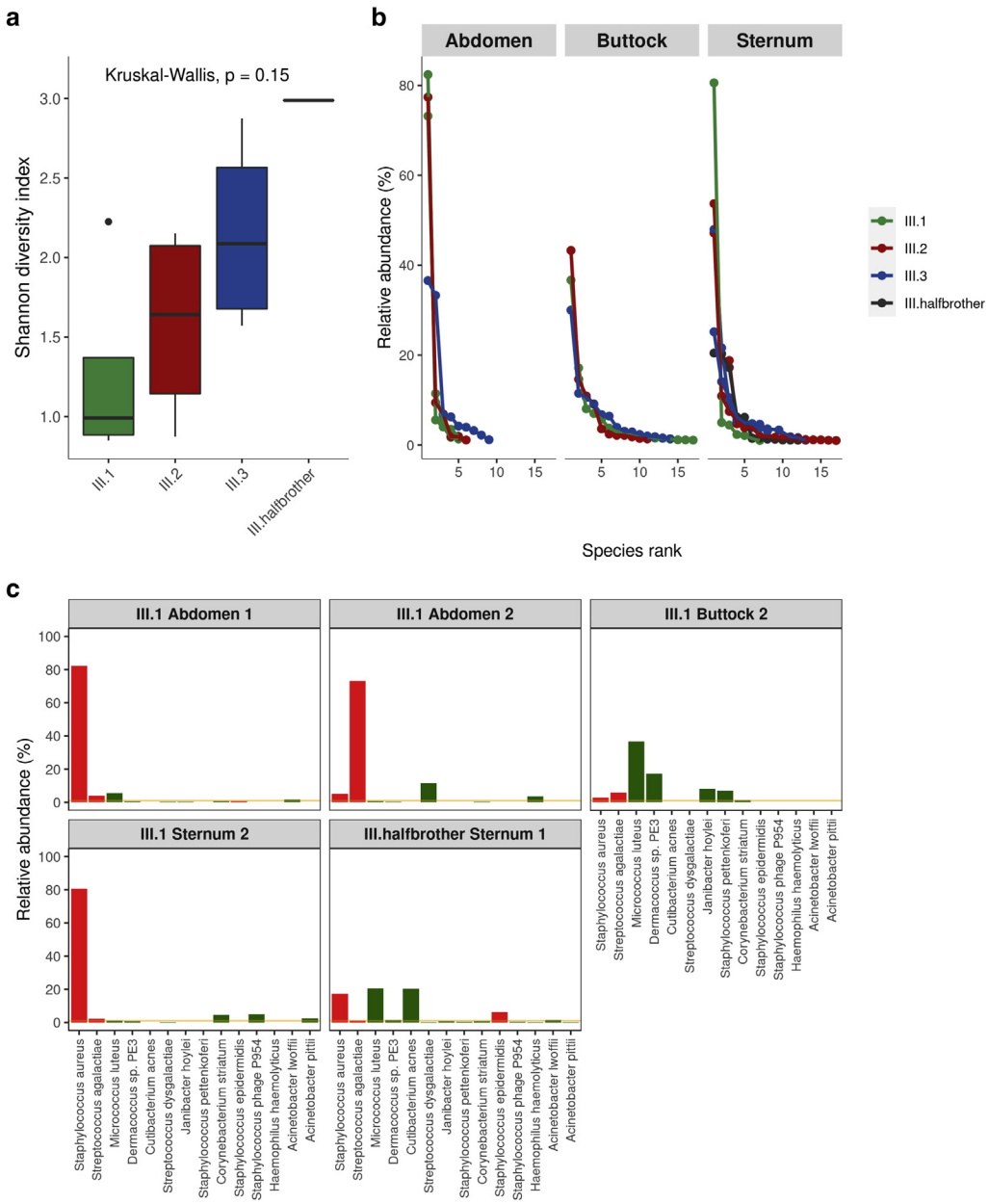

Figure 8. Skin microbiome of family III with three siblings with NS (III.1, III.2, and III.3) and HFC (half-brother). (**a**) Shannon diversity index of the family. (**b**) Relative abundance by the number of species in different skin sites. (**c**) Most abundant species of the patient III.1 with NS in the different skin site. Infectious sp. indicated in red ($P = 0.15$). HFC, healthy family control; NS, Netherton syndrome.

could contribute to the rapid allergic sensitization observed in patients with NS (Hannula-Jouppi et al., 2014). However, the results of this study suggest that skin microbiota does not associate with allergic sensitization, a result possibly influenced by the limited number of patients.

Because the role of *Sta. aureus* is central both in atopic dermatitis and NS skin inflammation (Liu et al., 2017; Nakagawa et al., 2017; Nakatsuji et al., 2016; Williams et al., 2019, 2017), we also investigated the *Sta. aureus*–derived cysteine proteases ScpA and SspB along with *Sta. epidermidis* extracellular cysteine protease. Williams et al. (2020) recently reported that these proteases contribute to the increased protease activity in NS skin. We found *Sta. aureus* ScpA genomic DNA to be elevated in most NS skin samples, and a high frequency was also detected in one healthy skin sample, whereas the SspB protease showed up at lower levels in some HFC skin samples.

Williams et al. (2020) found a strong correlation between the presence of staphylococcal *PSMα* and NS disease severity. They also demonstrated how *Sta. aureus* PSMα toxin peptides exacerbated proteolytic activity in NS skin lacking LEKTI, an epidermal serine protease inhibitor. We found a statistically significant correlation between PSMα abundance and the history of antibiotic usage in infancy. We also found a correlation between ScpA abundance and a history of recurrent bacterial

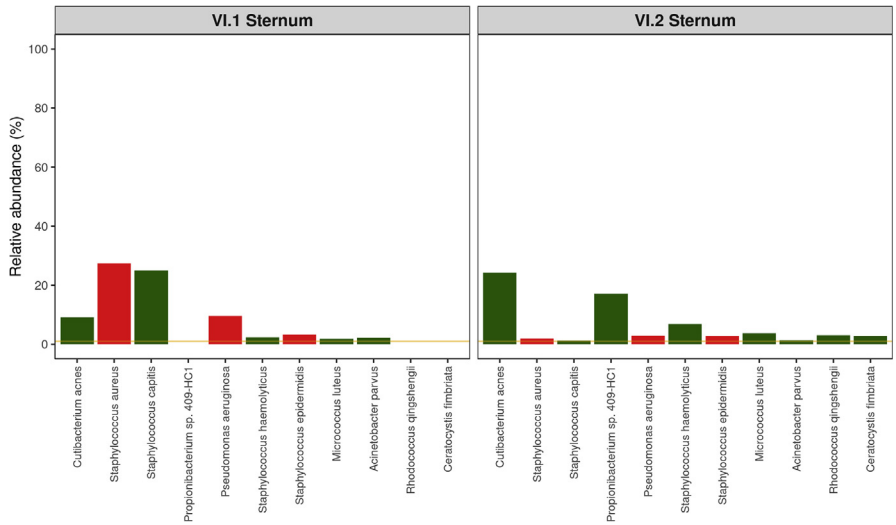

Figure 9. Skin microbiome of family VI with two patients with NS (brothers, VI.1 and VI.2). The most abundant species in the family are shown. Patient VI.2 had antibiotic treatment at the time of sampling. The number of bacteria that cause infections (in red) is lower during antibiotic use. NS, Netherton syndrome.

skin infections. Likewise, the SspB abundance correlated with recurrent conjunctivitis and was increased in patients with a history of postneonatal sepsis caused by *Sta. aureus* or *Sta. epidermidis*. In summary, the clinical relevance of *Sta. aureus* dominance in the NS skin microbiome is clear and seems to be mediated through secreted staphylococcal virulence factors but also associates with circulating CD57+ T cell and NK cell values.

MATERIALS AND METHODS

Patients

Patients with NS (n = 12; 4 female, 8 male) aged 2–15 years and one adult aged 53 years were recruited from the Helsinki University Hospital, Finland and the Seinäjoki Central Hospital, Finland. HFC were recruited from among the family members. V.1 and V.2; VI.1 and VI.2; and patients III.1, III.2, and III.3 were siblings.

All patients from families I, II, III, IV, and V had the same serine protease inhibitor Kazal-type 5 gene SPINK5 mutation (c.652C>T (p.Arg218X)). Additional serine protease inhibitor Kazal-type 5 gene (*SPINK5*) mutations were found in families VI (c.652C>T (p.Arg218X) and c.1220+1 G>C (IVS13+1 G>C)), VII (c.1772delT p.(Leu591Glnfs*124)), and VIII (c.1048C>T p.(Arg350*) and c.2098G>T p.(Gly700*)). We previously reported that patients with the same mutation seemed to have a similar clinical phenotype (Hannula-Jouppi et al., 2014). The skin of all the patients was widely erythematous, scaly, and almost erythrodermic (Figure 1). Some of the

Table 4. Infections and Antibiotic Use in the Pediatric Patients with NS Studied

Family and Patient Code	Age (y)	Antibiotic Use in Infancy[1]	Antibiotic Prophylaxis[2]	Bacterial Skin Infections[3]	Conjuctivitis[4]	External Otitis[5]	Lifetime Infection Score[6]
I.1	11	1	2	1	1	1	6
II.1[7]	10	1	2	3	2	0	8
III.1	13	1	0	2	0	1	4
III.2	10	0	0	0	0	0	0
III.3	7	0	0	0	0	0	0
IV.1	3	1	1	0	0	1	3
V.1[7]	7	1	1	1	0	0	3
V.2	4	1	2	1	0	1	5
VI.1	6	1	1	1	2	1	6
VI.2	3	1	2	1	0	1	5
VIII.1	17	1	1	1	0	1	4

Abbreviation: NS, Netherton syndrome; PVL, Panton-Valentine leucocidin.

Source: modified from Eränkö et al. (2018).

[1]0 = no, 1 = yes.

[2]0 = no, 1 = yes, for 1–1.5-year period, 2 = yes, for longer than 1.5 years.

[3]0 = as in the age group usually, 1 = recurrent infections at 1–1.5 years of age, 2 = recurrent infections at age >1.5 years, 3 = recurrent infections at age >1.5 years, additionally recurrent *Staphylococcus aureus* (PVL) abscesses.

[4]0 = as in the age group usually, 1 = recurrent infections at 1–1.5 years of age, 2 = frequent conjunctivitis or constant need for antibiotic eye drops.

[5]0 = as in the age group usually, 1 = frequent otitis or constant need for topical antibiotics; case VIII.1 had recurrent otitis media.

[6]Sum of the following scores: antibiotics in infancy, antibiotic prophylaxis, bacterial skin infections, conjunctivitis, and external otitis.

[7]Suffered from sepsis after the neonatal period caused by *S. aureus* or *S. epidermidis*.

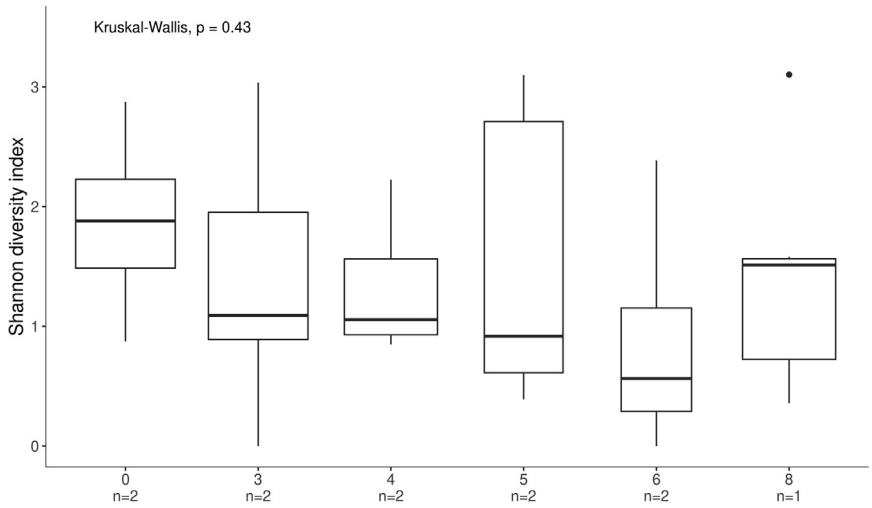

Figure 10. Association between lifetime infections and skin microbiome diversity in patients with NS. Association between lifetime infection score (x-axis: sum of the scores of 0–3 for antibiotic use in infancy, antibiotic prophylaxis, bacterial skin infections, conjunctivitis, and external otitis) and microbiome diversity measured by the Shannon index (y-axis) ($P = 0.43$). NS, Netherton syndrome.

patients received oral or topical antibiotics for 2 weeks before sampling (Table 1).

The study was approved by the Coordinating Ethical Review Board of Helsinki and Uusimaa Hospital District, Helsinki, Finland (number 91/13/03/00/11) and conducted according to the principles expressed in the Declaration of Helsinki. The parents of the underaged patients or the adult patients themselves gave written informed consent.

Infection history

Data were collected from patient records of the Helsinki University Hospital and Seinäjoki Central Hospital, covering the time period April 2003 to October 2017. Adult patient VII.1 had to be excluded from the infection analyses owing to limited childhood patient record. We summed and scored each patient's recurrent systemic, skin, ocular, and ear infections and the need for long-time prophylactic antibiotics (Table 4), as has been done in a previous study with this Finnish cohort (Eränkö et al., 2018).

Collection of skin microbiome

Skin samples were collected from seven siblings with NS of three families and five additional cases with NS with HFC as reference. The sampling areas included sternum, abdomen near the umbilicus, and buttocks. One patient was also sampled at the posterior thigh (severe lesional area) (Table 1). Samples were taken with no prior cleaning or preparation of the skin surface using sterile gloves. After placing a sterile plastic cylinder (2.5 cm in diameter) onto the appropriate skin area, 1.5-ml PBS (pH: 7.4) was supplemented into the cylinder and the skin was gently rubbed with a glass rod in a circular motion 10 times to the left and to the right. Subsequently, the microbiome-enriched PBS was collected with a sterile pipette and stored at −80°C.

Serum IgE levels and Immunocap ISAC microarray

Serum immunoglobulin levels for total IgE and specific IgE to 112 allergen components (ImmunoCAP ISAC microarray) were

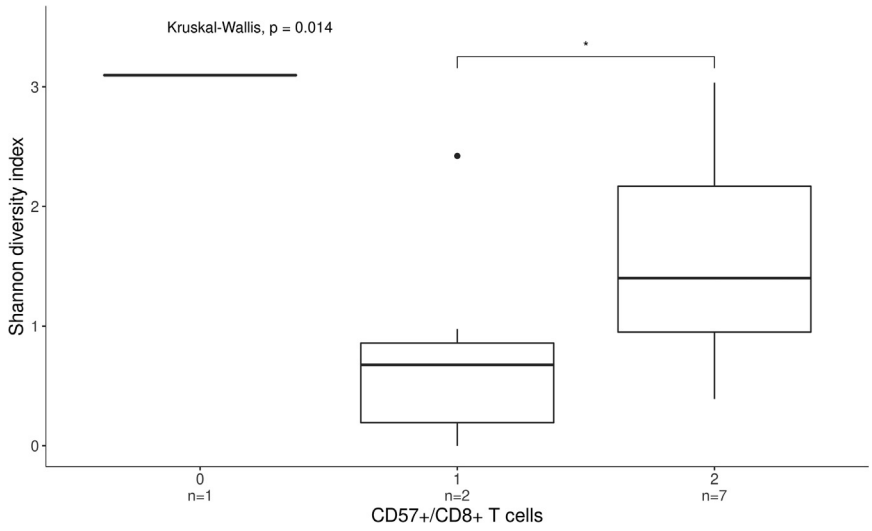

Figure 11. Association between CD57+ and/or CD8+ T cells and microbiota diversity measured by the Shannon index. The x-axis indicates the proportion of CD57+ and/or CD8+ T cells: 0 = within reference values, 1 = below reference value, 2 = above reference value ($P = 0.014$). The reference values have been described in detail by Eränkö et al. (2018). *Wilcoxon, $P < 0.05$.

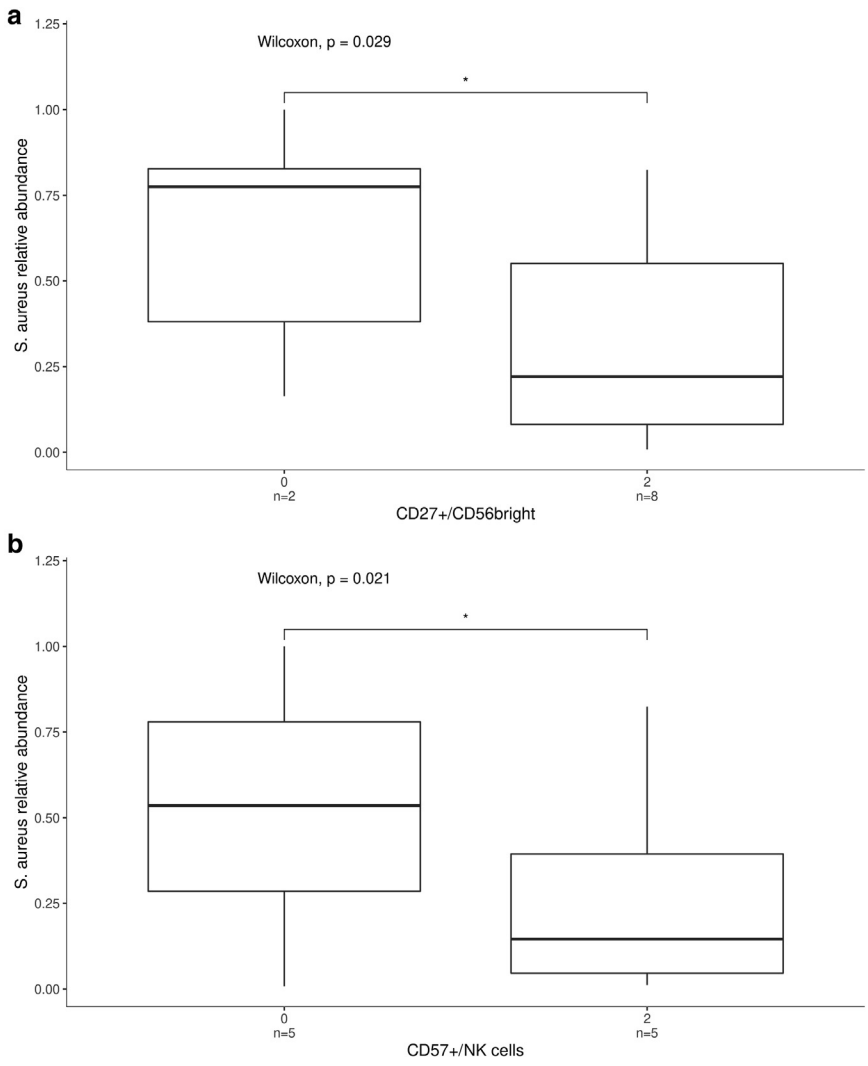

Figure 12. Association between lymphocyte or NK cell subclass cells and *Staphylococcus aureus* relative abundance. (**a**) CD27+ and/or CD56bright ($P = 0.029$) and (**b**) CD57+ and/or NK cells show a significant association with *S. aureus* abundance ($P = 0.021$). 0 = normal limits, 2 = above reference value. The reference values have been described in detail by Eränkö et al. (2018). *Wilcoxon, $P < 0.05$.

determined according to the manufacturer's instructions (Thermo Fisher Scientific, Uppsala, Sweden). Data were analyzed with the Microarray Image Analysis Software (version 1.2.4). Allergen-specific IgE-antibodies were expressed in arbitrary ISAC Standardized Units. We developed an allergen score for the analyses: the raw points for all food allergens, aeroallergens, and other allergens were summed separately and combined as follows. The ISAC Standardized Unit values < 0.3, representing negative to very low, were scored 0; values 0.3–0.9, representing low, were scored 1; values 1.0–14.9, representing moderate to high, were scored 2; and values ≥ 15, representing very high, were scored 3. Thereafter, the participants were divided into three groups: not allergic (low score), moderately allergic (medium score), and very allergic (high score).

Generation and analysis of metagenomics shotgun data

The microbiome-enriched PBS samples had the DNA extracted by Qiagen's Pathogen Lysis Tubes (catalog number: 19092; Qiagen, Hilden Germany) and the QIAamp UCP Pathogen Mini Kit (catalog number: 50214; Qiagen) according to the manufacturer's instructions. Libraries of 350 base pairs for shotgun sequencing were prepared using the ThruPLEX DNA-seq kit (Rubicon Genomics, Ann Arbor, MI) and sequenced using the Illumina HiSeq 2500 technology (2×150 base pairs; Illumina, San Diego, CA). The resulting reads were quality controlled and adapter trimmed by Trim Galore (version 0.4.1), followed by the removal of mapped reads to the human genome GRCh38 (GenBank accession number: GCA_000001405.15) using Bowtie (version 2.3.2) (Langmead and Salzberg, 2012).

Species-level taxonomic profiling was performed with Kraken (version 2.07—Beta-diversity) (Wood et al., 2019) by using a database built with reprDB (Zhou et al., 2018). The abundance of species was computed using Bracken (version 1.0.0) (Lu et al., 2017). Microbial community richness and diversity measures were calculated with the vegan R package (Oksanen et al., 2019). Bacterial aerobes versus strict anaerobes were deter-

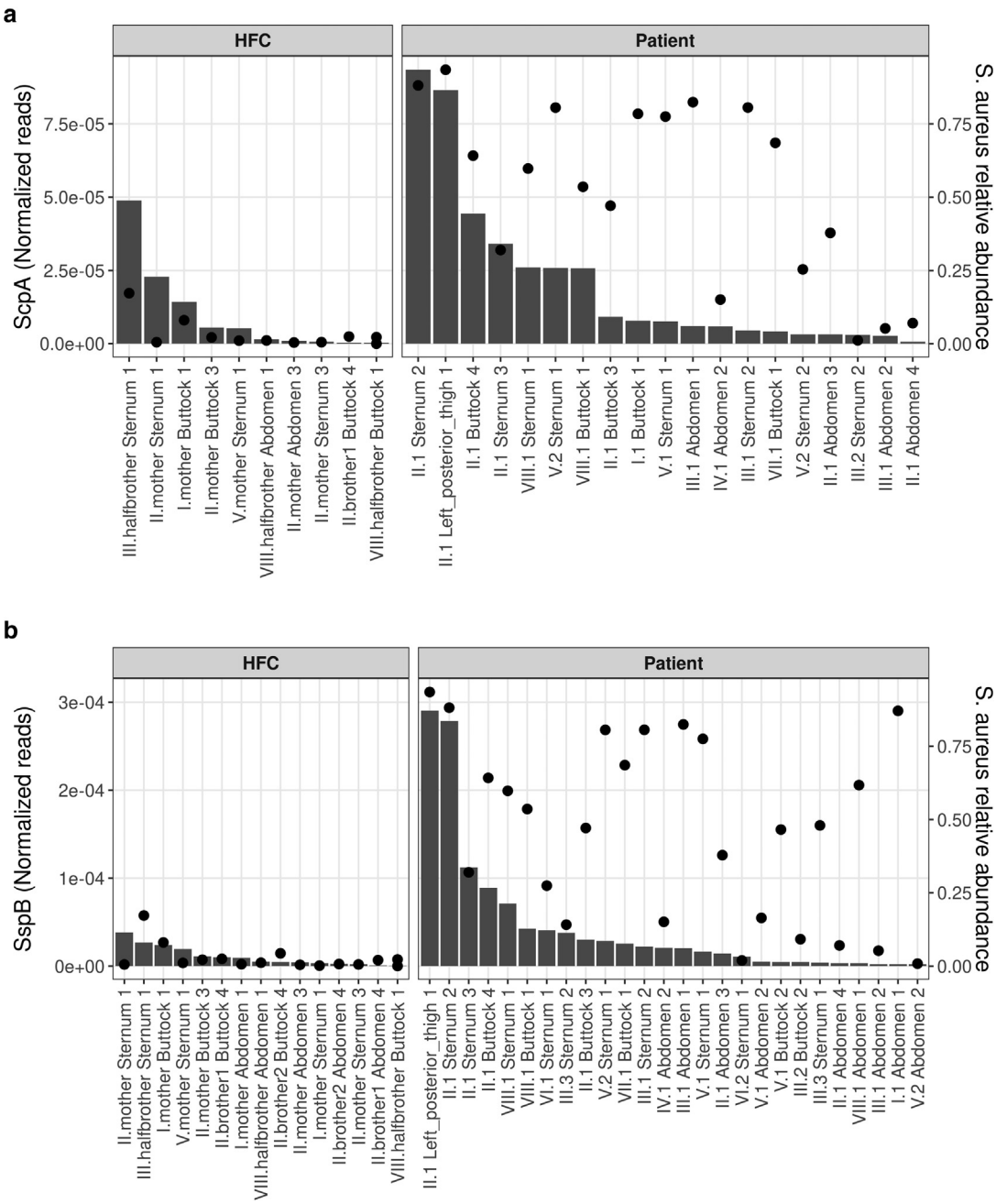

Figure 13. *Staphylococcus aureus* staphopain A and B are increased in NS compared with HFCs. Number of reads corresponding to (**a**) *ScpA* and (**b**) *SspB* genes normalized per library size for each sample. The points show the abundance of *S. aureus* in the samples. HFC, healthy family control; NS, Netherton syndrome.

mined according to Bergey's Manual of Systematic Bacteriology (Ruan, 2013). The distance between samples was estimated using Mash (version 2.0) (Ondov et al., 2016). A differential abundance testing of taxa across clinical groups was conducted using ANCOM from R package version 2.1 (Mandal et al., 2015), using the skin site as random effects and the family number as covariant. The test was run with default parameters and Benjamini–Hochberg for multiple comparison correction. The correlation between the species was analyzed using the proper R package with rho metric (Quinn et al., 2019). Associations between skin infections, allergic symptoms, and NK cells were examined with the microbiome Shannon diversity index and the *Sta. aureus* amount by performing the nonparametric Kruskal–Wallis test followed by the paired Wilcoxon test with Holm multiple comparison correction using the ggpubr R package (Kassambara, 2020). To identify the staphylococcal virulence factors among the samples, the filtered reads were mapped against four genes: (i) PSMα (GenBank: BK006301.1), (ii) ScpA (GenBank: AJ538362.1), (iii) SspB (GenBank: AF309515.1), and (iv) extracellular cysteine protease (GenBank: AJ298299.1) using Bowtie (version 2.3.2) (Langmead and Salzberg, 2012). The Kruskal–Wallis test was used for continuous variables, and the Wilcoxon test was used for the paired tests.

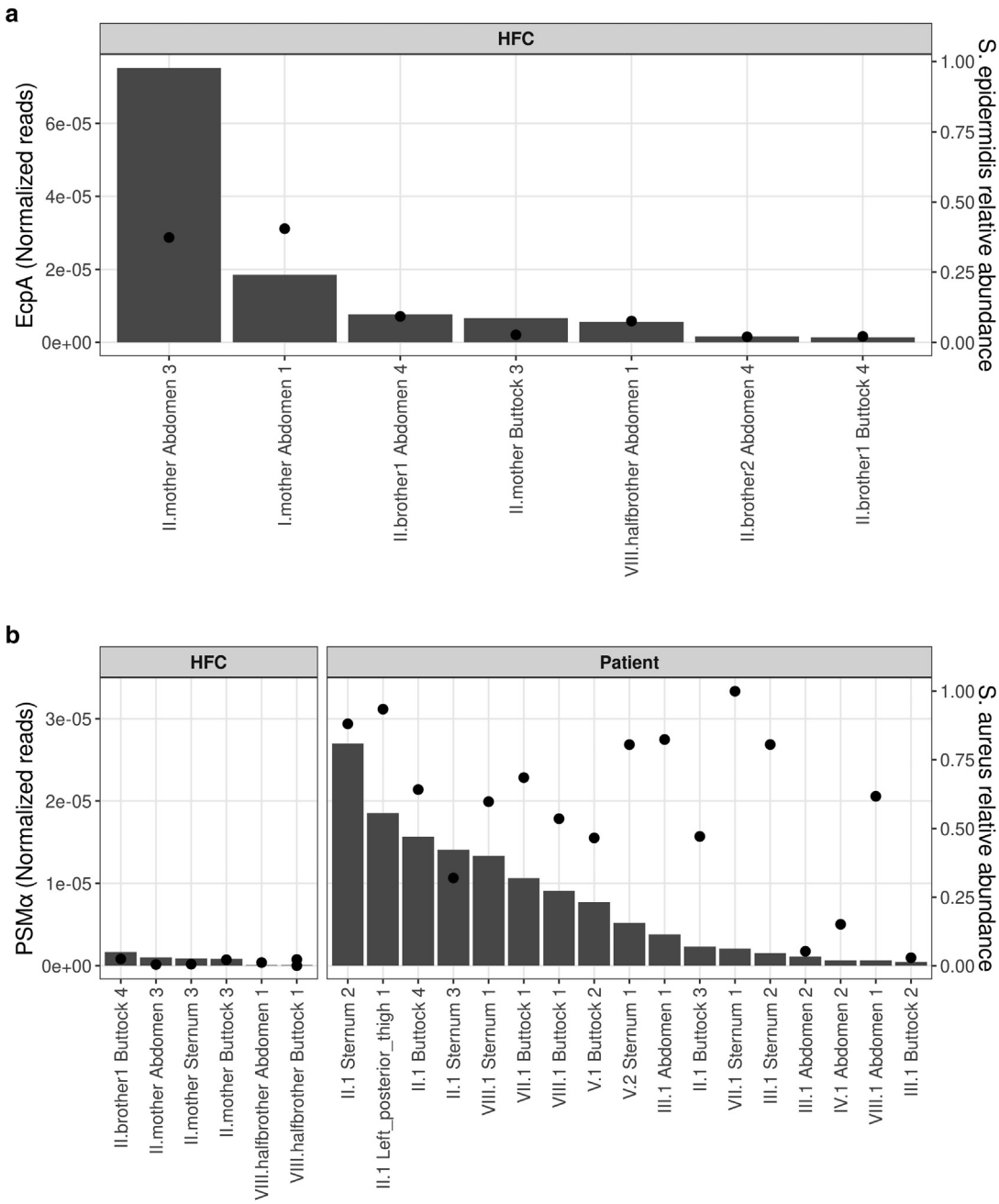

Figure 14. *Staphylococcus epidermidis* is increased in HFCs and *S. aureus* PSMα is increased in NS skin. Number of reads corresponding to (**a**) *EcpA* and (**b**) *PSMα* genes normalized per library size for each sample. The points show (**a**) *S. epidermidis* and (**b**) *S. aureus* abundance in the samples. HFC, healthy family control; NS, Netherton syndrome.

Data availability statement

The data analyzed during this study are included in this published article. Data sets related to this study have been submitted to the European Nucleotide Archive under the study reference number PRJEB39614.

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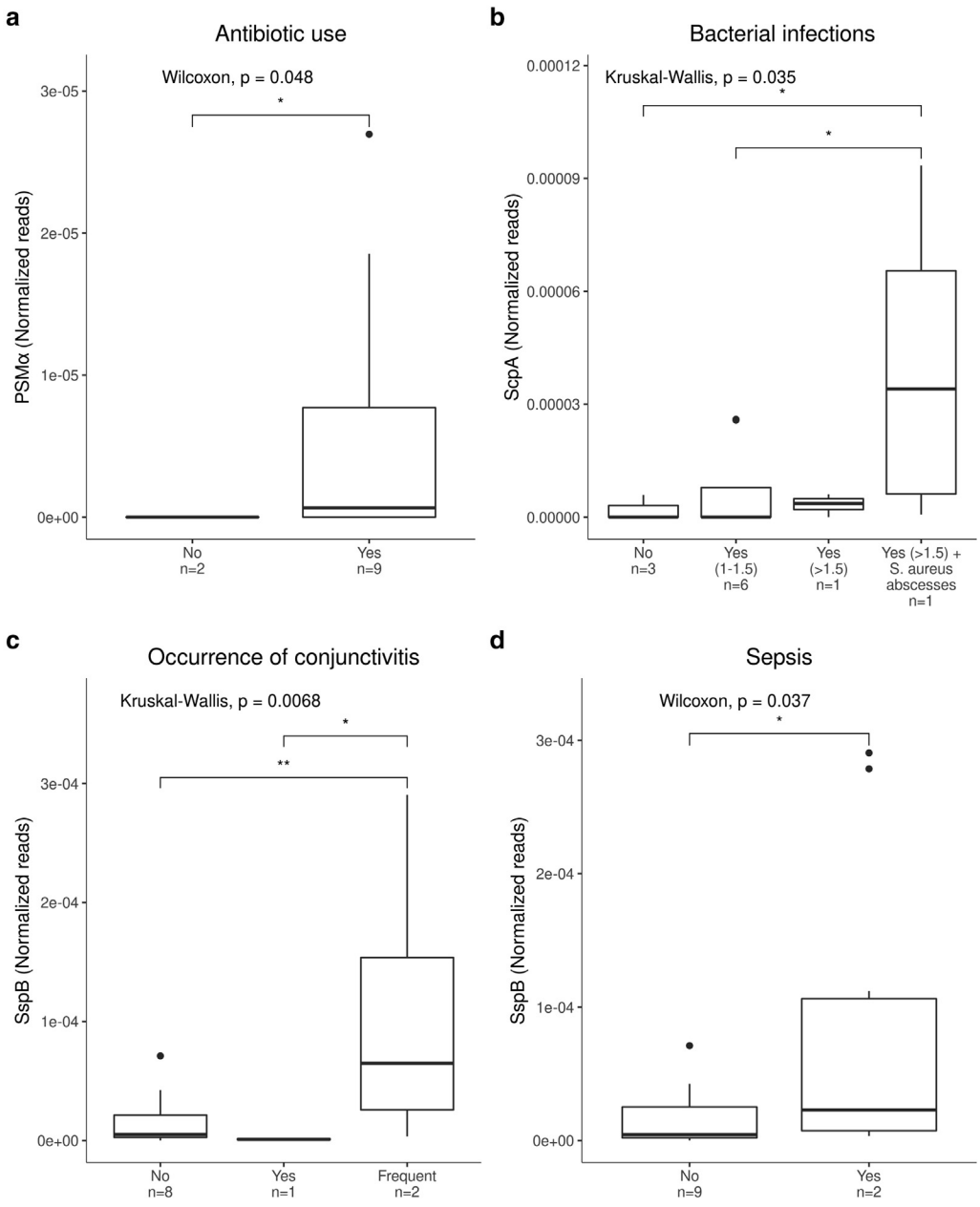

Figure 15. Association between infection data and *Staphylococcus aureus* genes. (**a**) *PSMα* is increased in patients with antibiotics in infancy ($P = 0.048$). (**b**) The *ScpA* gene is more abundant in patients with recurrent skin bacterial infection. No = as in the age group usually, Yes = recurrent infections at 1−1.5 years of age, >1.5 years and at age > 1.5 years, respectively ($P = 0.035$). (**c**) *SspB* is increased in patients with conjunctivitis. No = as in the age group usually, Yes = recurrent infections at 1−1.5 years of age, Frequent = frequent conjunctivitis or constant need for antibiotic eye drops ($P = 0.0068$). (**d**) *SspB* is more abundant in patients with sepsis caused by *S. aureus* or *S. epidermidis*. Although the statistical calculation has shown significance, the sample numbers are relatively small ($P = 0.037$). *Wilcoxon, $P < 0.05$ and **Wilcoxon, $P < 0.01$.

EE, MSB, TATS, BA; Writing - Review and Editing: VS, TATS, EE, MBS, KHJ, BA, AR

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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